ORIGINAL ARTICLE

Biomolecular and bioinformatics characterization of salivary matrix metalloproteinase-9 as oral cancer potential targeted therapy

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ABSTRACT

Introduction: Oral cancer is the 11th most common cancer worldwide. Previous studies have reported that saliva contains biomarkers for oral cancer. MMP-9, found in saliva, is a marker that indicates and facilitates tumor development. However, comprehensive biomolecular and bioinformatics characterization of salivary MMP-9 remains limited. This study aimed to analyze the biomolecular and bioinformatics characteristics of salivary MMP-9 as an oral cancer biomarker and a potential target for therapy because it plays an important role in tumor development and management. **Methods**: The features of MMP-9 were obtained from the NCBI database (www.ncbi.nlm.nih.gov). Protein analyses were performed using Protparam site, PROTSCALE, TMM application, PEPTIDE CUTTER, NETNGLYC and TARGETTP. The protein model and structure of MMP-9 were evaluated using SWISSMODEL. **Results**: Based on the study, it showed that MMP-9 is located on 20q13.12. It is involved in extracellular matrix breakdown, and this enzyme degrades type IV and V collagens. Based on the instability index (II), the protein was found to be unstable. The hydrophobicity range of the MMP-9 protein was 0–7654. Among the 20 amino acids analyzed, the hydrophobicity of arginine was -4.500, while isoleucine was 4.500. Transmembrane helix analysis indicated the MMP-9 protein isoform is predominantly located outside of the cell. The predicted N-glycosylation site was at the 28th amino acid. The structural model analysis indicated that the protein model was of good quality. **Conclusion**: Biomolecular and bioinformatics characterization of salivary MMP-9 demonstrates strong potential as an oral cancer biomarker. The biomolecular and bioinformatics characteristics identified in this study support the potential utility of MMP-9 as an oral cancer biomarker and provide a foundation for future studies investigating its therapeutic relevance.

KEYWORDS

Biomarker, computational biology, matrix metalloproteinase, mouth neoplasm, saliva

INTRODUCTION

Oral cancer affects a large number of individuals worldwide.[1] The National Cancer Institute reports that in 2024, there were 3.6 new cases and 0.7 deaths from oral cancer per 100,000 individuals. Based on cases from 2017 to 2021 and fatalities from 2018 to 2022, these numbers were adjusted for age.[2] Oral squamous cell carcinoma accounts for 90% of oral cancer cases. This malignancy has an 80–90% survival rate when discovered early.[3] The World Health Organization reports that oral cancer has one of the highest fatality ratios among malignancies, with a with a 5-year mortality rate of 45% despite significant advancements in therapy.[4] Delayed diagnosis is a major contributor to this high mortality rate. Oral cancer is now diagnosed at a later stage due to a lack of national screening programs and a lack of reliable molecular biomarkers for early

detection.[5] Saliva has been demonstrated to be a diagnostic fluid for an expanding variety of systemic diseases and disorders, including oral cancer.[6] Additionally, using saliva as a diagnostic fluid meets the need for accessible, affordable, and noninvasive diagnostic techniques.[7]

Saliva is an oral biological fluid consisting of more than 99% water and less than 1% proteins, electrolytes and other low-molecular weight components.[8] Saliva has been used to detect infections such as HIV and HPV, diagnose oral diseases such as caries and periodontitis, and detect drug abuse, such as the use of addictive substances.[9] The parameters used in examining human saliva include salivary pH, buffer capacity, flow rate, microbes, autoantibodies, and proteins. In oral cancer, specific proteins increase in human saliva.[10]

Yani *et al* reported that the identification of novel molecular markers (DNA, RNA, and protein markers) for the detection and monitoring of oral cancer has been made possible by newly developed molecular biology tools.[11] Wang *et al* reviewed that saliva is a biomarker that could be used to detect the presence of cancer.[12] Changes in salivary biomarker concentrations may facilitate early detection of oral cancer. Saliva can also be used to monitor the effectiveness of oral cancer therapy and other diseases.[9,10]

Information on bioinformatics analysis and biomolecular characterization of saliva is needed to develop methods for the early detection of oral cancer and to discover cancer drugs. Currently, oral cancer treatments have serious side effects, such as anemia, hair loss, fatigue, nausea, and so on. Selective oral cancer treatments have been widely developed.[2] The development of these selective oral cancer drugs requires sufficient knowledge of the protein structures found in saliva and their relationship to oral cancer.[12]

Bastias *et al* reported that matrix metalloproteinase-9 (MMP-9) was one of the most promising salivary biomarkers for oral cancer.[13] Saini *et al* reported that MMP-9 is a reliable prognostic marker because it is highly expressed on immunohistochemistry in advanced stages of oral cancer.[14]

Matrix metalloproteinases (MMPs) play important roles in normal tissue and extracellular matrix remodeling.[15] Their enhanced activity promotes tumor invasion lead to oral cancer progression.[16] Since molecular changes frequently occur before any obvious clinical symptoms or changes become apparent, MMP-9 may be a useful predictor of disease progression, survival time, and recurrence risk. MMP-9 contributes to tumor progression through several key mechanisms.[15,16]

Its mechanisms include extracellular matrix degradation, regulation of gene polymorphisms, promotion of epithelial-mesenchymal transition, and stimulation of adhesion molecule expression.[15] By understanding MMP-9 as an oral cancer biomarker, researchers may facilitate the development of more selective therapies for oral cancer.[16]

Molecular and bioinformatics characterization analysis of proteins is widely used by researchers to obtain a comprehensive understanding of genes related to biological functions, signaling pathways, mutation profiles, and drug sensitivity in oral cancer. Califf reported that bioinformatics information provides valuable insights for further research and the development of oral cancer therapeutic management.[17] Dewi *et al* concluded that selective therapy for the disease could be achieved by understanding the biomolecular and bioinformatics characteristics of target protein.[18]

Although the molecular structure and biological functions of MMP-9 have been extensively characterized, a comprehensive bioinformatics assessment integrating physicochemical properties, post-translational modification prediction, subcellular localization, and structural modeling of salivary MMP-9 in relation to oral cancer remains limited. Therefore, this study aimed to provide a systematic computational characterization of salivary MMP-9 to generate hypotheses for future functional and translational investigations.

The novelty of this study lies in providing comprehensive information on genes, molecular perspectives, disease mechanisms, and biomolecular approaches to oral cancer therapy. This study aims to analyze the biomolecular and bioinformatics characteristics of salivary MMP-9 as an oral cancer biomarker that plays an important role in tumor development and to identify its potential for targeted therapy. The findings of this study are expected to support the development of more selective oral cancer therapies by identifying novel therapeutic strategies that modulate proteins playing important roles in tumor invasion and metastasis.

METHODS

This study was designed as an in silico bioinformatics investigation using publicly available MMP-9 nucleotide and protein sequence data retrieved from the NCBI database. No human participants, clinical samples, or laboratory experiments were involved in this study. The genetic features of matrix metalloproteinase 9, known as MMP-9, were retrieved from the National Center for Biotechnology Information's website www.ncbi.nlm.nih.gov. This website provided information on the genome data viewer, location and region, nucleotide identification, and protein sequence.[19]

Protein analyses were performed utilizing the ProtParam tool for physicochemical evaluation (https://web.expasy.org/protparam/).[20] Parameters assessed included molecular weight, theoretical isoelectric point (pI), amino acid composition, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). All analyses were performed using default settings. Hydrophobicity analysis was performed using ProtScale.

The link ProtScale tool was accessed at https://web.expasy.org/protscale/.[18] Default parameters were applied for all calculations. TMM application was used to predict protein membrane topology. From the prediction, the analysis of target protein location of transmembrane protein were performed. This application was accessed through https://services.healthtech.dtu.dk/services/TMHMM-2.0/.[21] The settings were used to evaluate the probability of transmembrane regions and protein orientation. The protein structure was further evaluated by analyzing the prediction of protein cleavage by protease using PeptideCutter, predicting glycosylation index, and evaluating the role of proteins in metabolic pathways.

PeptideCutter was accessed via https://web.expasy.org/peptide_cutter. NetNGlyc (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/) was used for glycosylation index prediction. Protein stability and immunogenicity of this enzyme were also analyzed. The location of the protein target was assessed using Target (https://services.healthtech.dtu.dk/service.php?TargetP-1.1).[18] Ultimately, the model and structure of matrix metalloproteinase 9 were analyzed using SWISS-MODEL (https://swissmodel.expasy.org/interactive).[18,21] The best model was selected based on GMQE and QMEAN scoring functions provided by the platform.

The analytical workflow began with sequence retrieval, followed by physicochemical characterization, hydrophobicity profiling, topology and localization prediction, post-translational modification analysis, and finally structural modeling. This pipeline was designed to provide a comprehensive computational characterization of the MMP-9 protein relevant to oral cancer research.

RESULTS

Based on the National Library of Medicine, matrix metalloproteinase-9 (MMP-9), also known as gelatinase B (GELB), Collagenase type IV B(CLG4B), is found at 20q13.12. (Fig.1). It indicates that MMP-9 is located on chromosome 20 long hand, region 1, band 3, and has 13 exons. The nucleotide ID was NC_000020.11.

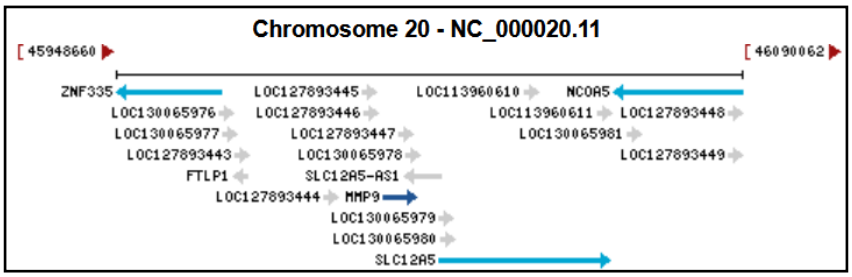

Figure 1. Location of MMP-9.

The RNA-Seq of MMP-9 in *Homo sapiens* was performed from 35 human fetal samples from 6 tissues collected between 10 and 20 weeks of gestation, using Illumina TruSeq Stranded Total RNA. It showed that the tissues analyzed included the stomach, intestine, adrenal, lung, and heart (Fig.2).

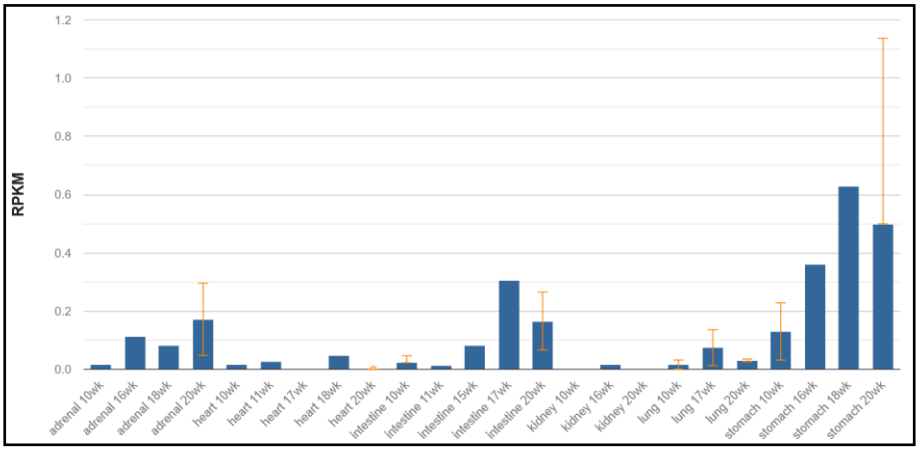

Figure 2. RPKM (Reads per kilo-base of transcript per Million mapped reads) of MMP-9

Salivary MMP-9 protein is involved in extracellular matrix breakdown in both physiological and pathological processes. Extracellular proteinases cleave MMP-9, which is released as inactive pro-proteins. This enzyme degrade type IV and V collagens. The amino acid sequences could be seen on Fig.3.

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AGACACCTCTGCCCTCACCATGAGCCTCTGGCAGCCCCTGGTCCTGGTGCTCCTGGTGCTGGGCTGCTGC
TTTGCTGCCCCCAGACAGCGCCAGTCCACCCTTGTGCTCTTCCCTGGAGACCTGAGAACCAATCTCACCG
ACAGGCAGCTGGCAGAGGTGGGCAAACACCTAGTCTAGAGTTGGGGAGGGCTGTCCGTGAGGGTGTTGAG
TGTCCCAGAGAGGATGCAGGGCCTCAGAGGAGATGCTTTAGGGGTGTGTTGGTGGTGATGGGCGTATCTG
AAGAACAGAGGTGTCCAGGGTTAGGCAGTGGGGGGTCTTGTGGAGGCTTTGAGCAGTGATGGCCAGAAAT
GGGCAATGGGGCTTTCCTAGGTGGGAAATGGGAAATGGTTTGGGGTGGGGGAGGCATTGGAGGGTTCTGG
GGTAAGCATAGGCTGGGAGTGAACAGGGGCAAACCTTATGCAGCTGTGGGGTAGAAATGGGCTAGAGGCA
TCCAGGGGTGAGAAGGAGCTGAGGATGTCTAAGGAGGGGAGATCCCTGGGTGGTCAGAAAGCACTGGTGT
CTGGAAAGCATTTAATGCTTTATTAAATGTTAGTCCCTGCTGGGCATGACGGCTCACACTTGTAATCCCA
GCACTTTGGGAGGCTGAGGTGGTAGGATCGCTGAAGCTCAGGAGTTTGAGCCCAGCCTAGGCAACATAGT
AAGATCCTGTCTCTACAAAAAAATTAAAGAAATAGCCAGGCACAGTGATGTGCACCTGTAGTTCCAGCTA
TGCAGAAGGCTGAGATGGGAGGATCGCTTGAGTCCAGGAGGTCCAGGCTGCAGTGGGCTGATACCGTCTC
TCCGAAAAAGAAAAAGAAAAAAGACTCCCTCCATGAGTGTCTGGAGGGAGTCCTTTGGCCCCAGCTGGGC
AGAGAAAGGGGTCAGAGATCTGGCATGTGTGTGTCCCTTCATCCACAGGAATACCTGTACCGCTATGGTT
ACACTCGGGTGGCAGAGATGCGTGGAGAGTCGAAATCTCTGGGGCCTGCGCTGCTGCTTCTCCAGAAGCA
ACTGTCCCTGCCCGAGACCGGTGAGCTGGATAGCGCCACGCTGAAGGCCATGCGAACCCCACGGTGCGGG
GTCCCAGACCTGGGCAGATTCCAAACCTTTGAGGGCGACCTCAAGTGGCACCACCACAACATCACCTATT
GGTGAGCCGGGGCCGTGGGGGCAGCGGGGTGGGGCGGGGAGGCCAGGTCTGGCTCTTGGGCCAGCGGTGA
ACATGTCCTGTCTTGGACGCGTCCCTGGGTTTCACTATTTAATGTGTGGCCCCTGGGGAGTGTCCCCACC
TCTGAGCCTCTGTTTCTCCTTCAGGGAAATGGCTCTTGCAATCCAAGTCCTCCTGCCAGGGCCATTGTGA
GGGTCTAAGTAGACAAAAAAAAAAAAAAAAAAAAACAGTCTGGAAGCAATTTATAGATGAGAGCGTGGACG
GCAGAGAGCATTGTGTATGTTGAAGTCTCTGCGATATGGGGTGTCCCTGCTGCCCCGCTCCAGCCTTTCA
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Figure 3. Sequences of MMP-9

The formula of MMP-9 was $C_{22547}H_{37442}N_{7654}O_{9423}S_{2175}$. Physicochemical characteristic analysis of MMP-9 showed that this protein has 7654 amino acids,

with a molecular weight of 636251.43 amu and Theoretical pI: 4.58 (Table 1). The amino acid composition is presented in Table 2. The aliphatic index of MMP-9 was 19.96, and the grand average of hydropathicity (GRAVY) was 0,794. The total number of negatively charged residues (Asp + Glu) was 0. The total number of positively charged residues (Arg + Lys) was also 0. The instability index (II) was 53.19. It indicated that the protein was unstable.

Table 1. Atomic composition of *homo sapiens* MMP-9.

Atom	Molecular Weight
Carbon (C)	22547
Hydrogen (H)	37442
Nitrogen (N)	7654
Oxygen (O)	9423
Sulfur (S)	2175

Table 2. Amino acid composition of *homo sapiens* MMP-9.

Amino acid	Composition	Percentage
Ala (A)	1528	20%
Arg (R)	0	0%
Asn (N)	0	0%
Asp (D)	0	0%
Cys (C)	2175	28.4%
Gln (Q)	0	0%
Glu (E)	0	0%
Gly (G)	2183	28.5%
His (H)	0	0%
Ile (I)	0	0%
Leu (L)	0	0%
Lys (K)	0	0%
Met (M)	0	0%
Phe (F)	0	0%
Pro (P)	0	0%
Ser (S)	0	0%
Thr (T)	1768	23.1%
Trp (W)	0	0%
Tyr (Y)	0	0%
Val (V)	0	0%
Pyl (O)	0	0%
Sec (U)	0	0%

Hydrophobicity analysis using the PROTSCALE method showed that the amino acid MMP-9 protein had a hydrophobicity range of 0–7654. (Fig.4). From the 20 amino acid values, it was observed that the hydrophobicity of arginine was -4.500, while isoleucine was 4.500. (Fig.5).

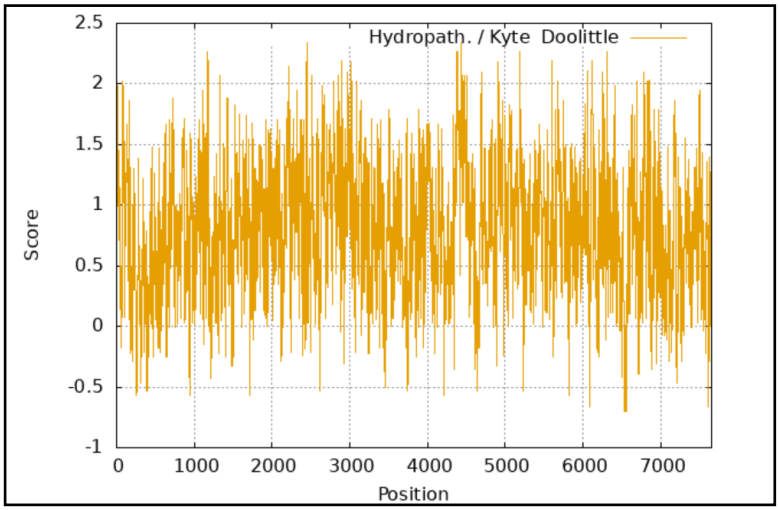

Figure 4. Proscale outputof hydrophobicity

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Ala:  1.800  Arg: -4.500  Asn: -3.500  Asp: -3.500  Cys:  2.500  Gln: -3.500
Glu: -3.500  Gly: -0.400  His: -3.200  Ile:  4.500  Leu:  3.800  Lys: -3.900
Met:  1.900  Phe:  2.800  Pro: -1.600  Ser: -0.800  Thr: -0.700  Trp: -0.900
Tyr: -1.300  Val:  4.200  : -3.500  : -3.500  : -0.490
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Figure 5. Individual values of MMP-9 amino acids

Transmembrane helices protein analysis shown in Figure 6 showed that MMP-9 protein isoform was located outside the cell, inside the cell, and within the transmembrane region. Positions outside the membrane were 0-7654 amino acid positions, inside the cells were 6340-7654 amino acid positions, and transmembrane positions were 3925-7010 amino acid positions.

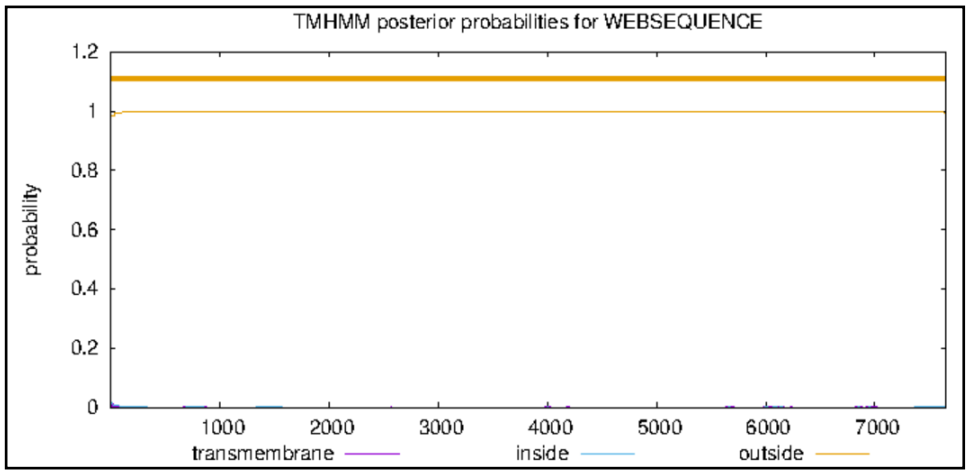

Figure 6. Transmembrane helices protein of MMP-9

The MMP-9 structure was evaluated using the PeptideCutter webserver. The tool was used to predict some that cut MMP-9 protein. Figure 7 showed that the cleavage prediction of MMP-9 included 39 enzymes. The enzymes were Arg-C proteinase, Asp-N endopeptidase, Asp-N endopeptidase + N-terminal Glu, BNPS-Skatole, Caspase-1, Caspase-2, Caspase-3, Caspase-4, Caspase-5, Caspase-6, Caspase-7, Caspase-8, Caspase-9, Caspase-10, Chymotrypsin-high specificity (C-term to [FYW], not before P), Chymotrypsin-low specificity (C-term to [FYWML], not before P), Clostripain, CNBr, Enterokinase, GranzymeB, Factor Xa, Formic acid, Glutamyl endopeptidase, Hydroxylamine, Iodosobenzoic acid, LysC, LysN, NTCB (2-nitro-5-thiocyanobenzoic acid), Pepsin (pH1.3), Pepsin (pH>2), Proline-endopeptidase, Proteinase K, Staphylococcal peptidase I, Tobacco etch virus protease, Thermolysin, Thrombin, Trypsin.

Prediction of the N-glycosylation site was done using NETNGLYC 1.0. N-glycosylation occurred on Asparagines and affected protein stability. The graph shown in Figure 8 described the graph of X-axis of the protein length from the N- to X-terminal. The output typically came from predicted N-glycosylation sites. The 28th sequence of amino acids had a 0.6526 score. While the 959th sequence of amino acids had a 0.3269 score. Using a cutoff score of 0.5, N-glycosylation was predicted to occur at that position. It was therefore determined that the 28th amino acid was a highly predicted site of N-glycosylation, whereas the 959th amino acid was not predicted to be an N-glycosylation site.

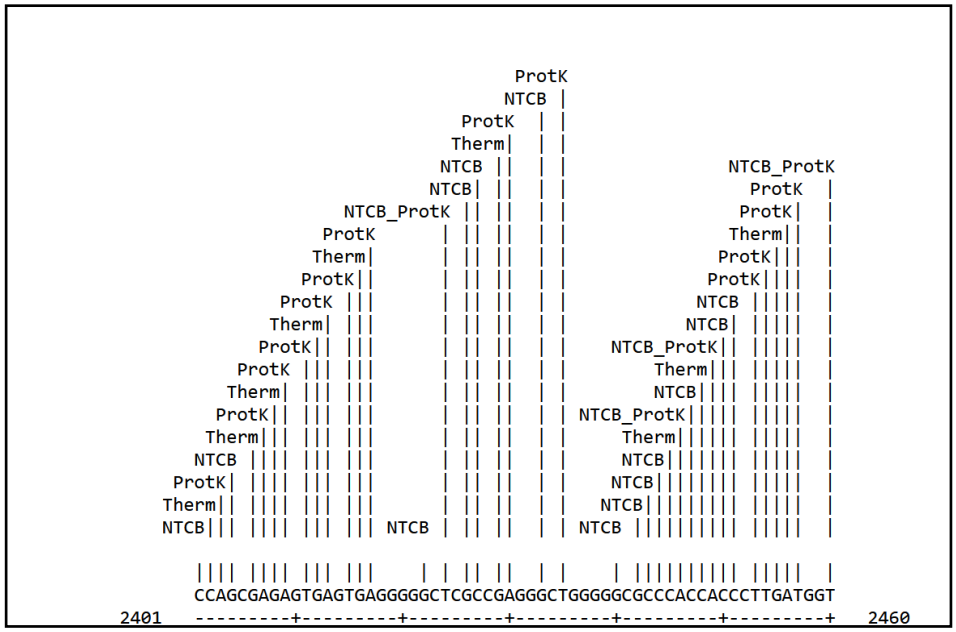

Figure 7. Cleavage sites of enzyme 2401-2460

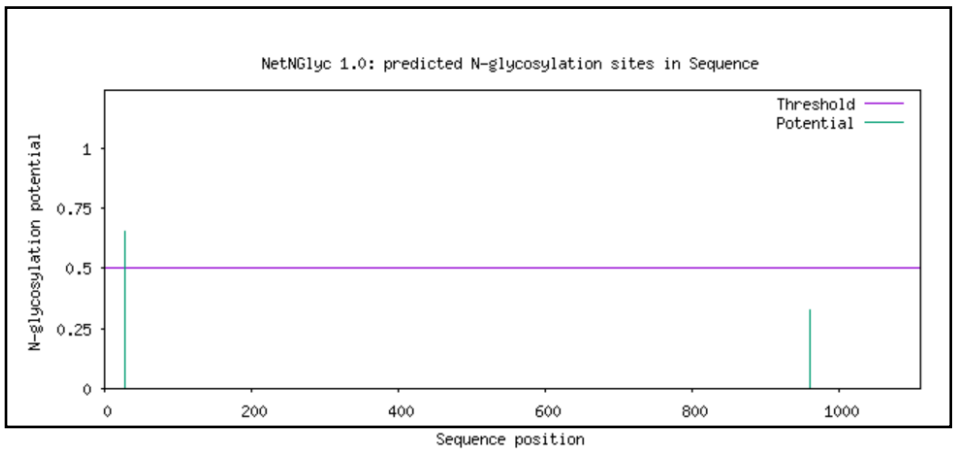

Figure 8. Prediction of N-glycosylation site of MMP-9

The protein structure of salivary MMP-9 was analyzed (Figure 9). Generally, it was found that Ramachandran favored 90.22%. This indicates that the bioinformatics metric showed the total amino acid residues in a protein structure model of MMP were in the most stereo chemically favorable bond angle region. It was also considered a very good or high quality protein structural model. On the other hand, the Ramachandran Outliers was 2.52% in protein structure analysis. It indicates that 2.52% of amino acid residues in the 3D structure model of the protein had dihedral angles outside the sterically permitted region. A value of 2.52% indicates the number of residues that were "wrong" or structurally unreasonable. However, a value of 2.52% was considered acceptable for structural modeling that without high-resolution experimental data.

The integration of the present findings suggests a biologically plausible role of MMP-9 in oral cancer progression. The predicted extracellular localization of MMP-9 is consistent with its established function in extracellular matrix remodeling. The ability of MMP-9 to degrade type IV and V collagens may facilitate basement membrane disruption, which is a critical step in tumor invasion and metastasis. Furthermore, the identified N-glycosylation site may contribute to protein folding, secretion, and enzymatic stability, while the structural modeling results support the preservation of functional domains required for proteolytic

activity. Collectively, these observations provide a comprehensive molecular profile of MMP-9 that may serve as a foundation for future functional studies investigating its role in oral cancer pathogenesis.

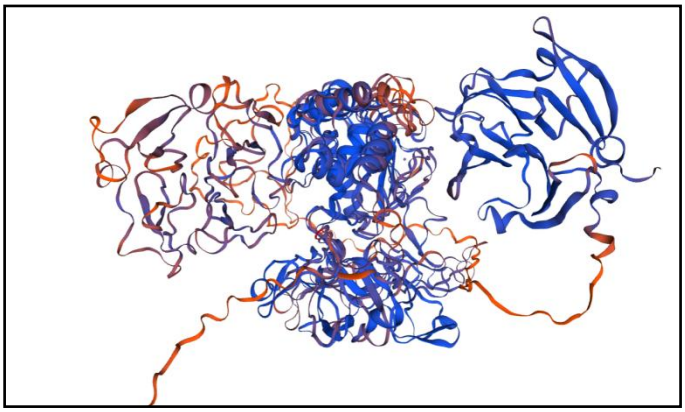

Figure 9. Model structure of MMP-9

DISCUSSION

This study provided information on the location and structure of the salivary MMP-9 gene, which is located on chromosome 20q13 (MP-9 is located on chromosome 20 long hand, region 1, band 3, and has 13 exons.). By knowing the location of the MMP-9 gene, researchers can track the gene in DNA research, distinguish it from other similar genes, assist in research related to diseases associated with the gene, and observe the presence of mutations or changes in genes in that area.[22] In addition, information obtained from the analysis regarding how genes are made, arranged, and analyzed for errors in the protein formation process is also needed to develop selective cancer therapies and appropriate dosing strategies.[23] MMP-9 is a protein that plays a crucial role in the invasion and metastasis of oral cancer. Knowledge of the specific chromosomal location of MMP-9 can aid in the design of specific inhibitors that target the DNA repair pathway in cancer therapy. Knowledge of genetic location information, combined with biotechnology for cancer treatment, is crucial for the management of oral cancer.[24] Sheikh-Hosseini *et al*. stated that understanding genes as one of the gene therapy strategies in cancer patients contributes to cancer management without damaging or affecting the normal activity of surrounding healthy tissue, such as in the management of breast cancer.[23] An example of other research developments related to knowledge of biotechnology and molecular is the therapeutic management of Thalassemia, which is still under development.[25]

The results of the RNA sequencing analysis in Figure 2 showed that MMP-9 in *Homo sapiens* was expressed in the stomach, intestine, adrenal gland, lung, and heart. MMP-9 is related to glands, one of which is the salivary gland. MMP-9 in oral cancer is used to detect and monitor the disease non-invasively. In oral cancer, salivary MMP-9 levels increase.[26] MMP-9 promotes the ability of cancer cells to penetrate the surrounding tissue and invade it. The higher the salivary MMP-9 level, the higher the level of malignancy and the worse the prognosis. MMP is produced in large quantities in the gastrointestinal tract, where the oral cavity is the gateway for food and drugs to enter the digestive system. MMP-9 in saliva is used as a faster and simpler method for detecting oral cancer, reducing the need for invasive procedures, assisting doctors monitor the patient's condition regularly.[27] Illumina TruSeq Stranded Total RNA is a laboratory method for analyzing RNA comprehensively (total RNA sequencing) using next-generation sequencing (NGS) technology from Illumina.[28] This method is used to identify all types of RNA in a sample (mRNA, rRNA, and non-coding RNA), determine which genes are active (gene expression), identify gene changes in certain conditions,

such as cancer, and determine the direction (strand) of RNA originating from which DNA.[29] This is very important for more accurate gene analysis, especially for overlapping genes. This examination aims to determine gene expression profiles, detect genes such as MMP-9, identify changes in cancer (including oral cancer), discover new biomarkers, and compare healthy and diseased conditions. Palomares *et al* stated that total RNA sequence analysis confirmed that MMP-9 plays an important role in digestive cancer.[30] Therefore, the results of the Illumina TruSeq Stranded Total RNA analysis indicated that MMP-9 had potential in the detection of oral cancer.[29,30]

Reading the amino acid sequence is important because the sequence is the "code" that determines the shape and function of proteins in the body.[31] The usefulness of displaying the amino acid sequence is that it allows researchers to study the protein function of salivary MMP-9, determine protein function, detect changes or mutations, develop drugs, compare proteins between species, understand protein structure, and identify proteins.[32] Figure 3 showed the amino acid sequence of MMP-9. The results of the amino acid sequence analysis of MMP-9 showed that the amino acid sequence of MMP-9 followed the general principle of proteins that had unique characteristics. MMP-9 is a protease enzyme that depends on metal ions (Zn^{2+}).[33] Modification of this metal ion is predicted to help in finding therapeutic management strategies for oral cancer.[32,33]

This protein consisted of 7,654 amino acids, indicating a very long protein. The more amino acids a protein contains, the more complex its structure is likely to be. The relationship between cancer markers and complex proteins is quite close, as many cancer markers are actually proteins (or parts of proteins) that have complex structures and change during cancer.[34] This is because proteins interact in complex networks to form signaling pathways that support tumor growth, angiogenesis, and metastasis.

The molecular weight of MMP-9 was 636,251.43 amu. This indicates that MMP-9 is a very large protein. This weight is derived from the total amino acids that constitute the protein. Protein molecular weight has an important relationship with therapy, especially in the development of protein-based drugs or cancer therapy targets, but it is not a direct determinant of therapeutic efficacy.[35] The size of a protein affects the action, distribution, and administration of a drug or protein.[36] Based on the molecular weight of the MMP-9 protein, it predicted that the types of anti-cancer drugs that might be developed are monoclonal antibodies, therapeutic enzymes, and enzyme inhibitors. Skarlis *et al* found that MMP-9 has complex protein structure. Proteins with large molecular weights are more difficult to penetrate cell membranes, so the drugs developed are preferably administered by injection. Therapeutic targets such as MMP-9 (an enzyme) can be inhibited with small inhibitors, large antibodies, and RNA therapy.[36,37]

Table 1 explained that this protein was acidic, as its isoelectric point was 4.58. This number does not directly indicate cancer, but it may be indirectly related to the cancer process. Cancer cells produce a lot of acid (due to high metabolism), making the environment around the tumor more acidic.[38,39] Therefore, the MMP-9 protein may remain stable or active in this acidic cancer environment, supporting cancer cell activity, tissue invasion, and metastasis. Tafech and Stéphanou reported that an acidic environment influences cancer pathogenesis and therapy.[38] A management strategy that can be developed is to study more deeply how the protein interacts with this environment.

The aliphatic index of salivary MMP-9 was 19.96, and the grand average of hydropathicity (GRAVY) was 0.794. The Aliphatic Index (AI) indicates how stable a protein is at different temperature based on the number of aliphatic amino acids (such as alanine, valine, leucine, and isoleucine).[40] An AI value of 19.96 is considered low, indicating that the protein is less stable under temperature variation due to its relatively low aliphatic amino acid content.[41] This suggests that the MMP-9 protein is more easily structurally altered and is more susceptible to

environmental or drug changes. This could also aid in the design of drugs targeting MMP-9, potentially reducing cancer expression.

The GRAVY value of the salivary MMP-9 protein indicated that MMP-9 was hydrophobic, tended to be insoluble in water, and preferred to interact with non-aqueous environments such as lipids or membranes.[42] This information influences how the protein works and how researchers can target it as a therapy or biomarker. Therefore, this protein tends to be located in the cell membrane or has parts that are "embedded" in the cell structure. Therefore, it is necessary to choose an appropriate strategy for delivering the drug to bind to the protein and reach the target site.[43,44] The drug used must also have the ability to bind to the hydrophobic parts or prevent the interaction of these proteins. However, this protein was composed of hydrophilic and hydrophobic parts. This was indicated by the hydrophobicity of arginine being -4,500 and the hydrophobicity of isoleucine being 4,500. This indicated that this protein can interact with body fluids and maintain cell stability.

The MMP-9 protein can be found in the extracellular space, intracellularly, and associated with the cell membrane, reflecting its complex role in biological functions and diseases.[45] However, the protein is predominantly localized in the extracellular space. This indicates that MMP-9 functions intracellularly, at the cell membrane, and predominantly within the extracellular matrix, where it degrades surrounding tissues.[46] This indicates that MMP plays an important role in cell communication, tissue remodeling, and disease processes such as cancer. MMP-9 has the ability to facilitate cancer cell invasion and metastasis.[45,46] These findings suggest that MMP-9 may represent a target for cancer therapy because it is predominantly located outside the cell.

Protein cutter analysis revealed that there were 39 enzymes or proteases that had cleavage sites on MMP-9. These findings indicate that 39 enzymes can cleave or process the MMP-9 protein, highlighting the complexity of its biological regulation.[47] This suggests that MMP-9 had multiple regulatory points, its activity might be activated or modified by various other enzymes.[48] This finding also indicated that MMP-9 was a very complex protein in biological regulation.

Cutting the protein chain into smaller pieces may convert pro-MMP-9 (inactive) form into active MMP-9.[48] Activation of MMP-9 through cleavage increases tissue degradation, promotes cancer cell invasion, and accelerates metastasis.[47,48] Jia *et al* demonstrated that protein cleavage plays an important role in tumor expansion.[48] Therefore, effective therapeutic strategies for cancer patients should be developed based on this information. Protein cleavage by enzymes or proteases is highly relevant in treatment because this process often determines whether a protein is active, inactive, or functionally altered.[46,48] Cleavage acts as a switch for protein activity, meaning that if cleavage activates harmful proteins, the process should be inhibited, whereas if cleavage activates protective proteins, it should be promoted.[49]

In oral cancer, drug development strategies may focus on protease inhibitors, so that MMP-9 becomes inactive, thereby reducing cancer invasion.[50] In addition, this knowledge may also be applied to develop antibody-based therapies targeting the MMP-9 protein.[51]

N-glycosylation prediction is a computational approach to determine whether and where a sugar group (glycan) will be added to a protein at a specific amino acid (typically asparagine).[52] N-glycosylation prediction is used to predict the location and role of sugar additions in proteins, which is important for understanding protein function, detecting cancer and other diseases, and developing biological therapies and drugs. N-glycosylation is a protein modification process in which sugars (glycans) are added to proteins.[53] This occurs at specific motifs (usually Asn-X-Ser/Thr), and is crucial for protein function and stability. Frequently altered glycosylation patterns can be cancer biomarkers.[52,53] For example, changes in N-glycosylation have been associated with cancer progression and malignancy. This knowledge identifies potential drug and

antibody targets associated with glycosylation pathways, which is crucial for the development of cancer therapies.[53]

In the production of protein-targeted drugs, glycosylation prediction helps improve drug stability, reduce immune reactions, and extend the duration of drug action.[54] Thus, MMP-9 is predicted to undergo modifications such as glycosylation, which may affect enzyme activity, play a role in cancer invasion, and serve as a target or biomarker.[53,54]

The amino acid at position 28 of MMP-9 had a score of 0.6526. This score indicates a high probability of MMP-9 undergoing modification (e.g., glycosylation) or possessing certain features. Positions with high scores may influence protein activity and represent potential targets for research or therapy. On the other hand, the amino acid at position 959 had a score of 0.3269, indicating that glycosylation modification at that position was relatively low.[52,53]

Analysis of the structural model showed that MMP-9 is a three-dimensional protein composed of several important domains.[32] This structure determines its primary function as a tissue matrix-degrading enzyme. The structural model of MMP-9 consists of a pro-domain, a catalytic domain (Zn^{2+}-based), fibronectin-like repeats, and a hemopexin domain, which together form a complex 3D structure to carry out its enzymatic function.[55] Structurally, Figure 9 shows that MMP-9 has a globular shape with several folded regions. Its domains comprise a head (catalytic domain), a tail (hemopexin domain), and a flexible linker between these domains. This structural organization provides flexibility and the ability to interact with multiple molecular targets.

MMP-9 is not a single, simple protein, but consists of several parts (domains) arranged in a complex 3D structure, including the initial domain or pro-domain, the catalytic domain, the fibronectin-like repeats, and the hemopexin-like domain. The pro-domain is located at the N-terminal end.[56] This region functions to maintain the inactive state of the enzyme (pro-MMP-9), which contains a cysteine switch mechanism.[57] This mechanism specifically regulates enzymes such as MMP-9 (Matrix Metalloproteinase-9) to remain inactive until they are truly needed. The catalytic domain (head) is the core part that performs the enzyme's function and contains Zn^{2+} (zinc) ions, which are crucial for protein cleavage activity.[32,57] This cleavage functions to convert the inactive form of the protein into the active form in MMP-9.[57,58] Furthermore, this cleavage also plays a role in altering the shape (structure) of the protein and altering how the protein functions, as observed during oral cancer progression.

Fibronectin-like repeats are a characteristic domain of MMP-9, as not all MMPs possess this domain.[59] This domain plays a role in binding substrates such as collagen and interacting with extracellular tissue in various biological activities.[60] The hemopexin-like domain, also known as the tail domain, is located at the C-terminal region. This domain plays a role in target recognition and interactions with other proteins.[61]

By understanding this 3D structure, it becomes evident that MMP-9 is a potential biomarker in oral cancer and may serve as an important target in cancer therapy.[60,61] This structure explains the mechanism by which MMPs cleave the matrix, causing cancer invasion, stimulating metastasis, and facilitating tissue interaction.

The results of the biomolecular and bioinformatics characterization of salivary matrix metalloproteinase-9 analysis indicate that MMP-9 contained in saliva has considerable potential as a biomarker for assessing the degree of malignancy in oral cancer. Shaukat *et al* stated that salivary MMP-9 is a useful biomarker for detecting early-stage (stages I and II) oral cancer.[61]

Starska-Kowarska reported that saliva-based analysis of MMP proteins was a modern and promising method for the early diagnosis and improvement of treatment outcomes in patients with OSCC and oral potentially malignant disorders (OPMDs), with high diagnostic, therapeutic, and prognostic potential.[62] Gupta *et al* found that bioinformatics and biomolecular knowledge could result in better

margin assessment, help resolve issues related to the high recurrence rate of oral cancer, and eventually improve patient outcomes.[63,64] Bioinformatics feature analysis supports the analysis of complex transcriptomic data to investigate common and distinctive molecular characteristics, identify possible biomarkers, and explore therapeutic targets. Du et al. stated that biomolecular and bioinformatics analysis was very helpful in identifying biomolecular biomarkers for diseases such as oral cancer, due to limitations associated with conducting clinical trials because of ethical and other issues.[65]

Understanding bioinformatics and biomolecular biology plays a crucial role in identifying protein-based therapeutic targets for diseases such as in oral cancer. Bioinformatics analysis provides insights into large-scale biological data, such as DNA, RNA, and protein sequences.[11] This greatly assists researchers in identifying which genes or proteins undergo significant changes in specific disease states. Through techniques such as gene expression analysis, sequencing, and protein structure prediction, candidate therapeutic targets can be screened more quickly and accurately.[66] Zhang *et al* reviewed that bioinformatics tools and technologies in drug discovery include biological databases, molecular docking tools, and omics techniques for diagnosis and treatment.[67] This knowledge is very important to understand the protein function and drug design, identifying novel drug targets for cancer treatment.[22]

On the other hand, biomolecular approaches elucidate the mechanisms of protein action within cells.[68] Information about a protein's 3D structure, location within the cell, physicochemical properties (such as hydrophobicity and pI), and activation mechanisms such as protein cleavage or cysteine switches, provides insight into how proteins interact with other molecules and contribute to disease progression.[46,49,59] For example, proteins such as MMP-9 are known to play a role in the degradation of the extracellular matrix, which facilitates cancer cell invasion and metastasis.[55] Moreno-Vargas and Prada-Gracia stated that bioinformatics analysis and biomolecular characterization provide a strong basis for developing therapeutic delivery methods. This approach has the potential to bring about a paradigm shift in targeted therapeutic delivery systems.[69]

The integration of bioinformatics and biomolecular engineering enables researchers not only to identify target proteins but also to understand their vulnerable sites that can be exploited therapeutically.[64,69,70] For example, by predicting active sites, glycosylation sites, or transmembrane regions, specific drugs can be designed to inhibit the function of these proteins. Furthermore, this approach supports the development of biomarkers for early diagnosis and therapy monitoring.[67] Nelakurthi *et al* reported that RNA/DNA microarrays and bioinformatics approaches in proteomics research for large-scale gene expression studies have advanced biotechnology, thus enhancing the relevance of biomolecular tools. Molecular profiling of oral cancer is very important for both oral cancer research and its therapy management.[69] Another study of Matsuoka and Yashiro stated that the integrated implementation of bioinformatics and biomolecular analysis, together with and multi-omics datasets supported by computational modelling, significantly improves the identification of predictive and prognostic biomarkers and may play an important role in the development of targeted therapies.[71] Thus, the combination of bioinformatics and biomolecular engineering provides a comprehensive approach to identifying protein therapeutic targets, from candidate identification to the design of more effective, specific, and molecular mechanism-based treatment strategies.

Rather than serving as isolated observations, the identified molecular features collectively provide insights into the biological behavior of MMP-9. The predicted extracellular localization supports its established role in extracellular matrix degradation, while the presence of glycosylation sites and conserved structural domains may contribute to protein stability, secretion, and enzymatic activity. When interpreted together, these findings suggest that MMP-9 possesses molecular characteristics that are consistent with mechanisms involved in tumor

invasion and metastasis. Although further experimental validation is required, the present bioinformatics analysis provides a comprehensive molecular framework that may guide future studies investigating the role of MMP-9 in oral cancer pathogenesis.

This study has several limitations. First, bioinformatics analyses are computer-based predictions rather than direct experimental results. Therefore, these results do not always reflect real-world conditions or biological processes and are highly dependent on the quality of the underlying data and algorithms, meaning that they cannot replace direct biological experiments. Second, bioinformatics research relies heavily on data quality. If the data used are incomplete, biased, or incorrect, the analysis results will also be inaccurate. Third, bioinformatics research is limited in its ability to describe the complex interactions between proteins, the influence of the cellular environment, and the temporal dynamics of biological systems, which may lead to variability in protein prediction results. Furthermore, each software tool uses different calculation methods and assumptions, which may produce different results depending on the software used. Finally, bioinformatics research can identify correlations between proteins and diseases, such as between MMP-9 protein and oral cancer, but cannot always establish causality (direct cause). Therefore, to validate the present findings, laboratory experiments, including in vitro, in vivo, and clinical studies, are necessary in future research.

CONCLUSION

Biomolecular and bioinformatics characterization of salivary MMP-9 has strong potential as an oral cancer biomarker. The unique features of the MMP-9 protein provide insights into potential therapeutic strategies of oral cancer. Bioinformatics research on MMP proteins, particularly MMP-9, in saliva has important implications for the diagnosis and treatment of oral cancer. Through a bioinformatics approach, sequence, structure, and gene expression analyses can be performed to identify the role of MMP-9 as a non-invasive biomarker. Saliva is an ideal medium because it is readily available and reflects local conditions in the oral cavity. Therefore, increased MMP-9 expression can be used as an indicator of pathological processes such as cancer.

Another implication is the development of potential targeted therapies. By understanding the structure and properties of the MMP-9 protein, such as hydrophobicity, transmembrane location, and activation mechanism (the cysteine switch), researchers can design specific inhibitors that target the active site of the protein. In addition, bioinformatics can help screen potential drug candidates before laboratory testing, making the process more efficient in terms of time and cost. Because this bioinformatics research is a predictive study, future studies should validate these findings through laboratory experiments and clinical trials.

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