Review

Emerging trends in Sjögren's syndrome: basic and translational research

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ABSTRACT

This review will address the 'state of the art' of novel genomic and proteomic biomarkers for primary Sjögren's syndrome (pSS) and the current status and potential of gene transfer to salivary glands in restoring the function of salivary glands.

Introduction

Primary Sjögren's syndrome (pSS) is a complex autoimmune disease characterised by a progressive hypofunction of the salivary and lachrymal glands, frequently associated with a variety of extraglandular manifestations, including malignant lymphoproliferative disorders (1). To date, pSS is a challenging disorder, as the underlying pathophysiological mechanisms remain obscure, disease activity is challenging to evaluate, and no specific treatments are known to be effective. Recently, however, new light has been shed on pSS pathogenesis, diagnosis and treatment (2-6). These new insights are mainly due to the latter advances of novel high throughput genomic and proteomic technologies which have appeared as new potential tools for generating pathogenetic, diagnostic and prognostic biomarkers for pSS (7-10). Genomic and proteomic technologies are widely considered to be complementary in their potential scientific application: the former exploring the gene expression profiling and the latter, being a large-scale study of the proteins expressed by the genome. Both high-throughput gene expression assays and mass spectrometric proteomics have provided promising results particularly in utilising saliva to explore biomarkers for diagnostic purposes giving clues as to how to work with saliva as a pSS diagnostic fluid (9, 11, 12). Joint efforts are also under way looking to improve the knowledge on the pathogenesis of the disease and to identify new therapeutic targets in pSS (2, 13-19). This review will address the "state of the art" of novel genomic and proteomic approaches to pSS and the current status and potential of gene transfer to salivary glands in restoring the function of salivary glands. For the genomics the focus will be in microRNAs (miRNAs) as they represent one of the most promising class of molecules in both biomarker and pathogenesis studies.

A Medline search of English language articles published in the PubMed database (from 1950 to date), using the following words: "Saliva" [Mesh]) and "Biological Markers" [Majr: NoExp]; ("Proteomics" [Mesh]) and "Sjögren's Syndrome" [Mesh] ("Proteomic"[Mesh]) and "Sjögren's Syndrome" [Mesh]; ("Sjögren's Syndrome" [Mesh]) and "MicroRNAs" [Mesh]; ("Genomics" [Mesh]) and "Sjögren's Syndrome" [Mesh] formed the data sources. These were combined with our clinical and experimental experience in this field.

microRNAs: state of the art

The importance of microRNAs (miRNAs) as regulatory keys in a variety of physiological and pathological processes has been well established in the past few years. Among others they are instrumental in regulating immune development, normal immune function and autoimmunity (20). MicroRNAs are small regulatory non-coding RNAs of 19–25 nucleotides in length. MicroRNAs are generated as primary microRNAs (pri-miRNAs) in the nucleus by the RNA polymerase II and are cleaved into pre-miRNAs in the nucleus by an RNase III enzyme called Drosha (21, 22). Through Exportins, the pre-miRNAs are then transported to the cytoplasm (23) where a cytoplasmic endonuclease DICER, along with RNA

binding proteins, cleave both strands of the pre-miRNA hairpin duplex thus generating a double stranded RNA, 19–24 nucleotides long (24). The loading of the miRNA in the complex RISC, a ribonucleoprotein complex consisting of the RNA helicase A and other proteins such as argonaute 2 and TRBP (25), is fundamental for the binding of the miRNA and the target mRNA. The post-transcriptional regulation by miRNA is mediated by either degradation of the target messenger RNA or just by repression of translation (26). The miRNA can target the messenger in both the 3'UTR and in the 5'UTR, as well as in the coding sequence (27). During the last few years, there has been an increasing interest in miRNAs in clinical studies due to their intrinsic stability compared to mRNAs and also due to their preservation in many kind of stored samples (such as FFPE tissues) along with their easy retrieval from those samples. These characteristics together make miRNAs very useful and easily accessible candidates for biomarkers. For autoimmune diseases such as pSS, the functional importance of miRNAs is currently being explored. Their roles in pSS should be investigated in two major directions: First, miRNA alterations might be associated with the autoimmune characteristics of lymphocytes, *i.e.* increased immune activation or loss of immune regulation. Second, miRNAs might be playing a role in the altered exocrine gland function.

Recently, the salivary gland mirnome has been characterised in the salivary gland of pSS patients. Using the microarray approach, the miRNA signature in pSS patients has been separated from the healthy controls (15). Among other differentially expressed miRNAs, hsa-miR-768-3p and hsa-miR-574 were found to be markers of inflammation and with very distinct expression levels correlating with the inflammatory status of the salivary glands (15). A new and very interesting role of miRNA has been proposed lately in the regulation of the immune response acting directly on the transcriptional level of Ro/SSA and La/SSB mRNA (7). In this paper a differential expression has been shown of certain miRNAs in the SG tissues, SGECs and peripheral blood mononuclear cells (PBMC) of pSS patients and controls (7).

The functional role and the downstream effect of differentially expressed miRNAs in pathological states are also of interest. In the case of pSS miRNAs can help identify putative pathways involved in the pathogenesis or in the cascade of factors responsible for the immune-pathologic phenotype. There has been a number of prediction algorithms of the miRNA-mRNA binding sites an example being RNA22 (27). MicroRNAs are also been shown to target cytokines, and recently it has been demonstrated that hsa-miR-146a significantly upregulates phagocytic activity in THP-1 cells and negatively regulates pro-inflammatory cytokines as TNF-α, IL-1β, MIP-1α and IL-6 as demonstrated in human monocytic THP-1 cells (28).

The advent of new emerging techniques such as Deep Sequencing marks a new era for the characterisation of miRNA signature in different human samples. Next generation sequencing is already being applied to study a variety of diseases and offers the opportunity to sequence entire genomes or transcriptomes within days (20). One of the main advantage of this approach over other high-throughput techniques is the possibility to discover novel miRNA sequences but the real challenge with this approach is the analysis of the large amounts of data generated. Next generation sequencing has been used to characterise the small RNA population in minor salivary glands of pSS patients. Several previously unidentified miRNAs have been discovered. Specifically, so far, six previously unidentified miRNAs were first identified and reported in minor salivary glands and then validated in other cell lines (20). The goal of identifying known and novel miRNA candidates responsible for the pathogenesis of the disease is a crucial step in the development of new therapeutic cures. One of most interesting emerging approaches that can become a new frontier of the medicine is the use of viral vectors such the Adeno-associated Virus (AAV) family to deliver, in a tissue-specific manner, a miRNA or its anti-miRNA in order to modify the cellular balance (29).

Proteomics: state of the art

Proteomics is the large scale study of the proteins expressed by the genome. Since biological and pathological phenotypes are ultimately determined by proteins, in the last few years, several proteomic methodologies have been applied to the search for novel and specific biomarkers of disease processes (30-34). More specifically, recent advances in mass spectrometry (MS) have provided promising results in utilising saliva and tears to discover novel protein biomarkers for pSS (35). The application of different mass spectrometry techniques to human saliva and lachrymal fluids was guided by the working hypothesis that these biological fluids might closely reflect the underlying pathological involvement of salivary and lachrymal glands which are the main target organs in pSS. From this point of view, therefore, it was suggested that putative salivary or lachrymal proteins might eventually represent more sensitive and specific biomarkers to reliably describe the different pathophysiologic aspects of pSS. The additional advantages of biofluids which might be collected easily and non-invasively represented a further impulse to the proteomic research (36). At present, while studies on lachrymal fluids are relatively scarce (37), the most consistent data are those related to the identification of proteomic diagnostic biomarkers in salivary samples of pSS patients (38). More specifically, since 2005 eight studies have been performed on saliva (12, 39-44). A number of different, complementary biotechnologies have been employed in these studies including two-dimensional electrophoresis (2DE) and different mass spectrometry (MS) techniques coupled with different mass analysers (*i.e.* matrix-assisted laser desorption ionization time of flight (MALDI-TOF-MS), electrospray ionization (ESI), surface enhanced laser desorption ionization time of flight (SELDI-TOF-MS)) (8) or immunoassays and quantitative real-time polimerase chain reaction (qPCR) for preclinical validation (17, 41). In addition, different protocols for salivary col-

lection and analysis have been adopted (whole saliva *versus* parotid saliva; pool samples *versus* individual samples; unstimulated *versus* stimulated saliva). Nonetheless, despite heterogeneous and preliminary, taken together, the available data indicated that secretory proteins of acinar origin were reduced in pSS patients, while inflammatory phase proteins were increased when compared to normal subjects (35). All the available data also converged in outlining that a combination pattern of several biomarkers rather than a single one may define a specific disease such as pSS. Among candidate proteins to characterise the proteomic profile of pSS the vast majority of the studies recognised an important role as disease potential biomarkers to proteins such as β2-microglobulin, Immunoglobulin κ light chain, Immunoglobulin γ light chain, α-enolase, cathepsin D, S-100A9, epidermal fatty acid binding protein (E-FABP), α-defensin, cystatins and α-amylases. Recently, three mRNA biomarkers have also been pre-validated in pSS and namely myeloid cell nuclear differentiation antigen (MNDA), Guanylate binding protein 2 (GIP2) and low affinity IIIb receptor for the Fc fragment of IgG (FCGR3B), which was significantly elevated in patients with pSS compared to both SLE patients and healthy controls (41). From a pathogenetic point of view, these mRNA are involved in the IFN pathway and GIP2 gene was reported to be up-regulated at the mRNA level also in minor salivary glands from patients with pSS (45). On the other hand, it has been hypothesised that the increased expression of β2-microglobulin, Immunoglobulin κ light chain and Immunoglobulin γ light chain in pSS saliva might reflect both systemic B-cell activation and an increased intra-glandular immunoglobulin synthesis, which are peculiar aspects of the disease (44). Similarly, the increase in inflammatory proteins might be correlated both to the chronic inflammation of the salivary glands and to the persistent damage of the oral environment (12). Different considerations should be made for the observed decrease of secretory proteins in pSS saliva which might more closely reflect the functional impairment of the salivary secretion process due to pSS exocrinopathy. In fact, the decrease in cystatins, carbonic anhydrase VI, α-amylases and other smaller secretory proteins such as proline-rich proteins, histatins, and statherin might been ascribed also to damage of the acinar cells (44) and to the presence of fragmentation processes which might be in turn related to an unbalanced expression of proteases and protease inhibitors (17, 42, 46). Interestingly, from this point of view, Hu *et al*. and ourselves found a peculiar abundance of α-amylases precursor fragments in the saliva of pSS patients (17, 46). Comparable findings have also been described in patients with secondary SS (sSS) associated to other systemic autoimmune disorders, the latter showing an overall protein profile that was intermediate between that of the pSS patients and the healthy subjects (17, 38).

Overall, although heterogeneous, these preliminary results have indicated that a number of proteins and peptides can be considered pSS biomarkers, being 2-3-fold up- or down-regulated compared to normal subjects or having an exclusive presence in the saliva or tears of pSS patients. Despite these encouraging results, however, the identification of reliable proteomic biomarkers of pSS as well of sSS is still in its infancy and a number of critical factors need to be overcome in the near future in order to make it possible to translate proteomic research into clinical settings. Although it is certainly true that saliva, in contrast to blood, is easy to obtain by non-invasive means, salivary flow, composition and protein concentration are quite variable among individuals, and even within individuals are influenced by many confounding parameters, such as time of sampling, flow rate, food intake, age and stress (47). Moreover, salivary components are subject to enzymatic degradation at the moment they enter the oral cavity and become exposed to enzymes of host and microbial origin (36). Therefore, larger and standardised validation studies are still required in order to obtain reliable pSS diagnostic biomarkers.

Another challenge in proteomic research for pSS is the search for prognostic and therapeutic biomarkers. It is widely accepted that pSS is generally a benign chronic disease characterised by sicca symptoms and fatigue; however, excess mortality related to the increase in risk for developing non-Hodgkin's lymphoma (NHL) has been described (48-51). The discovery of any eventual proteomic biomarker able to early identify the 'high-risk' population, which are more prone to develop systemic and lymphoproliferative complications, would improve the management of pSS patients making it possible to make an early characterisation of the different disease phenotypes and to adopt a targeted and tailored treatment for the patients. Promising data came from a recent transcriptomic and proteomic analysis of human parotid glands from patients with pSS and patients with pSS and MALT lymphoma. In this study, a panel of 8 candidate genes (GRB2, ARHGDIB, CD40, proteasome subunit, aldolase A, peroxiredoxin 5, PARC, and cyclophillin A) was apparently able to distinguish pSS from pSS/ MALT. Moreover, proteomic analysis showed that 70 proteins were up-regulated in pSS/MALT lymphoma compared to normal subjects and pSS, and 45% of these proteins had mRNA transcript concordantly expressed (52). Isolated case reports have also outlined a correspondence between salivary proteomic profile and clinical course in patients with pSS syndrome and NHLs (42, 43).

When entering the field of "personalised medicine", which should allow us to optimally match patient with treatment, besides robust prognostic biomarkers that predict the disease outcome, a growing interest also arose in the identification of novel pathogenetic biomarkers. The possibility of integrating the protein identification processes with proteomic functional analysis and genomic may allow us to better clarify the critical pathogenetic pathways underlying the different subsets of pSS, leading to the development of new concepts for curative therapies.

Overall, in summary, the scenario of the proteomic research in pSS nowadays is characterised by a number of promising hints which still require a larger validation and integration processes in order to be translated into clinical practice.

Gene therapy: state of the art

For long term management of chronic autoimmune diseases such as SS, gene therapy approaches have the potential of delivering a therapeutic gene in the tissue of interest for improving the symptoms or correcting underlying pathological alterations.

To date, there has not been any human gene therapy study for pSS, even though there have been several reports about the successful and efficacious use of viral vectors in animal models resembling SS phenotype. One of the advantages of gene therapy in pSS is that the two most affected exocrine organs (lachrymal and salivary glands) can be treated with local deliveries, diminishing the both the quantities of vectors and the multiorgan effects associated with systemic deliveries.

There are several viral vectors that can be used for gene therapy, with AAV family being the most commonly used in clinical trials. The choice of viral vector also determines the length of expression of the gene of interest. If local therapy for the salivary glands is to be considered, vector choice will also affect which type of cells will be infected, for example ductal or acinar cells or both. The immune response induced by the various types of viral vectors is another factor that has to be considered, especially in the context of autoimmune diseases. In highly inflamed ducts activating the immune system might not only be detrimental for the targeted cells, but it might also alter the disease progress.

One of the very first published works in gene therapy of salivary glands of SS-like mice examined the effect of a viral vector encoding the cytokine Interleukin-10 (IL10) (53). IL10 is a potent anti-inflammatory cytokine (53) that has been suggested as potential therapeutic for pSS (54). This study compared local delivery in the salivary glands through retrograde cannulation with systemic intramuscular delivery and found that local delivery of IL10 induced a statistically significant increase in salivary flow and a statistically significant decrease in the focus scores compared to intramuscular vector delivery.

Since that study, several others have been published in animal models of SS mainly targeting immune-modulatory molecules (55-58).

The first clinical trial of gene transfer for improvement of xerostomia is currently taking place at the National Institutes of Health in the USA (http://www.clinicaltrials.gov/ct/show/NCT00372320). In this study, the water channel AQP1 encoded by an adenoviral vector is transferred to the parotid glands of radiation-induced salivary hypofunction subjects, locally through retrograde cannulation, in an effort to reengineer the salivary ducts to allow for fluid movement and restore salivary flow (59). The results of this Phase I/II study will heavily influence the future of salivary gland gene therapy for xerostomia treatment associated with several diseases as AQP1 does not interfere with the immune regulation of the glands, but rather changes the physiological characteristics of ductal cells, making them capable of secreting fluid in an exocrine fashion.

Besides viral vectors that encode genes that get translated by the cellular mechanisms in proteins, gene therapy vectors for the transfer of non-coding RNAs are being developed, such as for short hairpin RNAs (60, 61) for the treatment of hepatitis B. As miRNAs are characterised for their role in pSS, their use in gene therapy applications might prove highly beneficial due to their characteristics of regulating the expression of several genes simultaneously.

Future directions

Over the last few years, emerging biotechnologies have shown great complementary potential for characterising different subsets/phenotypes of pSS. At present, nonetheless, individual efforts looking at single diagnostic parameters have not led to findings that can be translated in a clinical setting, partly because complex diseases like pSS might require a more systemic multidimensional approach that can efficiently capture the most relevant factors associated with this syndrome. Nowadays, the available data retrieved from genomic and proteomic studies represent a challenging starting point for the more thorough characterisation of biomarkers that reliably describe the different pathophysiologic aspects of pSS, helping to establish the diagnosis and to predict the outcome of the disease. In the near future, the ultimate goal of the integration of genomic and proteomic studies could be the development of novel targeted therapies for pSS which might replace the currently adopted treatments that are mainly focused on symptomatic relief. A step forward in this direction is represented by gene therapies which offer the possibility to re-engineer the glands, thus restoring the function of damaged salivary glands. In summary, emerging biotechnologies appear to offer promising tools to approach the challenge of the discovery of new, accurate biomarkers for pSS from a novel perspective and, ultimately, to adopt new "personalised" therapeutic strategies for pSS.

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