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STABILITY INSIGHTS OF ABERRANT GLYCATED MUCIN1 (MUC 1) VARIABLE NUMBER OF TANDEM REPEAT (VNTR) REGION THROUGH A MOLECULAR DYNAMIC APPROACH

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Abstract

Mucin 1 (MUC1) is a membrane-bound glycoprotein that is present on the apical surface of epithelial cells. A lot of studies have connected it to epithelial adenocarcinomas growth, metastasis, and poor prognosis. Carbohydrate groups' numerous structural alterations associated with the protein backbone are one of the qualitative alterations in MUC1 during epithelial adenocarcinoma. Importantly glycan structures play a crucial function in the cellular connection. Here in this study, we constructed 3D MUC1-VNTR structure and Core 1-4 were added to their appropriate locations thereby designed $MUC1^{Wild}$ type and $MUC1^{Cancer}$. Different bioinformatics analysis such that Root Mean Square Deviation, Root Mean Square Fluctuations, Radius of Gyration, Hydrogen bonding, Secondary Structure Determination, Clustering, and Principal Component analysis was performed on the 100 ns molecular dynamic simulation trajectories. We found out that RMSD, RMSf, and Rgyr significantly differ between the $MUC1^{Wild}$ and $MUC1^{Cancer}$. More H-bonding and strong protein stability and folding were seen in the $MUC1^{Wild}$ and PCA analysis further confirms $MUC1^{Wild}$ compact motion in the parallel direction during MD simulation. These research findings revealed structural dynamics of the $MUC1^{Wild}$ and $MUC1^{Cancer}$ VNTR Mucin1 areas that could not be investigated experimentally, allowing us to build a more complete picture of their carcinogenic effects. Furthermore, glycation differences in protein structure can impact the overall conformational interaction network and give structural insight for drug discovery and biomarker development.

Keywords: MUC1, VNTR, MD simulation, RMSD, RMSf, Rgyr, Principal Component Analysis, interaction networks.

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1. INTRODUCTION

Mucin 1 (MUC1) is membrane-bound glycoprotein expressed on the epithelial cell apical surface. Several positive shreds of evidence are reported of MUC1 in correlation with cancer proliferation, metastasis, and bad prognosis [1]. Among qualitative changes in MUC1, one of them is carbohydrate groups multiple structural changes attached to the protein backbone. The difference is called with the term “aberrant glycosylation”. Normal Glycan structures have an important role in cellular interaction as the outer exposed residues form interaction to the closely residing extra cells called vicinity of sugar residue [2]. These glycans are also attached with binding protein and perform cell to cell signaling and migration of the antigens in the cell body [3]. As a result, glycosylation alterations in tumor cells are critical for the survival of tumor cells. This dynamic shift of glycans is particular characteristics of specific disease and act as a treatment target [4]. The protein structure consists of two domains i.e. (1) MUC1-N composed of PTS and SEA domains. Also, the variable number tandem repeat (VNTR) region, is found in the N-terminal domain where heavily O-glycosylation (core 1, 2, 3 & 4) are occurred, followed by N-glycosylation at SEA domain. (2) MUC1-C terminal domain. Heavily glycosylated site of the PTS domain provides a mucus gel and induces the protection of the intestinal walls [5]. However, In cancer, reduced production of core 3 {(GlcNAcβ1-3GalNAcα-Ser/Thr) [core 3 synthase]} and core 4 {[GlcNAcβ1-3(GlcNAcβ1-6) GalNAcα-Ser/Thr] [core4 synthase]} structures were reported due to loss of transferase enzyme [6]. Furthermore, nothing is known about the MUC1 protein's differential glycans, which may be critical to their function. Because of the advancements in computational biology, many tools are available to develop different core structures on the miniaturized VNTR glycoprotein and compare their protein folding stability in the solution phase through molecular dynamic approach [7]. Over the past decade targeted drug treatment were unsuccessful due to resistant to drug therapy, while recent research has shown that MUC1 glycosylation alters with cancer. Therefore, the current study is specifically designed to investigate the association of aberrantly glycosylated MUC1 with tumor progression and metastasis, through stability of a protein. Which can provide a base for future research for the diagnostic, prognostic and therapeutic modalities of cancer.

2. METHODS

2.1 Structure Preparation

The UniProt database was used to obtain the MUC1 FASTA sequence (UniProt ID: P15941), where the VNTR regions of the MUC1 were also identified. The Glyconnect database was utilized to better understand O-linked glycation with threonine or serine residues common in VNTR repeat areas. Chimera software was used to create a 3D homology model of the VNTR sequence (GSTAPPAHGVTSAPDTRPAP). Furthermore, the 3D structure was optimised for energy efficiency using the Molecular Operating Environment (MOE) software package® 2015. The homology-modeled structure was subjected to charge correction and ionisation, followed by the insertion of hydrogen bonds

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using the MOE software package® 2015. This technique was used to alter the pH and H-bonding of the produced structure in order to reduce atomistic conflicts. Glycam.org (https://dev.glycam.org/) introduced carbohydrates to the 3D MUC1-VNTR structure and Core 1-4 were added to their appropriate locations. The difference between $MUC1^{Wild}$ and $MUC1^{Cancer}$, carbohydrate cores are represented in **Figure 1.0**. General Force Field (CGenFF) tool V 2.2.0 was used to create Carbohydrate topology and to neutralise charges, the Monte Carlo ion placement approach was applied. Water particles were added to the already produced protein-sized structure in a rectangular fit at the distance of edge 10.

2.2 Molecular Dynamic Simulation

Energy minimization (EM) was used to optimize the entire system, including carbohydrate-protein complex. EM was performed in several phases, including the Steep integrator, the Verlet cutoff method, and the LINCS constraint algorithm. Before the last molecular run, the system was equilibrated to adjust the solvent molecules with protein in carbohydrate -attached configurations. Aberrant MUC1 protein structure was equilibrated using an isothermal-isochoric ensemble (NVT). To approach NVT Equilibration ensemble, MD integrator, 500000 Nsteps, LINCS constraint-algorithm, VDW type cut-off, and Nose-Hover temperature coupling with a reference temperature of 300K were utilized. The complicated system was built under constant temperature using the V-Rescale t-coupling group and constant pressure using Perriello-Rahman (isotropic, p-coupltype). Prior to running the final 100 nanosecond MD simulation for all reduced and equilibrated structures, the VDW type cut-off and LINCS constraint-algorithm were performed in linear order. The GROMACS software, Origin Pro Lab V2018, PyMol v1.7, and VMD were used for trajectory analysis and graphical representations.

2.3 Molecular Dynamic Analysis

RMSD of the normal $MUC1^{Wild}$ type and their least fit Cα aberrant glycated isoform were computed. RMSf analysis was used to highlight the fluctuation of MUC1 residues, which may be brought about by any hypothesis questions raised throughout study design. Moreover, radius of gyration (Rgyr), assessed the overall protein structure in the specific protein box. Hydrogen bonding between the residue at the distance of 0.35 nm were calculated. Geometric clustering was performed to locate similar structures gathered during the MD simulation. The DSSP secondary structure determination programme was used to determine secondary structure outcomes. Ramachandran plot displaying the angle of torsion Phi and Psi of the residues in a protein on the x-axis and y-axis were plotted. Principal component analysis (PCA) was used to reduce the large data sets into a smaller set that preserves the bulk of the information easier to inspect and visualize.

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3 RESULTS

3.1 Structural Validation of MUC1

The stability of the MUC1 bound complex for both the native $MUC1^{Wild}$ and aberrant $MUC1^{Cancer}$ were measured in terms of root mean square deviation (RMSD) of the alpha-carbon (Cα) atomic coordinates. In $MUC1^{Cancer}$ structure was initially stable up to 60 ns and then moves to 1.3 Å by 65 ns, the same higher path was then followed afterward. Similarly, the native isoform was at 1.5 Å initially unstable until 25 ns, and moves to stability (0.5 Å) by until 60 ns, after the 60 ns it behaves like a $MUC1^{Cancer}$ for some time, but downgrade quickly (0.6 Å) by 70-100 ns. The interpretation of the RMSD is further plotted in **Figure 1**.

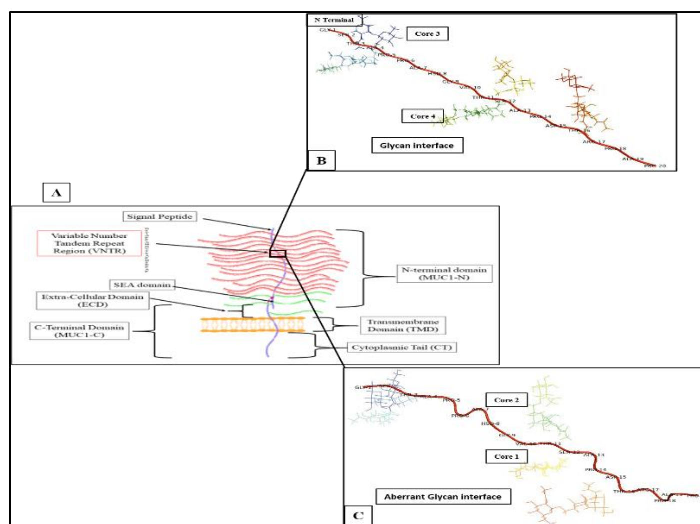

Figure: 1 MUCIN protein with VNTR region. (B) Normal MUC1-VNTR homology modeled structure with Core-3 2D Structure attached to Serine VNTR Region. (C) Aberrant glycosylated MUC1-VNTR structure with the attached Core-2 2D Structure at Threonine VNTR Region

3.2 Structural Exaggeration of MUC1

To further explore, how different structures exaggerated the Cα of the atoms in MDS, the graph was plotted to obtain each residue flexibility/disordered regions of protein structures during a 100ns MDS. $MUC1^{Wild}$ was used as a reference structure, and to increase the accuracy of the plot, stable phase trajectory was trimmed from the structures and aligned. Moreover, calculation was done ahead of 4 ns. The scatter RMSf plot representing different colour schemes for the specified trajectories, such that red (wild protein part) and black (aberrant isoform). X-axis denoted 20 amino acid VNTR region and Y-axis suggest Å fluctuations. The residue behavior pattern of fluctuations is similar; however, the $MUC1^{Cancer}$ has high Å fluctuations compared to the $MUC1^{Wild}$ (**Figure 2**). This suggested that aberrant glycan can bring un-stability towards protein structures. Moreover, gyrate (Rgyr), **Figure 2** indicates red line (wild type) maintains its linear

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trajectory, implying higher compactness and optimal protein folding, whereas the black line (aberrant form) changes over ps time, implying protein unfolding. In conclusion, this finding demonstrated that the MUC1^{Wild} is more compact than the MUC1^{Cancer}. The same mechanistic approach was suggested by Lemkul, J [8].

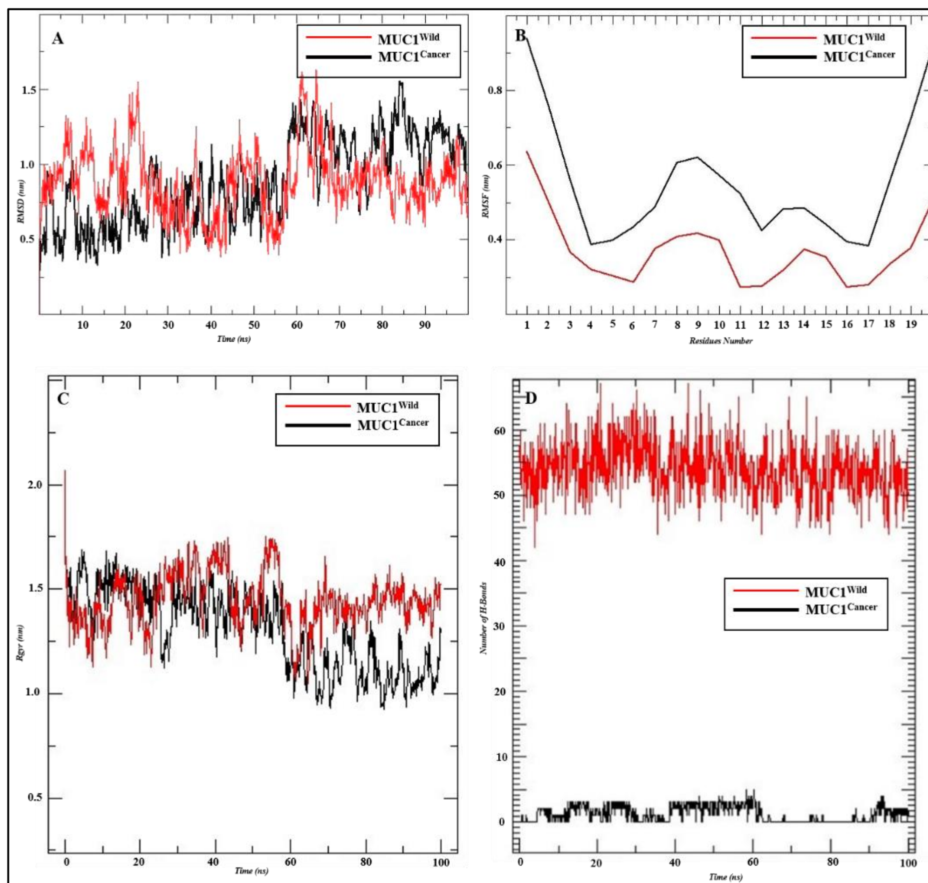

Figure: 2 Molecular Dynamic Analysis (A) RMSD scatter plot of the protein stability wild type and cancer isoform. (B) RMSf scatter plot of the protein stability wild type and cancer isoform. (C) Rgyr scatter plot of the protein stability wild type and cancer isoform. (D) Inter-protein H-bonding between protein and solvent

3.3 Glycation Interfere with Structure Stability

MUC1, introduce earlier has been surrounded by neighbored residues with H-bonding. And these residues are playing a significant role in complex stability. After subjection of the complex to MDS, Hydrogen bond analysis in the MUC1^{Cancer} suggested a decrease in the number of H-bonds in both the Intra-protein & inter-protein H-Bonding. This reflection was further validated by the output files showed increased progression of the total number of H-bonds between the MUC1^{Wild} groups over time. This result assumption further supports Rgyr analysis, such that MUC1^{Wild} protein has a stable structure. In-

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addition Ramachandran plot was utilized to see the difference between the two secondary structures after the simulation run. By taking $MUC1^{Wild}$ as the reference plot analysis showed that $MUC1^{Cancer}$ deviates at both right and left-handed α-helix, these are further elaborated by the DSSP algorithm (**Table 1**).

Table 1: Secondary structure determination after the final simulation run for wild type and cancer isoform

Residue	Initial Before MD run	Wild type after MD run	Cancer Isoform after MD run
	Structure, SASA		
GLY (Neutral)	Coil, 136.7	Coil, 643.1	Coil, 522.6
SER (Neutral)	Coil, 125.7	Coil, 641.1	Coil, 492.2
THR (Neutral)	Coil, 144.5	Coil, 649.4	Coil, 525.4
ALA (Hydrophobic)	Coil, 82	Coil, 601.3	Coil, 468.5
PRO (Neutral)	Turn, 110.7	Coil, 627.4	Coil, 500.8
PRO (Neutral)	Turn, 132.2	Turn, 602.9	Coil, 493.7
ALA (Hydrophobic)	Turn, 93	Turn, 631.2	Coil, 472.5
HSD (Neutral)	Coil, 183.2	Turn, 695.5	Coil, 574.6
GLY (Neutral)	Coil, 52.8	Turn, 553.9	Coil, 491.6
VAL (Hydrophobic)	Coil. 138.7	Coil, 644.2	Coil, 519.1
THR (Neutral)	Coil, 139.3	Coil, 637.1	Coil, 476
SER (Neutral)	Coil, 109.5	Coil, 621.3	Coil, 448.9
ALA (Hydrophobic)	Coil, 106.9	Coil, 598.8	Turn, 504.8
PRO (Neutral)	Coil, 120	Coil, 639	Turn, 538.8
ASP (Hydrophilic)	Coil, 125.3	Coil, 673.8	Turn, 480.5
THR (Neutral)	Coil, 140.4	Coil, 648.2	Turn, 589.1
ARG (Hydrophilic)	Coil, 133.6	Coil, 724.7	Coil, 515.2
PRO (Neutral)	Coil, 115.2	Coil, 614.8	Coil, 462.8
ALA (Hydrophobic)	Coil, 65.2	Coil, 598	Coil, 601.9
PRO (Neutral)	Coil, 182.4	Coil, 727.8	Coil, 598

3.4 Principal Component Analysis (PCA)

The PCA was performed and plotted to identify the most prominent structural changes induced by the binding of the aberrant glycan complex. The wild form exhibited cluster motion, $MUC1^{Wild}$ PC1 + PC2 cluster around -2 & +1, -1 & +1. Which denoted a more compact and stable protein folding over the 100 ns molecular dynamic simulation. On the other hand cancer isoform. The $MUC1^{Cancer}$ PCA plots, on the other hand, PC1 + PC2 residue, cluster moves around -4 & +4, -4 & +4. This suggested a highly unstable and randomness in the protein VNTR structure in specialized cancer isoform (**Figure 3**). In Conclusion, by keeping in view all the results. $MUC1^{Wild}$ protein with normal glycation pattern stabilized the protein with normal protein folding capability, while on the other

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hand evident variations were seen in MUC1^{Cancer}, however that just differ by the glycation pattern (aberrant glycans).

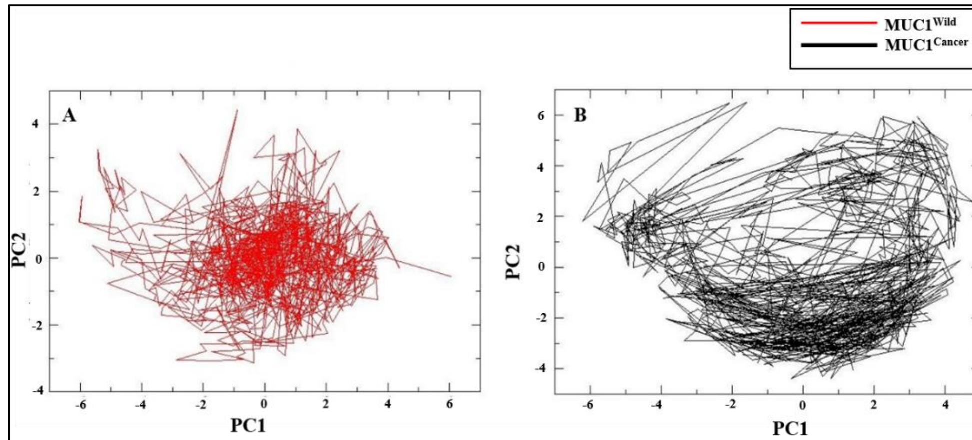

Figure: 3 A) PCA Plot of wild-type VNTR protein residues (B) Cancer isoform PCA Plot

4. DISCUSSIONS

Mucin 1 (MUC1) is a transmembrane glycoprotein found on the apical surface of epithelial cells. It has been linked to CRC proliferation, metastasis, and poor prognosis in several studies [2]. During CRC, the MUC1 gene is overexpressed relative to its normal levels, forming a competitive interaction with E-cadherin. Carbohydrate groups' numerous structural alterations associated with the protein backbone are one of the qualitative alterations in MUC1 during CRC. The distinction is referred to as "aberrant glycosylation." Importantly normal glycan structures play a crucial function in cellular connection because the exterior exposed residues establish contact with the nearby additional cells known as proximity of sugar residue [9]. These glycans are also coupled to binding proteins and perform cell-to-cell communication and antigen movement in the cell body. MUC1 is commonly expressed with core 2 O-glycans. While in malignancy, Core 1 O-glycans are present, this is owing to the absence of the Core 2 6-GlcNAc-transferase enzyme. As a result, glycosylation alterations in tumor cells are critical for cell survival. Furthermore, nothing is known about the MUC1 protein's differential glycans, which may be important in their function. Since, advances in computational biology, Molecular Dynamic Simulations allow us to build several core structures of the miniaturized VNTR glycoprotein and assess their protein folding stability in the solution phase. As a result, the current study's goal was to define the MUC1 protein's distinct glycosylation pattern. By utilizing cutting-edge computational techniques valuable information was aided to our understanding of the mechanistic stability of the MUC1^{Cancer} and its MUC1^{Wild} VNTR region protein [10].

Over the last decade, targeted drug treatment has been specific to focused un-glycosylated protein structures, but glycan-protein structure changes (aberrant) with cancer stages, making a potential marker for therapy, so computational approach to find

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the structural changes that glycosylation brings. Hence this offered an idea for future drug-pharmacokinetics investigations. It is questioned that MUC1's variable glycosylation pattern plays a key role in the advancement of epithelial cancer such as colorectal cancer, potentially lowering survival outcomes. To answer this, several bioinformatics tools analysis was carried out that confirm the above mentioned susception. Such that, RMSD of the $MUC1^{Wild}$ was 0.5 Å more stable than $MUC1^{Cancer}$. Stephanie Berger et al. study found that many cancer cells overexpress the family proteins, and their RMSD is greater than the natural type. Such events are closely connected with protein stability [11], demonstrating that the research results are positively related to the study outcomes. When compared to the $MUC1^{Wild}$, the $MUC1^{Cancer}$ exhibits a high RMSf, similar to a study [12]. Also, the gyration radius was compact in the $MUC1^{Wild}$. However, not all disruptive changes appear to have the same effect on backbone dihedral angles and hydrogen bonding. While, all structural glycans mutations resulted in large alterations in backbone dihedrals, whereas hydrogen bonding was significantly hampered. Cancer mutations, on the other hand, led to more confined alterations in the backbone dihedrals. The local hydrogen-bonding network, however, both directly and indirectly, was significantly disrupted.

When compared to other in silico approaches for predicting protein stability, the molecular dynamic technique performed above average. Cancer mutations that result in considerable declines are destabilizing, whereas those that result in small alterations or increases are neutral/stabilizing. This approach has an overall prediction accuracy of 89 percent if earlier research is right [13], [14], [15]. This compares favorably to identifying the effect of glycan substitutions on VNTR stability; nevertheless, future investigations in this domain may benefit from a focused meta dynamics-based approach [16]. Computational modeling and simulations based on molecular dynamics modeling might be an efficient way to understand the biomarker binds that are present in the human body as detection methods. The current study simulated the individual behaviors of MUC1 natural protein and modified aberrant-MUC1 complex using the GROMACS molecular dynamics package. Extensive molecular dynamics simulations were carried out once these distinct behaviors were established. The entire behavior of the $MUC1^{Wild}$ and $MUC1^{Cancer}$ system was viewed from a molecular perspective, which is not always achievable in wet lab studies, and the dynamical simulation findings were visually and statistically evaluated for conformational changes. Based on a biomarker detection technique, the study and display of the natural course of aid developments. The present study used extensive MD simulations to better understand the aberrant-MUC1 complex and MUC1 natural protein folding. Due to a lack of computing resources, the MUC1 VNTR areas were specifically examined in this work. Despite the requirement for a long-term dynamic study, it show that how the whole MUC1 protein-protein binding activity altered. The current MD simulations, analyses, and discussions demonstrate that the MUC1 VNTR region with post-translational modification may be simulated and studied utilizing computational modeling. The findings of the dynamic simulations have a high agreement with the association and disassociation found in the restricted wet lab trials, according to a preliminary comparison. The proven ability provided and addressed in this thesis

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increases the possibility to improve the selection of unknown molecular combinations while also serving as fundamental research for computational modeling and simulations for selection in new biomarkers developments for different epithelial cell cancer like colorectal cancer.

5. CONCLUSIONS

In summary, we used a variety of computational techniques in our in-silico investigations to get a deeper understanding of the glycated core1-4 normally and aberrantly glycated isoforms, as well as the implications of structural stability. According to the findings of this study, glycation differences in protein structure can impact the overall conformational interaction network. Overall, our findings support the experimental findings and give structural insight for drug discovery and biomarker development.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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References

1) Duncan, T.J., et al., The role of MUC1 and MUC3 in the biology and prognosis of colorectal cancer. World journal of surgical oncology, 2007. **5**(1): p. 31.

2) Kasprzak, A., et al., Differential expression of mucin 1 and mucin 2 in colorectal cancer. World journal of gastroenterology, 2018. **24**(36): p. 4164.

3) Saeland, E., et al., Differential glycosylation of MUC1 and CEACAM5 between normal mucosa and tumour tissue of colon cancer patients. International journal of cancer, 2012. **131**(1): p. 117-128.

4) Jiang, Y., et al., Aberrant O-glycosylation contributes to tumorigenesis in human colorectal cancer. Journal of cellular and molecular medicine, 2018. **22**(10): p. 4875-4885.

5) Gendler, S.J., et al., Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. Journal of Biological Chemistry, 1990. **265**(25): p. 15286-15293.

6) Vavasseur, F., et al., O-glycan biosynthesis in human colorectal adenoma cells during progression to cancer. European journal of biochemistry, 1994. **222**(2): p. 415-424.

7) Mihalache, A., et al., Structural characterization of mucin O-glycosylation may provide important information to help prevent colorectal tumor recurrence. Frontiers in oncology, 2015. **5**: p. 217.

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8) Lemkul, J., From proteins to perturbed Hamiltonians: a suite of tutorials for the GROMACS-2018 molecular simulation package [article v1. 0]. Living Journal of Computational Molecular Science, 2018. **1**: p. 5068.

9) Qu, D., et al., The effects of diet and gut microbiota on the regulation of intestinal mucin glycosylation. Carbohydrate Polymers, 2021: p. 117651.

10) Shi, J., K. Aihara, and L. Chen, Dynamics-based data science in biology. National Science Review, 2021. **8**(5): p. nwab029.

11) Berger, S., et al., Computationally designed high specificity inhibitors delineate the roles of BCL2 family proteins in cancer. Elife, 2016. **5**: p. e20352.

12) Wilson, C.J., et al., KEAP1 Cancer Mutants: A Large-Scale Molecular Dynamics Study of Protein Stability. International Journal of Molecular Sciences, 2021. **22**(10): p. 5408.

13) Hast, B.E., et al., Cancer-derived mutations in KEAP1 impair NRF2 degradation but not ubiquitination. Cancer research, 2014. **74**(3): p. 808-817.

14) Cloer, E., et al., p62-dependent phase separation of patient-derived KEAP1 mutations and NRF2. Molecular and cellular Biology, 2018. **38**(22).

15) Khan, H., et al., Molecular effects of cancer-associated somatic mutations on the structural and target recognition properties of Keap1. The Biochemical Journal, 2015. **467**(1): p. 141.

16) Zhang, H., E.L. Kai, and L. Lu, Investigating the stability of dengue virus envelope protein dimer using well-tempered metadynamics simulations. Proteins: Structure, Function, and Bioinformatics, 2020. **88**(5): p. 643-653.