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Salivary Peptidomic Analysis -The Extension of Proteomics

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Abstract

Nowadays, proteomic-related studies have developed rapidly and improvement has been made in the scientific and technological fields. Besides the proteome, the peptidome with low molecular weight has apparently attracted increasing attention in recent years too. The peptidome performs a large array of vital biological functions to contribute to homeostasis in the certain tissues. However, due to the interference by high-abundance proteins in complex biological body fluids such as saliva and serum, selective enrichment of peptides with low molecular weight is the first and most important step before analyzing the peptidome. This review mainly illustrates the general concept of the peptidome, specific methods of saliva collection, advanced peptidomic technologies and the recently developed peptidomic-related reports, as well as some limitations of the application in salivary peptidome. The future prospects of the peptidome are also involved.

Over the last few decades, mass spectrometry (MS)-based proteomics has emerged and expanded its vital role in the research areas of science and technology [1]. Interest in the salivary proteome has accordingly increased [2], and saliva-based proteomics research has become a recent focus due to the advantages of saliva analysis [3]. It offers an alternative to blood, plasma, serum, or urine for diagnostic or detective purposes [4] due to its noninvasiveness, convenience, and low cost [5]. Interest in saliva as a diagnostic fluid has attracted many researchers in recent decades, leading to the development of diagnostic tools based on saliva analysis [6]. In addition, significant improvement in MS instrumentation has enhanced the sensitivity, efficiency, and accuracy of biological analysis [7]. These advances have provided a powerful approach to investigation and identification of differential expression patterns in protein profiles of different samples under various conditions, particularly between disease and normal controls [8]. In other words, proteomic strategies are used increasingly to discover novel and specific biomarkers of physiological and pathological conditions, facilitating the early detection, diagnosis, and prognosis of related diseases [9]. Besides, saliva has recently become popular for use in some othernon-peptidomic, classical clinical tests. For example, Grynderup MB et al. [10] promoted that there was some relation between the salivary cortisol concentration and the risk of depression. The study indicated the low mean and a small difference between morning and evening cortisol concentration may be risk factors of depression.

Salivary Peptidome

In addition to the "proteome" or the descriptive term"proteomic," another term is often seen: the "peptidome."These are two distinct concepts that should be clearly distinguished. Saliva is mainly derived from the secretions of the major salivary glands, numerous minor salivary glands, and constituents of gingival crevicular fluid, food debris, oral microflora, and desquamated epithelial cells [11]. Therefore, saliva contains various informative constituents including proteins, peptides, small molecules, and other compounds [12].The proteomics implicate the analysis of all the expressed proteins in a precise timeframe, and it has been an important developing research field during the past two decades [13]. However, as the conventional gel-based proteomics presented a lot of limitations, the peptidomics strategy was introduced in 2001 with the aim to resolve molecules below 15 kDa [14]. More than 2000 peptides consist the salivary peptidome which performs many biological functions to contribute to homeostasis in the oral cavity [14]. The peptidome represents the whole peptides in a cell, tissue or organism, including the body fluid of course, under a certain physiological or pathological condition [15]. Though there is some overlap between the proteome and the peptidome, the latter one comprises approximately 40–50% of the total secreted proteins in the whole saliva, originating directly from gene expression and post-translational modification in addition to the breakdown products of proteins and peptides generated from the proteolysis of proteins of different sources [16]. That means although the peptidome in whole saliva is derived mainly from proteolysis, it is more than simply the sum of the protein degradation products. Thus, the association between changes in saliva peptide composition and health can be elucidated [17]. For example, the composition of saliva could reflect the level of drugs or hormones in the blood because there is sufficient exchange between the saliva and blood content [18].

On the other hand, the informative constituents in blood or urine reflect more about the systematic response of diseases, thus saliva is a better subject for detecting oral diseases than other kinds of body fluids. For an example, it is difficult in some sense to detect the difference in the blood and urine samples between the patients with oral diseases and the healthy ones, as there exists dilution effect in the blood and urine. Distinctly, the salivary peptides originate partly from the desquamated epithelial cells and their degradation products. What's more, the secretion and content of the saliva could change when the salivary

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glands are damaged. Therefore, the differences of salivary peptides between the patients and healthy individuals are more significant.

The salivary peptidome also include the secretion of oral microflora. There have been more than 600kinds of identified microorganisms at present, besides the types difficult to culture and identify. The microflora play an important role in maintaining the harmony of oral microecology and in directly reflecting the defense against outside and condition of the body organism [19,20]. Lots of studies suggested that the oral microorganisms played an vital role in the onset of dental caries and periodontitis. Moreover, some research pointed that the oral microflora were different between the OSCC patients and the healthy [21], and some other reports indicated the differences in saliva microflora between IBD children and the controls [22]. Thus the differences of oral microflora leaded to certain specific secretion and then were reflected in the peptidomic spectrums. Obviously, this difference is specific in saliva not in the blood and urine.

Certainly, the salivary peptidome is somehow complicated because of the diversity of salivary constituents. However, the sensitivity and resolution of analysis are improving as the mass spectrum techniques have been developing well. Thus the advanced MS-based strategies nowadays are capable of analyzing the complicated samples or constituents. Above all, the salivary peptidome could play a more and more important role in the molecular diagnosis in the future.

One of the core procedures for salivary peptidomic analysis is salivary sample collection [23]. The following factors should be given special attention: the collection time, saliva pattern, status of the individual, and sample pretreatment. The saliva secretion volume, flow rate, and constituents could be influenced by intrinsic and extrinsic factors, including gender, age, inter-individual variation, and so on. Besides, the informative molecules could fluctuate according to the status of the individuals, including the jaw movement or tooth brushing. Hence, efforts should be made to standardize sample collection and pretreatment techniques to enhance the reliability and reproducibility of salivary peptidome profiling. Thus, a comprehensive protocol should be made and the researchers should tell the subjects to agree and obey before the saliva samples were collected. Which is better to exclude the influence of the body movement and chewing action during the daytime. All individuals were asked to rest for 15 min before saliva collection between 8:00 am to 10 am, and not to eat or drink after dinner the previous evening or to brush their teeth on the collection day morning. The subjects sat upright in a quiet room and were required to put the tip of their tongue against the sublingual caruncle without straining. Thus, the saliva, which was received in a paper cup for the first 5 min, could run from the mouth, and we collected 6 mL of the spontaneous whole saliva flow in a 50-mL centrifuge tube. We suggest collecting the unstimulated whole saliva not the secretion from the single gland, as the latter one is more difficult to get and couldn't represent well the certain condition in the whole oral cavity since it only reflects the condition of single salivary gland. During the collection procedure, patients were asked not to speak. As the saliva contains various different active enzymes, it needs to suppress the activities that could produce peptide fragments as artifacts. Immediately after collection, the unstimulated whole saliva samples were kept on ice and then centrifuged at 9000 × g for 7 min at 4°C to remove insoluble materials, cells, and debris. Ethylene diamine tetraacetic acid and phenylmethyl sulfonylfluoride should be added to the sample supernatants to inhibit protease activity [24,25].

Mass Spectrometry Techniques

Saliva is a typical yet complex body fluid containing many abundant proteins, such as amylase and mucin [26], which mask the existence of low-molecular-weight proteins. This could interfere with the detection and identification of potential biomarkers of relatively low abundance [27]. Thus, advanced techniques are required to enrich the less abundant proteins/peptides. Proteomic studies of body fluids have used both conventional gel electrophoresis and gel-free approaches [28]. Initial studies applied mainly two-dimensional gel electrophoresis (2-DE) [29], in which proteins are separated in terms of their molecular weight for protein separation and quantitation, followed by mass spectrometry (MS) for identification [30]. However, this strategy possesses several significant disadvantages, including complex procedures, the large sample size required, and that the informative low-molecular-weight peptides are likely outside the detection range of 2-DE [28]. Thus, peptidomic studies rely generally on the gel-free approach to overcome the above limitations. Gel-free MS includes surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS [31,32], matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [33], liquid chromatography triple quadrupole mass spectrometry (LC–MS/MS) [34], CE-MS [35] and MS approach using electrospray ionization [36] and so on. The SELDI-TOF MS approach has been reported predominantly for profiling of high-molecular-weight proteins (10–20 kDa) [37]. In contrast, MALDI-TOF MS approaches, which do not use on-target peptide/protein purification, have been applied to identification of low-mass proteins and peptides (1–15 kDa). Besides, LC–MS/MS not only performs high throughput, it also requires minimal sample preparation and a small sample volume. All these features make it another attractive method to use in a clinical setting. Besides, MS approach using electrospray ionization (ESI) is preferred for molecules with high polarity, This ionization techniques are commonly used for the analysis with LC–MS/MS. Moreover, CE-MS has been used popularly in the last 5 years. It has been developed for large molecules, while analyses of smaller molecules are moving on the way. The ESI has its own specific advantages and disadvantages. The biological molecules produced from ESI always carry more than 10 electric charges, thus it has a relative wide detective range. Besides, ESI has a high sensitivity and could be used together with LC-MS/MS and is suitable for peptide analysis. However, the salt-tolerance capability is somehow low, and it is difficult to clean when polluted.

As stated above, advanced MS techniques are needed to obtain reliable information. MALDI-TOF MS is one such technique. It can detect low-molecular-weight peptides with adequate resolution and sensitivity, making it a useful tool for peptide pattern profiling [7]. In contrast, for pretreatment of body fluids before MS analysis, beads with a library of peptides, mesoporous silica particles [38], or beads with a magnetic core [39] such as weak cation-exchanger magnetic beads are used for selective enrichment of low-molecular-weight peptides. Magnetic beads constructed on nanomaterial have been considered to be a promising material among the various types of separation beads. The combination of magnetic beads and MALDI-TOF MS enables efficient and sensitive detection of peptides specific for certain conditions. This explains thesuccess of the magnetic bead-based approach in identifying serum peptide profiles and in our pilot studies [24,25].

In other words, a seemingly well-defined procedure has come into being. It comprises weak cation-exchanger magnetic beads for sample separation, MALDI-TOF MS for peptide profile detection, and a database for construction of condition-specific peptidome models

that may represent powerful tools for the early detection, diagnosis, or determination of the prognosis of various conditions [40].

Related Studies

Many recent studies have characterized changes in saliva composition associated with various conditions and disorders, including systemic diseases such as breast cancer, rheumatoid arthritis, and Sjögren's syndrome, as well as oral pathologies such as dental caries, periodontitis, cleft palate, and oral cancer. These studies made use of specific markers, such as inflammatory factors, that play a vital role in certain disease (Table 1).

Peptidomics has for many decades been used for analysis of blood markers [54]. It has also been used to detect and identify specific biomarkers, including of gastric cancer [55], pancreatic cancer [56], renal cell carcinoma [57], colorectal cancer [58], and excessive alcohol consumption [59]. However, serum-based peptidomics focuses on systemic diseases. Besides the saliva, serum and urine, development have also been made in the diagnosis-oriented studies of other body fluids such as cerebrospinal fluid (CSF), tear fluid and so on[60-64]. For example, CSF is a kind of valuable body fluid that can serve in diagnosis, prognosis or monitoring of specific conditions. Moreover, apparent advances in mass spectrometry (MS) now have allowed the detection of large array of peptides using CSF [60, 65]. Thus many studies have identified some biomarkers of related diseases, such as the Alzheimer's disease [66].

The table shows that certain proteins or peptides were detected and identified as biomarkers indicating different diseases. Biomarkers, which represent informative signals, can be inflammatory factors, hormones, cytokines, growth factors, or other factors related to a specific status [67]. Their specificity and sensitivity are important for early detection, diagnosis, prediction, or determination of prognosis [68]. A meaningful biomarker should be measurable in a body fluid such as serum, urine, or saliva [69]. It can be seen that some not all studies validate the primary results from MS analysis, using western blotting, ELISA or immunoblotting and so on. Moreover, some studies are moving toward a potential clinical use and validated the primary results in new patients. For example, the breast cancer-related study verified the biomarker candidates by protein immunoblotting using the pre-validation cohort in 30 breast cancer patients versus 63 controls, yielding an accuracy of 92% (83% sensitive, 97% specific) on the preclinical validation sample set. On the other hand, a cluster of possible markers generally might represent a protein profile specific for a condition. However, the above-mentioned biomarkers, although they indicate the same disease, were somehow distinct from one another. The reasons for this may be divided into the following two aspects. First, significant diversity among samples was evident, as seen in the different

Disease	Sample (patients & control)	Pattern	Origin	Method	Biomarkers	Validation
HNSCC [41]	8&8	USS	WS	2D-DIGE	Beta fibrin S100 calcium-binding protein transferrin cofilin-1	Western Blotting
				LC/MS		
Cleft lip and palate [42]	31&20	USS	WS	MALDI-TOF/MS	Arpc3 Dermokine	
Gingivitis [43]	10&10	USS	WS	LC/MS MALDI-TOF/MS	Albumin Hemoglobin Immunoglobulin Keratins	
OSCC [44]	4&4	USS	WS		Myosin Actin	Western Blotting
Chronic periodontitis [45]	10&10	USS	WS	2D-DIGE MALDI-TOF/MS nLC-MS/MS	Immunoglobulin Hemoglobin Albumin Cystatin	
Dental caries [46]	16&16	USS	WS	2DE MALDI-TOF/MS	Amylase IgA Lactoferrin	
Sjögren's syndrome [47]	8&8	USS	WS	2DE ESI-MS/MS	Keratin Albumin 2 actin isoforms	
OSCC [48]	35&51	USS	WS		IL-1B IL-8 M2BP	ELISA
Gastric cancer [40]	23&18	USS	WS	MALDI-TOF/MS	4 proteins (1472.78 Da, 2936.49 Da, 6556.81 Da, and 7081.17 Da)	
Breast cancer [49]	10&10	USS	WS	2D-DIGE	CA6	Immunoblotting
Rheumatoid arthritis [50]	20&20	USS	WS	2DE MALDI-TOF/MS	2 S100A Apolipoprotein a-1	Immunoblotting
Type I diabetes [51]	31&31	USS	WS	LC-ESI-MS	S100A9 PRP-1/PRP-3	
Type I diabetes [15,52]	15&5	USS	WS	iTRAQ MALDI-TOF/MS LC-MS/MS	HbA1c BPI MMP-9	
Type II diabetes [53]	40&10	USS	WS	LC-MS/MS	5 metabolism proteins	ELISA

HNSCC: Head and Neck Squamous Cellcarcinoma; USS: Unstimulated Saliva; WS: Whole Saliva; OSCC: Oral Squamous Cellcarcinoma

Table 1

diagnostic approaches, sample sizes, and saliva collection methods. Second, diverse MS techniques were used. The MS techniques can be classified into "top-down" and "bottom-up" techniques [70], which differ from each other. In addition, the use of different applications might lead to generation of disparate data sets. Thus, standardization of the procedure is necessary.

Limitations and Perspectives

Salivary peptidome analysis could provide new insights into various pathological or physiological conditions by enabling identification of specific fingerprints for their diagnosis and determination of prognosis [4]. Besides its advantages, saliva analysis also has the following limitations. Saliva contains a large array of abundant proteins, which masks the presence of low-molecular-weight peptides of low abundance [14]. This may result in concealment of a medical condition. Moreover, many proteins or peptides exist in multiple forms [71]. They undergo complicated post-translational modifications (PTM),such as phosphorylation [72], during or after secretion [73]. And PTMs are very crucial in regulating protein function, localization, and interactions in the whole saliva [74]. PTMs are also necessary and required for appropriate folding of the protein. However, the big challenge in proteomics and peptidomics is to completely characterize the variously different or complicated PTM types and identify the dynamic features present at any given time in the saliva, as well as in the other body fluids. Recently, many modification sites can be identified in MS-based techniques and useful biological information could be reached, as modern advances have been present in the MS-relevant field. Thus we might catch more detail information in the future. Moreover, the saliva secretion volume, flow rate, and constituents could be influenced by intrinsic and extrinsic factors, including gender, age, inter-individual variation, and so on [75]. Hence, efforts should be made to standardize sample collection and pretreatment techniques to enhance the reliability and reproducibility of salivary peptidome profiling. Besides, for the peptidomic analysis suitable statistical methods such as the two-tailed t-test and Student's t-test should be used to compare peptide peak intensities between patients and controls. Then the data should be analyzed using specific statistical package. The establishment of a relatively complete peptide spectrum database will facilitate the determination of both the source of the salivary peptide profile variation and the mechanism thereof. Besides, a larger sample size is needed to confirm the significant differences in peptide mass peaks found in the related study, as its inherent challenge including the wide protein concentration range therein and the presence of multiple post-translational modifications.

In summary, body fluid-based peptidomic research currently plays an important role in a number of fields. However, challenges remain in terms of identifying truly specific and useful biomarkers for pathological conditions and elucidating the pathways in which they participate and their effect on diseases. Thus acquisition of detailed knowledge, including the advantages and disadvantages of this technique, is vital. In conclusion, the ongoing rapid development of biological technologies will inevitably result in accumulation of further useful data.

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