Ph.D. THESIS

MOLECULAR BIOLOGY APPROACH OF SJÖGREN'S SYNDROME

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INTRODUCTION

The group of the polysystemic autoimmune diseases consists of different members, such as systemic lupus erythematosus (SLE), mixed connective tissue diseases (MCTD), Sjögren's syndrome (Ss) etc.. Sjögren's syndrome is characterized by dry eyes and mouth caused by mononuclear cell infiltration of the lacrimal and salivary glands but other organs can also be involved. Ss exists in primary and secondary forms, the latter can be associated with other autoimmune diseases such as rheumatoid arthritis (RA) or SLE.

The etiopathogenesis of Sjögren's syndrome is multifactorial: different factors play a role in the induction of the diseases such as a complex genetic background, hormonal factors, and other external and internal factors (exogen, endogen and retroviruses, UV irradiation, chemical agents, drugs, etc.). Sera of patients with Ss usually contain antinuclear antibodies (ANAs). One of the targets of an ANA system is the nuclear autoantigen La/SS-B, which serves as a factor in hnRNA processing and it is a transcription termination factor of polymerase III. The role of marker autoantibodies, such as the La/SS-B, is unquestionable in the diagnostics of certain autoimmune diseases, since they are important markers of these diseases, however they may participate in the pathomechanism of these illnesses as well.

It has long been suspected that the La autoantigen possesses pathophysiological importance in the Sjögren's syndrome. The La protein is localized mainly in the nucleus but it could appear in the cytoplasm or in the cell membrane as a result of different stress factors (e.g. UV irradiation). The protein consists of 2 main domains that are connected by a protease sensitive amino acid chain of 130 pieces. It has enzyme activities, which is connected to its function as a transcription termination factor (mainly dsRNA and dsDNA unwinding and cutting activities). The gene of La is localized on human chromosome 2 and it codes a 1.47 kb long RNA with 11 exons. Later results indicate that 3 splicing variants of La (Fig. 1.) are

expressed in several different cell types and their expression could result in the synthesis of aberrant proteins as a result of certain circumstances. These aberrant proteins could serve as triggering factors in the etiology of autoimmune processes.

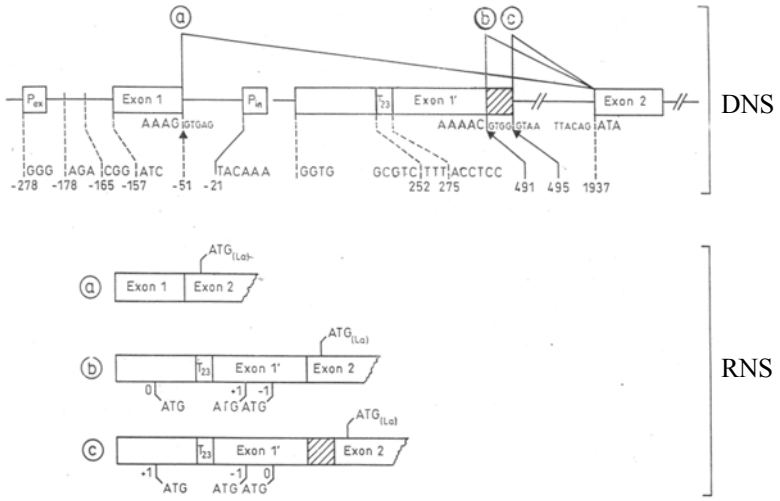
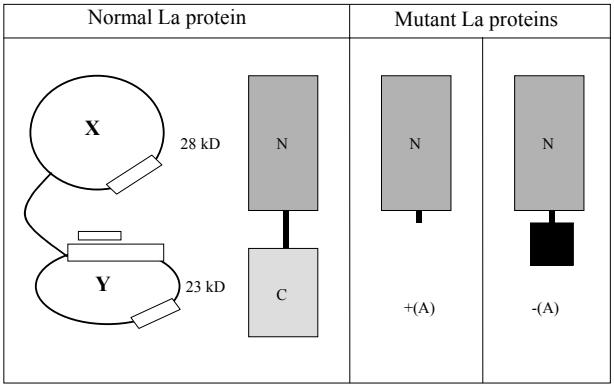


Figure 1. Structure of the 5' end of the La antigen and that of the 3 splicing variants of its mRNA.

a = exon 1; b = exon 1'; c = exon 1" variants. Starting ATGs are indicated where the $ATG_{(La)}$ is the normal starting site.

Moreover, a hot spot region in the exon 7 of La was also found, and mutations in this region could directly lead to new epitopes without the existence of other complement factors (Fig. 2.). This phenomenon could serve as a base for autoimmune reactions.



A

Figure 2. The normal La protein and the variants resulting from the effects of point mutations.

A. The –(A) mutation results in the formation of a neoepitope while a +(A) mutation leads to a cryptic epitope. The white bricks indicate the epitopes known.

B

CTGCAGTCTTAACTTTGTTCTCGTGAACTTAGCCTCTGTACTGTGTGTTGTTTAGGGACGATT
ACTTTGCC**AAAAAAAA**TGAAGAAAGAAAACAAAATAAAGTGGAAGCTAAATTAAGAGC
TAAACAGTAAGTATGTTGAACTAATCACGACATAATTTGAATTC

B. The exon 7 of La gene with its hot spot region. The exon, in its intron surroundings, is underlined and the hot spot ologoA region is indicated with bold letters.

The main causes of death in Sjögren's syndrome patients are the lymphoproliferative diseases, such as B-cell lymphomas. The majority of these lymphomas are follicular ones, which can be characterized with the persistence of the t(14;18) chromosome translocation. It is not clear yet what kind of role this translocation has in the Sjögren's syndrome.

AIMS OF THE STUDY

All the above results that characterize the La gene with sufficient details inspired us to approach the Sjögren's syndrome with the study of its molecular bases. Therefore our aims were as follows:

1. In order to study Sjögren's syndrome in a systematic way we wanted to find a method that is capable of revealing all the changes in gene expression as a function of the disease, this way all the genes that participate in the etiology of the diseases can be identified.

2. We wanted to study the mutations of hot spot region of exon 7 of the La gene whether these mutations could lead to the formation of proteins that serve as a base for the emergence of autoimmune reactions.

3. Our goal was to gather information with respect to one of the main causes of death in Ss, namely the lymphoproliferative syndromes, and their relationship with the t(14;18) translocation.

4. Since Ss arises at later ages more frequently our intention was to discuss the role of the aging process as a factor in the etiology of the illness. Moreover, we wanted to investigate

how aging and some diseases are coupled with certain genetic factors, such as single nucleotide polymorphisms (SNPs).

PATIENTS AND METHODS

All the patients suffering from Sjögren's syndrome, SLE and RA were patients of the 3rd Department of Medicine. They were diagnosed by using the international criteria accepted world wide. The mean age of the patients was 54.4 years and the mean time of disease persistence was 6 years. The control lymphocytes were isolated from blood of healthy donors who did not have any disease symptoms. Patients of follicular lymphoma are from the Haematological and 2nd Department of Medicine of Semmelweis Hospital in Miskolc. They were diagnosed in cooperation with the Malignant Lymphoma Reference Center of the University of Pécs.

Differential display

RNAs were isolated from lymphocytes of Ss patients as well as from those of healthy donors and the RNAs were transcribed into cDNAs using reverse transcriptase. Following that a special PCR procedure was conducted by using several random and oligo(dT) primers in order to amplify the mRNAs of the two populations. Electrophoresis technique was used to separate the DNAs amplified and this way the differences in the gene expressions of the two populations could be identified. [5].

Polymerase chain reaction (PCR)

Conventional PCR. Pieces of DNAs were amplified with the appropriate primer pairs using conventional PCR technique in 25 μl end-volume by Amplitaq polymerase in a Perkin Elmer

9600 PCR equipment. The amplified DNAs were usually visualized in agarose gel using ethidium bromide under UV light [11].

Nested PCR. In order to increase the specificity and sensitivity of a PCR amplification the once amplified DNA was used as a template in the subsequent PCR reaction using a new internal primer pair. The reaction conditions were similar to those of the conventional PCR depending on the primers [6].

Single stranded conformational polymorphism (SSCP)

PCR-amplified and denatured DNAs were separated by a special non-denaturing acrilamide electrophoresis at 16 ^{o}C. This method makes it possible to separate DNAs of identical length even if there is a polymorphism between the different DNAs. Under these conditions they run at different positions, although they have the same length or there is only one nucleotide mutation [11].

Sequencing

PCR products were separated by low-melting-point agarose and following purification they were inserted into a vector. This vector was transformed into E. coli, then after amplification the plasmid was separated. The sequencing was performed using Sequenase 2.0. GeneBank databases were used to help the sequence analysis.

RESULTS

1. Systematic approach of the Sjögren's syndrome

In the conventional candidate gene approach studies usually only one or a few genes are studied. The genes are selected after considering the data available regarding the appropriate phenomenon. This gene (or genes) is investigated from different point of views and one tries to find connections between the gene (genes) and the particular phenomenon, such as a disease. A systematic approach of a complex disease was not possible so far because of the numerous factors and parameters that one has to take into account. However, using the differential display one can compare two RNA populations and both the qualitative and the quantitative differences can be identified. We aimed to establish such a method in order to investigate the pathogenesis of the Sjögren's syndrome. Our results show that this method can be well used to compare the gene expression patterns of two populations, namely one can identify the differences in the RNA expressions of Sjögren's patients and healthy donors (Fig. 3.)

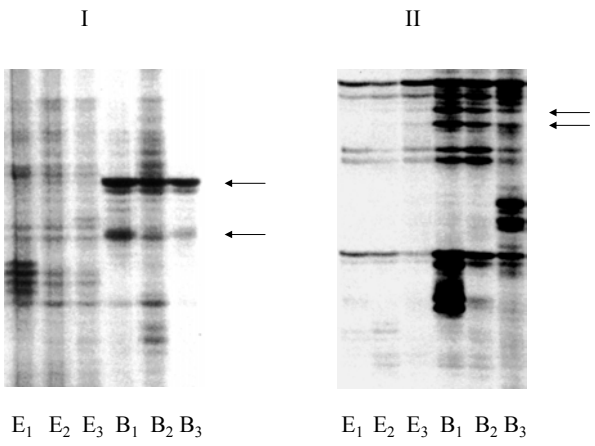


Figure 3. Examples of the identification of differentially expressed genes. The differences in the expression patterns of patients (B) and that of healthy donors (E) are indicated by arrows.

Beside the systematic approach the La gene was further investigated since data indicated that this gene plays not only a diagnostic role but it may participate in the pathomechanism of the Sjögren's syndrome as well.

2. Hot spot region of exon 7 of the La gene

Point mutations in a part of the La gene that later proved to be a hot spot region, could be identified from the lymphocytes of a Ss and a SLE patient during the preliminary experiments right after the cloning of the La gene from lymphocytes of a patient suffering from Sjögren's syndrome. Our aim was to identify further patients who carry these mutations using the SSCP method. Ten of each patients suffering from Ss, SLE and RA were studied and several patients carrying mutations could be identified (Fig. 4.).

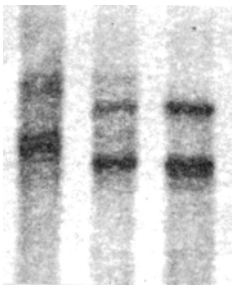


Figure 4. An example of a mutation in the oligoA hot spot region of exon 7 in the lymphocytes of a SLE patient using SSCP technique. 8A = normal form (oligoA8), S = SLE patient, 7A = mutant standard (oligoA7).

These results prove the existence of further patients who carry mutations in the hot spot region of exon 7 of the La gene.

3. Relationships of the t(14:18) chromosome translocation with Sjögren's syndrome

The identification of the appearance of the t(14:18) chromosome translocations was carried out not only in patients suffering from follicular lymphoma but from Ss patients as well, after establishing the method for translocation analysis, since it is noted that both the frequency of Sjögren's syndrome and that of t(14:18) translocation increase as a function of age. Five Ss patients out of 100 proved to be translocation positive in their peripheral lymphocytes. Therefore the translocation frequency in Ss patients was 5 times higher than in healthy donors. In two cases out of the 5, translocations were identified even in the lymphocytes of bone marrow and these translocations persisted during a couple of years. Our prediction is that it is possible that these patients will develop follicular lymphoma after a while.

4. Relationships of the t(14:18) chromosome translocation with lung carcinomas

Several malignant diseases can be traced back to alterations happened in certain gene(s). Patients suffering from follicular lymphoma can be well characterized with the presence of the t(14;18) chromosome translocation, where the bcl-2 gene translocates to the immunglobulin one, and this way the expression of the bcl-2 gene changes and in turn it will result in the disturbance of the normal apoptotic processes. The malignant types of diseases arise more frequently in several different illnesses, such as in Ss, right due to the above mentioned genetic alterations. The existence of the t(14;18) translocation and the alteration in bcl-2 gene expression could also play a role in other types of diseases, such as in certain lung carcinomas as well.

5. On the role of aging in the etiology of autoimmunity

Different types of diseases may be in close relationship with the normal aging processes, since e.g. Ss patients are from a well defined older age group, i.e. the frequency of the diseases increases as a function of age. Our immune system has a defense mechanism against two directions: against external agents and at the same time against the unwanted self. In the later case, however, one has to make a difference between normal (physiological) clearance and the autoimmune processes. It seems that the aging of the thymus is one of the key elements of autoimmunity besides aging of other cell types and different other factors can play an important role as well. Spontaneous genetic instability, mutations due to aging and the changes in the information level of the organism have an important role both in the etiology of physiological autoimmunity and in that of autoimmune diseases. The physiological autoimmunity is influenced by natural factors such as aging and apoptosis, however, the etiology of the primer autoimmune diseases could primarily be influenced by the genetic instability factor.

6. Relationships of genetic polymorphisms with aging and diseases

Single nucleotide polymorphisms (SNPs) lead to a more than 3 million base-pair difference when genomes of two individuals are compared. These differences are known in more and more details and several facts indicate that these differences are responsible for different biological phenomena. Individual polymorphisms may result in increased disease susceptibility, i.e. certain nucleotides are more frequent in patients suffering from various illnesses than other nucleotides in healthy people. These SNPs could lead to alterations in the cells e.g. the activity of an enzyme can change as a result of nucleotide exchange, in other

cases the effects of SNPs are not so pronounced. Numerous later results show that certain SNPs could be coupled with people who reach a higher mean life span than the average one. Even if these SNPs do not influence the maximum life span they would indicate which genes could play a substantial role in the regulation of the life span. In this respect the SNPs could be considered as further factors in the determination of the individuals' information level. Therefore the SNPs are important factors of the aging processes as well. Since there are age-related diseases discoveries of age- and illness-dependent SNPs could help us to find the common roots of aging and some diseases.

DISCUSSION

Besides the candidate gene approach of the Sjögren's syndrome we tried to find a possibility to approach the disease in a systematic way. The Differential Display (DD) technique makes it possible to compare the gene expression profiles of Ss patients with those of healthy donors. This way not only the role of a gene (or a limited set of genes) can be investigated as a disease-factor but all the significant differences could be identified that exist in the gene expression profiles. The results of our studies show that the genes, that could be responsible for the alterations in the gene expressions, can be well identified using DD. However one can face other difficulties during these experiments namely the correct identification of the genes is the hardest part of the studies. Nevertheless, the recent DNA chip technologies can overcome all these difficulties and they are more suitable for such a systematic approach. Using this technique one can save material, work force and time and the differentially expressed genes can still be identified more easily and quickly.

The mutations identified in our study in the hot spot region of exon 7 of the La gene could serve as an explanation for the emergence of the autoimmune reactions (Fig. 5.).

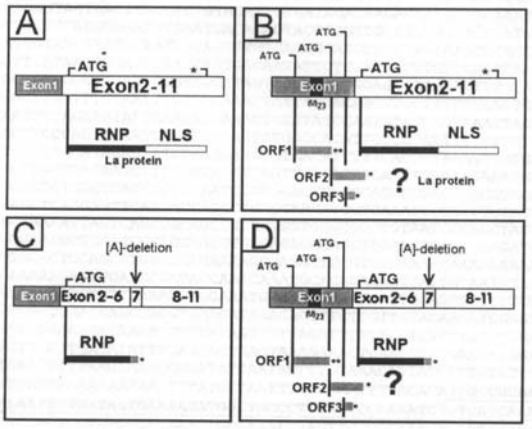


Figure 5. Appearances of the different La proteins as results of alterations in the mRNAs. **A**. A normal La protein. **B**. The possible proteins formed using the different splicing variant of the La mRNA, the La 1'. **C**. La protein as a result of the mutation in the exon 7 of the La gene. **D**. All of the possible La proteins formed.

The alternative splicing variants of the La mRNA (Panel B) could result in an altered protein only if a frame shift happens during the transcription process since there are stop codons not too far from the new starting sites. Such a frame shift could happen as a result of certain virus infections and in turn these altered proteins could lead to an autoimmune reaction. However, the mutations of the exon 7 of the La gene (Panel C) can directly lead to the formation of alternative La proteins. These proteins have either a neo or a cryptic epitope and these new epitopes can serve as the bases for the autoimmune reactions in the appropriate cells. An independent laboratory in the United States of America could also identify mutations in the hot spot region of exon 7 of the La gene in lymphocytes of patients suffering from Ss or SLE using a totally different technique.

We established that the frequency of the t(14;18) chromosome translocation that characterizes certain non-Hodgkin lymphomas is significantly increased in Ss patients compared to healthy donors. This phenomenon is paralleled by an increase in the appearance

of lymphoproliferative syndromes that are significantly responsible for the mortality of the Ss patients. These translocations can be identified in the peripheral lymphocytes of healthy donors as well, but in Ss patients these translocation are localized in lymphocytes with a bone marrow origin, too. Moreover the translocations in certain Ss patients persisted during a much longer period than it is usual in healthy donors.

These results make it possible to observe the signs of follicular lymphoma of early stages in patients of Ss. In turn the results could lead to a better handling of the follicular lymphoma and in certain cases the early treatment of the disease may prevent the outbreak of the heavy symptoms of the follicular lymphoma, which has been incurable so far.

Besides all the factors that play a role in the etiology of Ss (e.g. virus infection, hormonal effects, UV irradiation, etc.), some elements of the general processes of aging may contribute to the onset of the illness. The accelerated appearance of the genetic alterations due to aging that arise from external and internal factors - among others from the genetic instability of the genome - may also play a role in the pathomechanism of the disease. Nevertheless, it is highly possible that the personal differences, such as polymorphisms and the hormonal regulation (since the majority of the patients (90%) are women) could be important elements in the susceptibility of the diseases. As a conclusion of our results it seems that disease-susceptibility, aging, genetic factors due to other elements together with different unknown factors may result in the appearance of the Sjögren's syndrome.

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Sum of impact factors: 13.05

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