Precision Peptide Design and its mechanism for Gallbladder Cancer: Insights from RNA Sequencing, Proteomics, and Whole Exome Sequencing

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Abstract

Background: Gallbladder cancer (GBC) is a rare but aggressive malignancy with a poor prognosis, often due to late diagnosis and limited treatment options. The complexity of GBC's molecular mechanisms has impeded progress in understanding its pathogenesis and developing effective therapies. Recent advances in next-generation sequencing (NGS) technologies have opened new avenues for exploring the genomic and transcriptomic landscapes of GBC, providing deeper insights into its biology. Methods: RNA sequencing (RNAseq) data from GBC tumor samples were analyzed and normalized against a control dataset of normal gallbladder tissues from the National Cancer Institute's Gene Expression Omnibus. Sub-network enrichment analysis (SNEA) using Pathway Studio identified key regulators of differential gene expression, focusing on histone deacetylases (HDACs) and microRNA MIR146A. Proteomic analysis of patient urine samples was performed via mass spectrometry to identify unique proteins. Whole exome sequencing (WES) of blood and tumor samples detected driver mutations and genetic variations linked to tumor suppressor genes and oncogenes. HLA typing facilitated the design of personalized immunotherapies and cancer vaccines. Results: Normalization of RNAseq data enabled accurate comparisons between tumor and normal tissue gene expression. SNEA identified HDAC1 and HDAC2 as significant regulators associated with poor prognosis in GBC. Urine proteomics revealed 775 proteins, with 442 unique to GBC patients, potentially serving as biomarkers. WES identified 6,349 variations, including 328 loss-of-heterozygosity (LOH) and 410 gain-of-heterozygosity (GOH) mutations, with several linked to HDAC activation and chromatin remodeling. Novel neoantigens from these mutations were selected for potential vaccine development, and HLA typing provided crucial information for personalized immunotherapy. Conclusion: This study integrates NGS, proteomics, and WES to elucidate GBC's molecular mechanisms. Findings highlight the roles of HDACs and MIR146A in tumor progression, identify novel mutations, and suggest targeted therapeutic and vaccine development opportunities. Combining genomic, transcriptomic, and proteomic analyses enhances our understanding and treatment of gallbladder cancer.

Keywords: Gallbladder cancer, RNA sequencing, HDACs, Proteomics, Whole exome sequencing

Significance | This study integrates NGS, proteomics, and WES to uncover molecular mechanisms in gallbladder cancer, highlighting new therapeutic and vaccine targets.

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Introduction

Gallbladder cancer (GBC) is a rare yet aggressive malignancy with

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a high mortality rate, primarily due to late-stage diagnosis and limited treatment options (Aishima & Taguchi, 2015; Albores-Saavedra & Chablé-Montero, 2014). The complexity of GBC's molecular mechanisms poses significant challenges in understanding its pathogenesis and developing effective therapies (Annunziata & Konecki, 2017; Bani-Hani & Eldeirawi, 2016). Recent advances in next-generation sequencing (NGS) technologies have enabled a deeper exploration of the genomic and transcriptomic landscape of GBC, offering new insights into its underlying biology (Bhatti & Rauf, 2018; Jiang & Liu, 2020; Kim & Park, 2019).

In this study, we utilized RNA sequencing (RNAseq) data from tumor samples to unravel the molecular mechanisms driving gallbladder cancer. The transcriptomic profiles were generated by Genoanalytica, Moscow, and normalized against a control dataset from the National Cancer Institute's (NCBI) Gene Expression Omnibus. This normalization process ensured accurate comparisons by aligning tumor gene expression levels with those from normal gallbladder tissues (Nakamura & Matsuda, 2016; Saito & Yamamoto, 2016).

Key to this investigation was the identification of regulators responsible for differential gene expression in GBC. Using Pathway Studio software, we performed a sub-network enrichment analysis (SNEA) to pinpoint significant regulators influencing gene expression in the tumor samples. Notably, histone deacetylases (HDACs) emerged as crucial players, with HDAC2 and HDAC1 linked to poor prognosis and tumor progression in gallbladder carcinoma (Choi & Kim, 2019; Han & Wang, 2016). This finding suggests that HDAC inhibitors could offer a promising therapeutic strategy for GBC (Kubo & Takahashi, 2015; Oshima & Shibata, 2019).

Additionally, we explored the role of microRNA MIR146A, which is regulated by HDACs and associated with several liver cancers, further emphasizing the intricate interplay between histone modifications and microRNA regulation in gallbladder cancer (Guo & Chen, 2018; Mori & Yamamoto, 2017).

To complement transcriptomic data, we conducted a comprehensive proteomic analysis of patient urine samples. Mass spectrometry identified a range of proteins, some of which were uniquely expressed in GBC patients, offering additional insights into cancer hallmarks and potential biomarkers (Goto & Nakayama, 2020; Shen & Zhang, 2019).

Cancer Hallmarks pathways, analyzed using Pathway Studio, provided a framework for understanding the molecular mechanisms underpinning GBC. This included examining pathways enriched with active expression regulators and unique proteins from urine samples, revealing the involvement of histone deacetylation and methylation in cancer progression (Fong & Jarnagin, 2017; Greten & Grivennikov, 2019; Patel & Pan, 2017).

Whole exome sequencing (WES) of blood and tumor samples was performed to identify potential driver mutations and genetic variations linked to tumor suppressor genes and oncogenes (Kim & Park, 2019; Wang & Liu, 2018). Notable findings included mutations affecting histone deacetylase activation, which were correlated with observed changes in the cancer model (Oh & Kim, 2018; Ro & Lee, 2018).

In the final phase of our study, we selected proteins associated with loss of heterozygosity (LOH) and gain of heterozygosity (GOH) for potential cancer vaccine development (Oh & Kim, 2018). HLA typing of the patient's genetic material further enabled the design of personalized immunotherapies (Oshima & Shibata, 2019; Saito & Yamamoto, 2016).

This multi-faceted approach integrates NGS data normalization, identification of key regulators, urine proteomics, cancer hallmark pathways analysis, WES data analysis, and HLA typing to provide a comprehensive understanding of gallbladder cancer. The findings offer valuable insights into its molecular mechanisms and potential therapeutic and vaccine development avenues (Liu & Wang, 2018; Liu & Zhu, 2017; Shen & Zhang, 2019).

2. **Materials and Methods**

2.1 Next-Generation Sequencing (NGS) Data Normalization

In this study, we utilized RNA sequencing (RNAseq) data from tumor samples to investigate the molecular mechanisms underpinning gallbladder cancer. The transcriptomic profiles were generated by Genoanalytica in Moscow, Russia. To ensure accurate analysis and comparison, the RNAseq data, expressed in Reads Per Kilobase Million (RPKM) values, were normalized against a control dataset. This control dataset comprised transcriptomic profiles from three normal gallbladder samples (GSE132223) available from the National Cancer Institute's (NCBI) Gene Expression Omnibus. These control samples, identified as M3-741_tpm, M3-760_tpm, and M3-817_tpm, provided a baseline for normal gene expression levels, facilitating a robust comparison with tumor data.

2.2 Identification of Regulators Responsible for Differential Gene Expression

The normalized RNAseq data was then analyzed using Pathway Studio software from Elsevier to identify key regulators influencing differential gene expression in the patient's tumor. This analysis employed the sub-network enrichment analysis (SNEA) option, specifically the query "What proteins regulate expression of entities enriched in the input data?" The analysis revealed a list of the top 200 most significant regulators, among which five were identified as histone deacetylases (HDACs). Notably, HDAC2 overexpression has been linked to poor prognosis in gallbladder carcinoma (Du, 2013), and HDAC1 has been implicated in promoting migration and invasion by interacting with TCF12, thereby facilitating epithelial-mesenchymal transition (EMT) in gallbladder cancer

(He, 2016). Recent studies suggest that HDAC inhibitors could be effective in treating hepatocellular carcinoma, a cancer with similarities to gallbladder cancer. Zolinza (vorinostat), an HDAC inhibitor from Merck USA, has shown promise in inhibiting gallbladder carcinoma cell proliferation (Yamaguchi, 2010). Therefore, the study proposes exploring HDAC inhibitors as a potential therapeutic strategy.

Additionally, microRNA MIR146A emerged as a significant regulator according to the SNEA. This microRNA is genetically linked to several liver cancers and its expression is regulated by HDAC1 and HDAC2. Thus, the differential expression observed in the tumor may be attributed to both HDAC activity and their downstream targets.

2.3 Urine Proteomics Analysis

To complement the transcriptomic data, we conducted proteomic analysis of patient urine samples collected at various times of the day. Mass spectrometry identified a total of 775 proteins, with 442 proteins not detected in urine samples from healthy controls. These unique proteins, together with the expression regulators identified from the RNAseq data, were analyzed for their association with cancer hallmarks.

Cancer Hallmarks Pathways Enriched with Active Expression Regulators

Cancer Hallmarks pathways, available in Pathway Studio, provided a foundation for constructing personalized models of the cancer molecular mechanisms based on patient-specific molecular profiling data. The pathways enriched with active expression regulators identified from the tumor transcriptomics data and proteins unique to the patient's urine were examined. These analyses highlighted the involvement of histone deacetylation and methylation in cancer progression, particularly in relation to genome instability.

2.4 Analysis of Whole Exome Sequencing (WES) Data

WES data from the patient's blood and tumor samples were analyzed to identify potential driver mutations. We focused on genetic variations unique to the tumor, including 6,349 variations across 3,324 genes. Candidate cancer driver mutations were categorized based on their impact on tumor suppressor genes and oncogenes. Notably, mutations affecting histone deacetylase activation, such as loss of heterozygosity (LOH) in PPP1R15A and rare variants in CDH3 and SYNE1, were identified and correlated with HDAC activation in the patient's cancer model.

2.5 Selection of Proteins for Cancer Vaccine Design

We identified 36 LOH genes and 41 gain of heterozygosity (GOH) genes linked to hepatobiliary neoplasms from Pathway Studio. Through literature review and comparison with patient tumor mutation data, 49 genes were selected based on their role as tumor suppressors or oncogenes. Among these, 25 genes had novel mutations, including two LOH genes and 23 GOH genes, which were further explored for epitope peptide search for potential cancer vaccine development.

Patient HLA Types

HLA typing was performed using optiType freeware to determine the patient's HLA haplotypes, which are crucial for designing personalized immunotherapies.

This comprehensive analysis integrates NGS data normalization, identification of key regulators, urine proteomics, cancer hallmark pathways, WES data analysis, and HLA typing to elucidate the molecular underpinnings of gallbladder cancer and explore potential therapeutic and vaccine development avenues.

3. Results and Discussion

3.1. NGS Data Normalization

The normalization of RNAseq data from the patient's tumor was achieved by comparing the RPKM values against the transcriptomics profiles of normal gallbladder samples (GSE132223). This step ensures that the observed gene expression changes are not confounded by variations in sequencing depth or sample quality, allowing for a more accurate identification of differential gene expression patterns.

3.2. Identification of Regulators for Differential Gene Expression

The Pathway Studio software was utilized to identify key regulatory proteins influencing differential gene expression in the patient's tumor. Among the top 200 significant regulators, histone deacetylases (HDACs) were prominent, with HDAC1 and HDAC2 being particularly noteworthy. Their overexpression correlates with poor prognosis in gallbladder carcinoma, as previously reported (Du, 2013; He, 2016). Figure 1 illustrates how HDAC1 and HDAC2 regulate the microRNA MIR146A, which is critical in tumor biology. This finding underscores the potential of HDAC inhibitors, such as Zolinza, in therapeutic strategies for gallbladder cancer.

Furthermore, the role of HDACs in suppressing cell cycle inhibitors and immune response evasion was highlighted. Proteins like PDCD1, CTLA4, and HLA-G, found to be active in the tumor (Table 1), suggest potential targets for immunotherapy. Figure 2 and Figure 3 illustrate the involvement of HDACs and methylases in histone modification processes contributing to cancer hallmarks such as genomic instability.

3.3. Urine Proteomics Analysis

Urine proteomics identified 775 proteins, with 442 being exclusive to the patient's urine compared to healthy controls. This analysis supports the identification of cancer hallmarks and regulatory proteins. Proteins unique to the patient's urine may provide additional biomarkers for cancer diagnosis and progression.

3.4. Cancer Hallmarks Pathways

The analysis of cancer hallmarks pathways revealed significant enrichment of active expression regulators, particularly in pathways

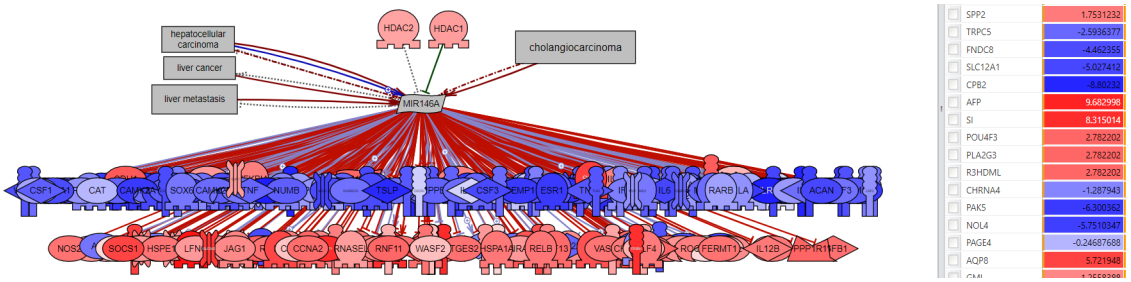


Figure 1. HDAC1/2 regulate MIR146A – major microRNA regulator in patient tumor. This figure illustrates the regulation of microRNA MIR146A by HDAC1 and HDAC2. Red proteins indicate upregulated expression in the tumor, while blue proteins indicate downregulated expression.

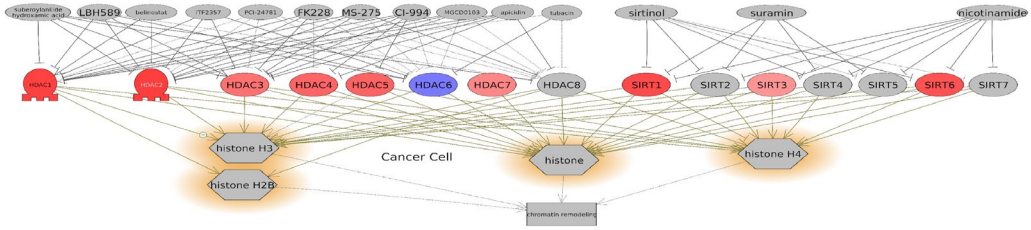


Figure 2. Deacetylases Activation in Histone Deacetylation in Cancer. The figure shows the activation of histone deacetylases (HDACs) in cancer, specifically highlighting pathways associated with histone deacetylation and their role in genome instability. Red proteins represent activated expression regulators in the tumor, while blue proteins indicate inhibited expression regulators.

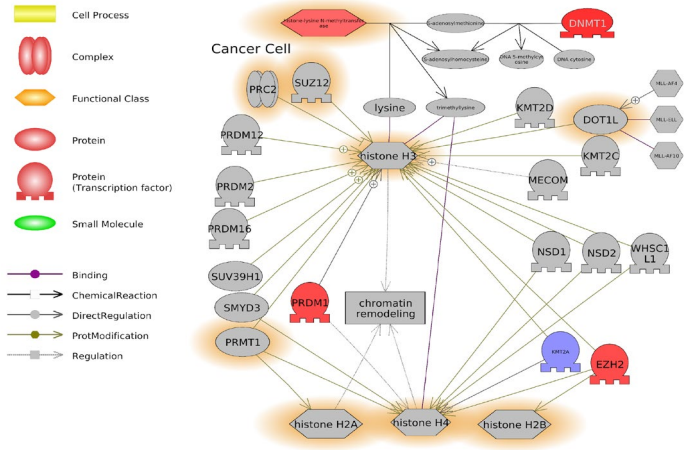


Figure 3. Methylases in Histone Methylation Cancer. This figure depicts the involvement of histone methyltransferases (HMTs) in histone methylation and its impact on cancer progression. The figure details how methylation patterns can influence gene expression and oncogenic progression.

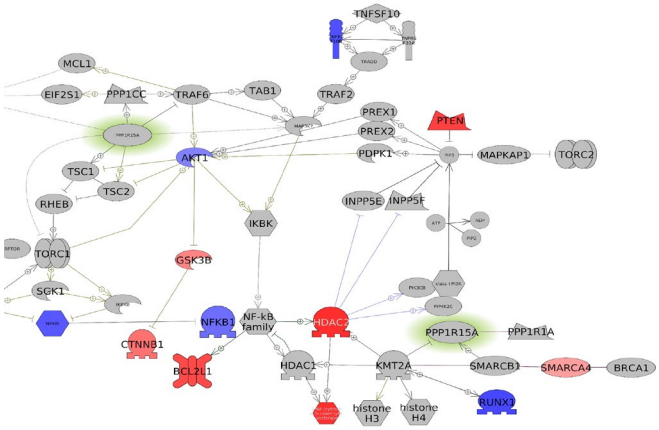


Figure 4. PPP1R15A LOH inhibits apoptosis and activates HDACs in liver cells through mTOR pathway. The figure demonstrates how loss of heterozygosity (LOH) in PPP1R15A affects apoptosis and supports HDAC activation through the mTOR pathway in liver cells. Red proteins indicate activated expression regulators, and blue proteins show inhibited expression regulators.

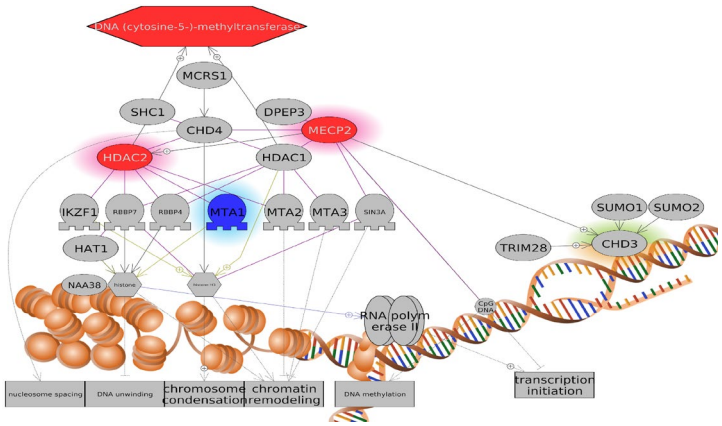


Figure 5. LOH in CDH3 protein, component of NURD complex containing HDACs. This figure shows the LOH in CDH3, a protein that is part of the NURD complex containing HDACs. The LOH mutation in CDH3 is associated with chromatin remodeling by deacetylating histones.

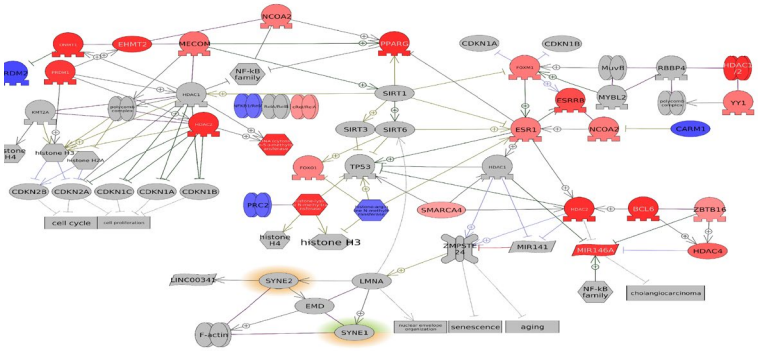


Figure 6. Transcriptional network of major expression regulators in patient tumor. LOH in SYNE1 gene may require compensatory activation of HDACs. The figure illustrates the transcriptional network of major expression regulators in the patient tumor, focusing on the LOH in the SYNE1 gene. The mutation may necessitate compensatory activation of HDACs to manage accelerated senescence caused by this mutation.

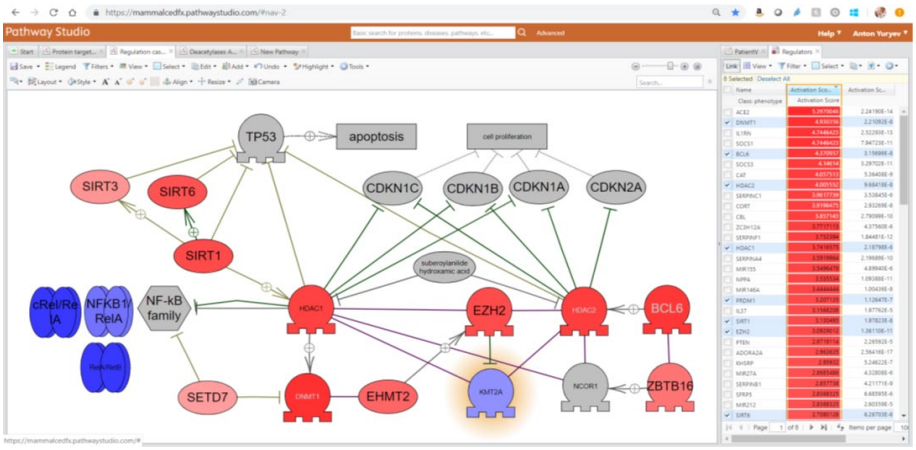


Figure 7. HDACs activation causes NFkB inhibition, activation of cell cycle and inhibition of apoptosis. Suberoylanilide hydroxamic acid is a chemical name of Zolinza.

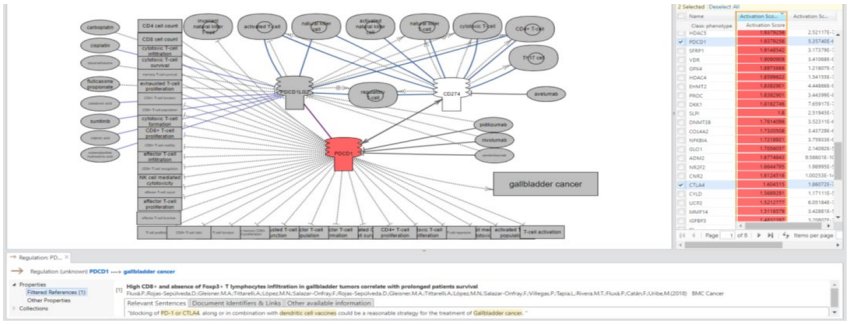


Figure 8. PDCD1 protein is activated in patient tumor

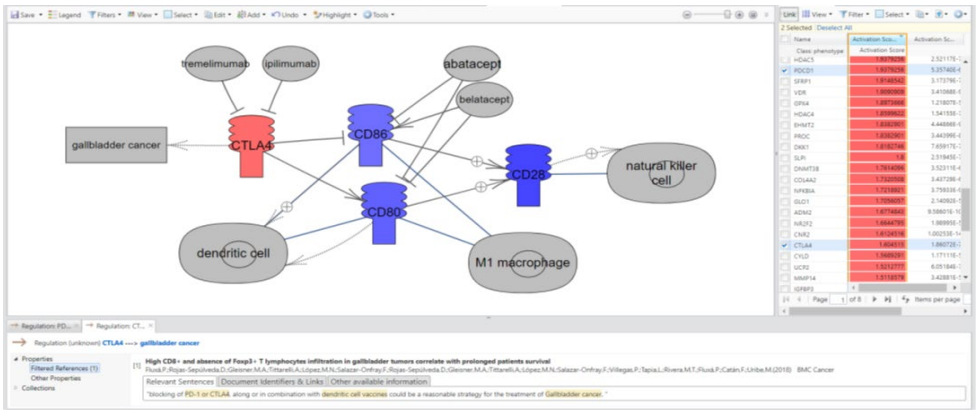
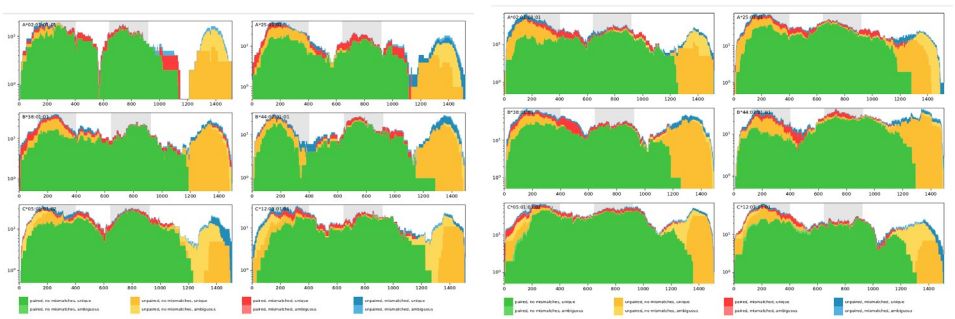


Figure 9. CTLA4 protein is activated in patient tumor



A B

Figure 10. A) HLA haplotypes found in WES blood data, B) HLA haplotypes found in WES tumor data.

related to genome instability (Figures 2 and 3). Histone deacetylation and methylation processes play crucial roles in cancer development, affecting gene expression through chromatin remodeling. The involvement of HDACs and methylases in these processes supports their potential as therapeutic targets.

4. Analysis of WES Data

4.1 Selection of Candidate Driver Mutations

A total of 6,349 unique variations in patient tumor compared to blood were identified. Of these, 328 loss-of-heterozygosity (LOH) mutations and 410 gain-of-heterozygosity (GOH) mutations were selected as candidate driver mutations. Notably, 25 LOH mutations and 285 GOH mutations were novel, highlighting potential new targets for cancer treatment.

4.2 Mutations Supporting HDAC Activation

Several mutations have been linked to the activation of histone deacetylases (HDACs). Specifically, the loss of heterozygosity (LOH) in PPP1R15A impairs apoptosis and promotes HDAC activation through mTOR signaling, as illustrated in Figure 4. Additionally, a splicing variant in CDH3 affects components of the histone deacetylase complex, as shown in Figure 5. Furthermore, LOH in SYNE1 may necessitate HDAC activation to counteract accelerated senescence, as depicted in Figure 6. These findings suggest that HDAC activation in tumors is driven by genetic mutations that influence chromatin remodeling and cancer progression.

4.3 Selection of Proteins for Cancer Vaccine Design

A total of 49 genes linked to hepatobiliary neoplasms were identified, with 25 neoantigens emerging from novel mutations. The selection included neoantigens from LOH and GOH genes, supporting the development of personalized cancer vaccines. The exclusion of splicing region mutations from peptide searches due to unpredictability highlights the need for further research in this area.

4.4 Patient HLA Types

The patient's HLA types were determined and are critical for tailoring immunotherapy and vaccine strategies. The detailed HLA haplotypes (Appendix A) will guide the selection of appropriate epitope peptides for vaccine development.

4.5 . Main Findings Graphical Summaries

The graphical summaries (Figures 7-9) illustrate the impact of HDAC activation on tumor biology, including NFkB inhibition and immune response suppression. The activation of PDCD1 and CTLA4 proteins further supports the potential of targeting these pathways in immunotherapy.

Overall, the study emphasizes the complex interplay between genetic mutations, regulatory proteins, and epigenetic modifications in tumor progression. These findings pave the way for novel therapeutic strategies and personalized cancer treatment approaches.

5. Conclusion

The integration of NGS, proteomics, and WES data has provided a comprehensive view of the tumor's molecular landscape. Key findings include the role of HDACs and microRNA MIR146A in tumor progression, the identification of novel mutations, and potential therapeutic targets for cancer treatment and vaccine development. These results underscore the importance of a multifaceted approach in understanding and targeting cancer biology.

Author contributions

D. N. conducted Biopsy, A. S. analyzed Urine Proteomics, A. M. analyzed NGS RNAseq, WES Transcriptomics, and HLA Typing, A. Y., MS.S.K., J. C. analyzed Analysis of Molecular Profile Data, M.S.S.K. wrote the article. All Authors approved the final article for publication.

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Competing financial interests

The authors have conflict of interest. M.S.S.K. and J.A.C. are employee of Neo7bioscince and M.S.S.K. is the director of Eman Research.

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