Molecular markers of systemic autoimmune disorders: the expression of MHC-located HSP70 genes is significantly associated with autoimmunity development

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Abstract

Objective

To analyse the expression regulation of two inducible HSP70 genes – HSPA1A and HSPA1B – located within the major histocompatibility complex (MHC) in patients with various systemic autoimmune diseases and to prove the reliability of MHC-located HSP70 genes as molecular markers reflecting the autoimmune process.

Methods

94 adult patients with idiopathic inflammatory myopathy (IIM, n=31), systemic lupus erythematosus (SLE, n=31) or systemic sclerosis (SSc, n=32) and 37 healthy individuals were analysed. The mRNA expression level was determined using quantitative real-time PCR method. The expression of intracellular HSP70 was established by flow cytometry, the extracellular HSP70 protein was measured in plasma samples using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA).

Results

*The expression of HSPA1A gene was significantly up-regulated in patients with autoimmune diseases (SLE: $p<0.01$; SSc: $p<0.01$; IIM: $p<0.0001$) compared to healthy controls. The expression of HSPA1B gene was increased only in patients with myositis ($p<0.05$). Furthermore, the HSPA1B gene expression is associated with the HLA-DRB1*03 risk allele in patients with IIM. In addition, we have found a relation between HSPA1A gene expression regulation and the presence of disease specific autoantibodies in patients with SLE and myositis. The level of intracellular HSP70 was not increased; however, the level of extracellular HSP70 protein was increased in patients suffering from SSc and IIM as compared to controls.*

Conclusion

The results suggest an involvement of the MHC-linked HSP70 genes in the pathology of studied autoimmune disorders. Therefore, the HSPA1A and HSPA1B genes might serve as an interesting candidate molecule for development of distinct types of autoimmunities.

Key words

autoimmune diseases, idiopathic inflammatory myopathy, HSP70, major histocompatibility complex

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Received on November 18, 2015; accepted in revised form on April 4, 2016.

Funding: this work was supported by the Internal Grant Agency of the Ministry of Health in the Czech Republic [MZCR NT 13699].

Competing interests: none declared.

Introduction

The primary function of the immune response is to distinguish between self and non-self antigens. Self-tolerance is essential for normal immune functions and loss of self-tolerance can result in autoreactivity. Autoimmune diseases occur when a specific adaptive immune response is established against self-antigens and these diseases affect approximately 5–7% of the human population. Susceptibility to autoimmune disease is known to be most consistently associated with the major histocompatibility complex (MHC) genes. Beside the genes encoding antigen presenting molecules, this gene region comprises also other genes involved in the regulation of immune response. Two of them – HSPA1A and HSPA1B – encoding the heat shock proteins (HSP) 70 – are inducible genes and are intensively expressed in cells under stress conditions (1, 2). The expression of the third MHC located HSP70 gene – HSPA1L – is tissue specific, and is expressed constitutively in the testes (3). HSP70 proteins play an essential role as molecular chaperones by assisting the correct folding of nascent and stress-accumulated misfolded proteins, and by preventing their aggregation. These proteins have a dual function depending on their cellular location. Intracellular HSP70 proteins have an anti-apoptotic and cell-protective role. Under normal conditions they act as molecular chaperones. In the presence of stress stimuli, the expression of HSP70 is induced in large quantities, which helps the cell to survive otherwise lethal condition. This is due to its important role in apoptosis. Increased levels of HSP70 block the apoptotic pathway and point out the powerful anti-apoptotic function. On the other hand, extracellular HSP70 proteins can mediate immunogenic function by transferring the immunostimulative signal from cell to cell.

The role of HSP70 was also shown in alloreactivity (4). Authors Jarvis et al. demonstrate that the expression of stress-inducible HSP70 is increased in human tissue model of acute graft vs. host disease (GvHD) and correlates with the degree of GvHD. Numerous studies have also shown that HSPs are over-expressed locally in inflammation areas (5). Current evidence also suggests that HSPs might play an important role in the pathogenesis of several biological processes, for example autoimmunity development (6), GvHD (4), or, at the cellular level, in apoptosis (7) and antigen presentation (8).

Inducible heat shock protein molecules may be suitable candidate molecular markers for monitoring of tissue damage, breakdown of the immunotolerance, autoimmune process, alloreactivity, or inflammation activity in general. In our study, we have focused on analysis of expression regulation of two inducible HSP70 genes located within the MHC in patients with various systemic autoimmune diseases and the quantitative determination of intra- and extracellular HSP70 protein. The aim of this study was to prove the reliability of MHC-located HSP70 genes as molecular markers reflecting or participating in the autoimmune process.

Methods

Patient cohorts

We have analysed 94 adult patients from the Czech Republic suffering from idiopathic inflammatory myopathy (IIM; n=31), systemic lupus erythematosus (SLE; n=31) or systemic sclerosis (SSc; n=32). Clinical and immunological details of this cohort are summarised in Table I. For the diagnosis of polymyositis (PM) and dermatomyositis (DM), Bohan and Peter (9, 10) criteria were used. All SLE patients fulfilled at least 4/11 features of the American College of Rheumatology (ACR) SLE classification criteria. SSc patients fulfilled the 2013 EULAR/ACR (11) classification criteria. All patients' groups included untreated as well as treated individuals. In patients suffering from IIM, the disease activity was assessed using myositis disease activity visual analogue scales (MYOACT) that individually scores each of the six organ systems: constitutional, articular, cardiac, pulmonary, gastrointestinal, cutaneous and muscle (12). Manual muscle testing of 8 muscle groups (MMT8) including one axial, five proximal (two upper extremity, three lower extremity), and two distal muscles (one upper, one lower

extremity) was performed (13). To assess the disease activity in patients with SLE, the Safety of Estrogens in Lupus Erythematosus: National Assessment (SELENA) version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (14, 15) was administered. In patients with systemic sclerosis, the disease activity was measured using ESSG (European Scleroderma Study Group) criteria for disease activity (16). The control group consisted of 37 age- and sex-matched healthy subjects from central region of Czech Republic. The study was approved by the Ethics committee at the Institute of Rheumatology and all patients signed an informed consent.

Autoantibody detection

Patients with IIM were divided into three groups based on the presence of myositis-specific autoantibodies (MSA) or myositis-associated autoantibodies (MAA) or both. Myositis-specific autoantibodies (MSAs) including anti-tRNA synthetase (anti-Jo-1), anti-Mi-2 and anti-signal recognition particle (anti-SRP), and myositis-associated antibodies (MAAs) such as anti-Ro/SSA, anti-U1RNP, anti-PM/Scl and anti-Ku as the prominent ones. The presence of other MSAs found in our patients was too low to perform any statistical analyses. Regarding the occurrence of autoantibodies before the diagnosis of SLE, we can divide these antibodies into two types. First type of autoantibodies (ANA) occurs frequently in other diseases or in low titer in healthy individuals and the second type, in healthy humans and other diseases rarely occurring autoantibodies (anti-dsDNA, anti-Sm), which are typical and probably pathogenic for SLE. While the first type of autoantibodies may occur for a long time (even years) before the onset of the disease, the second type begins to emerge usually around 2 years before the disease, wherein the anti-Sm is a question of the last year (17). Patients with systemic sclerosis were considered to be autoantibody positive if they had SSc-specific antibodies anti-Scl70 or anti-centromere (ACA) autoantibodies detected using immunoblot analysis; (Euroimmun, Germany).

Table I. Demographic and basic clinical data.

	IIM [n(%)] n=31	SLE [n(%)] n=31	SSc [n(%)] n=32	Controls [n(%)] n=37
Sex (female/male)	21 / 10	29 / 2	27 / 5	24 / 13
Age				
Mean ± SD	57 ± 16	45 ± 16	64 ± 9	41 ± 16
Disease duration				
Average years ± SD	-	3.8 ± 5.9	5.6 ± 5.6	-
Diagnosis				
DM (n)	12	-	-	-
PM (n)	19	-	-	-
Disease activity status				
Active	26 (84)	19 (61)	24 (75)	-
Inactive	5 (16)	12 (39)	8 (25)	-
Antibody status				
MSAs	10 (32)	-	-	-
MAAs	8 (25)	-	-	-
SLE specific autoantibodies	-	17 (54)	-	-
SSc specific autoantibodies	-	-	17 (53)	-

IIM: idiopathic inflammatory myopathies; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; DM: dermatomyositis; PM: polymyositis; MSA: myositis-specific autoantibodies; MAA: myositis-associated antibodies.

Table II. q-RT-PCR primer sequences, amplicon size, proximity to poly-A and efficiency coefficient in q-RT-PCR assays for the selected references genes and the target genes HSPA1A and HSPA1B.

	Primer sequence 5`- 3`	Amplicon (bp)	Proximity to poly-A (bp)	Efficiency coefficient (E)
HSPA1A	F: GCTGTTTTTGTTTTGGAGCTTCA R: TTCAACATTGCAAACACAGGAAA	87 bp	155	0,88
HSPA1B	F: GGCCTTTGTTCTTTAGTATGTTTGTCT R: AGCTGAAGCAGAAATGACATAGGA	92 bp	285	1,00
B2M	F: GAGCAGGTTGCTCCACAGGT R: CAAGCTTTGAGTGCAAGAGATTGA	128 bp	246	0,98
UBC	F: TTGGTCCTGCGCTTGAGG R: GGAATGCAACAACTTTATTGAAAGGA	95 bp	2	1,00
TBP	F: TGTTGAGTTGCAGGGTGTGG R: GCGGTGTTCTCAGTGCACAA	97 bp	393	0,83

The primers used for gene specific amplification were first tested for specificity and efficiency. To ensure the specificity of the PCR amplicons, PCR products of all primer pairs were sequenced with the ABI3130 sequence analyser (Applied Biosystems, USA; data not shown). The amplification efficiency of primers was tested by generating external standard curves and amplification efficiency calculation for all PCR products. For this purpose, we have used a pool of random 20 cDNA samples. Standard curves were prepared as ten-fold serial dilutions with 5 concentrations starting from 2x cDNA amount. HSPA1A: heat shock 70kDa protein 1A; HSPA1B: heat shock 70kDa protein 1B; B2M: beta-2-microglobulin; UBC: ubiquitin C; TBP: TATA box binding protein; F: forward primer; R: reverse primer.

Analysis of HSP70 expression at the mRNA level

The blood samples were collected into EDTA or PAXgene tubes (QIAGEN GmbH, Germany) under sterile conditions. The tubes with EDTA were used for PBMCs preparation performed by density gradient centrifugation using Ficoll-Paque (GE Healthcare Life Scences, Sweden), the PAXgene tubes were used for direct RNA extraction. The RNA preparation from PBMCs was carried out using the conventional TRIZOL® reagent (Invitrogen, USA) extraction procedure. The quantity of extracted RNA in both cases was measured with NanoDrop ND-1000 (NanoDrop Technologies, USA) and

the quality was controlled using microfluidic electrophoresis in Bioanalyzer 2100 (Agilent Technologies, USA). For the evaluation of mRNA expression, only RNA samples of similar quality were used (RIN 7.0-9.9).

For the mRNA expression detection, we have used gene specific primers for HSPA1A and HSPA1B genes (Table II). As endogenous expression control, three housekeeping genes – the UBC, B2M and TBP – were used. Amplification reaction was carried out in 96 well plate in 25µl reaction volume containing Power SYBR® green PCR master mix (Applied Biosystems, USA) with ROX as a reference dye. All reactions were performed in ABI 7900 PCR cycler and analysed with ABI Prism 7900 Sequence Detection System (Applied Biosystems, USA).

To assess possible differences in expression, we have compared the measured values of the expression of HSP70 genes in mRNA isolated from samples collected into EDTA and PAXgene tubes. We did not find any differences in expression values between these two methods of sample collection.

Analysis of HSP70 expression at the protein level

In our study, we have performed the analysis of HSP70 protein expression in both of its forms: intracellular and extracellular form.

Analysis of intracellular HSP70 protein

The presence of intracellular HSP70 was determined by flow cytometry in peripheral blood mononuclear cells (PBMCs) of patients suffering from autoimmune diseases (SLE, SSc and IIM patients) and healthy controls. PBMCs were performed by density gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences, Sweden). In all samples, membrane staining was performed with a panel of monoclonal antibodies including: CD8-APC (clone DK25) and CD45-CY (clone T29/33) (both Dako, Glostrup, Denmark), CD16-PE (clone 3G8), CD56-PE (clone NKH-1) and HLA DR-ECD (clone Immu-357) (both Beckman Coulter, Marseille, France), CD3-PerCp (clone UCHT1), CD4-PE-Cy7 (clone SK3) and CD14-APC-Cy7 (clone MøP9) (all BD Biosciences, San Jose, CA USA) and CD19 PB (clone HIB19) (BioLegend, San Diego, CA,USA). For intracellular HSP70 proteins determination, the IntraStain kit (Dako, Glostrup, Denmark) and anti-HSP70 antibody was used. After two washing steps, samples were acquired on the flow cytometer CyAn ADPTM and the analysis was performed using Summit 4.3 software (both Dako, Colorado, USA).

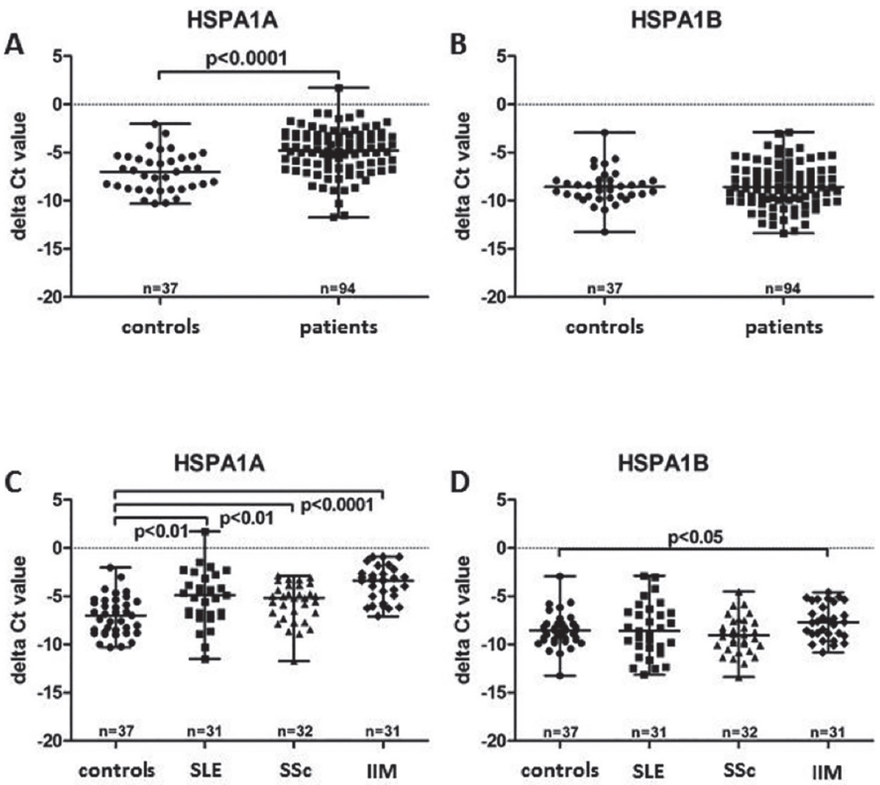

Fig. 1. Expression of the HSPA1A and HSPA1B genes in all patients (**A**, **B**) with studied systemic autoimmune diseases compared to healthy controls and in patients divided according to the three different diagnosis (**C**, **D**), respectively.

Statistical analysis

Allele and gene frequencies of HLA-DRB1 and -DQB1 loci were calculated using standard methods by direct counting. The significance of the differences in allele and gene frequencies were evaluated by χ^2 or Fisher's exact test. The regulation of expression of the two HSP70 genes was calculated according to the delta-delta Ct mathematical model. Statistical differences between the patients and control groups were established using Mann-Whitney U-test with corrected *p*-value calculation. Correlations were calculated using the Spearman's rank correlation coefficient with two-tailed *p*-value calculation. We have used GraphPad Prism 5 (GraphPad Software, California) for all statistical analyses. *p*-values <0.05 were considered to be statistically significant. Data are expressed as median with SD.

Results

mRNA expression of HSP70 genes is altered in patients with systemic autoimmune diseases.

In our study, we have analysed the mRNA expression regulation of HSP70 genes in patients with diverse systemic autoimmune disorders. The regulation of HSP70 mRNA expression was measured in 31 IIM patients, 32 patients with SSc and 31 patients with SLE. For the initial evaluation, we have analysed the expression regulation of HSPA1A and HSPA1B in all patients with systemic autoimmune diseases without dividing them according to their exact diagnosis, clinical features or immunologic characteristics. Our results indicate significant up-regulation of the HSPA1A ex-

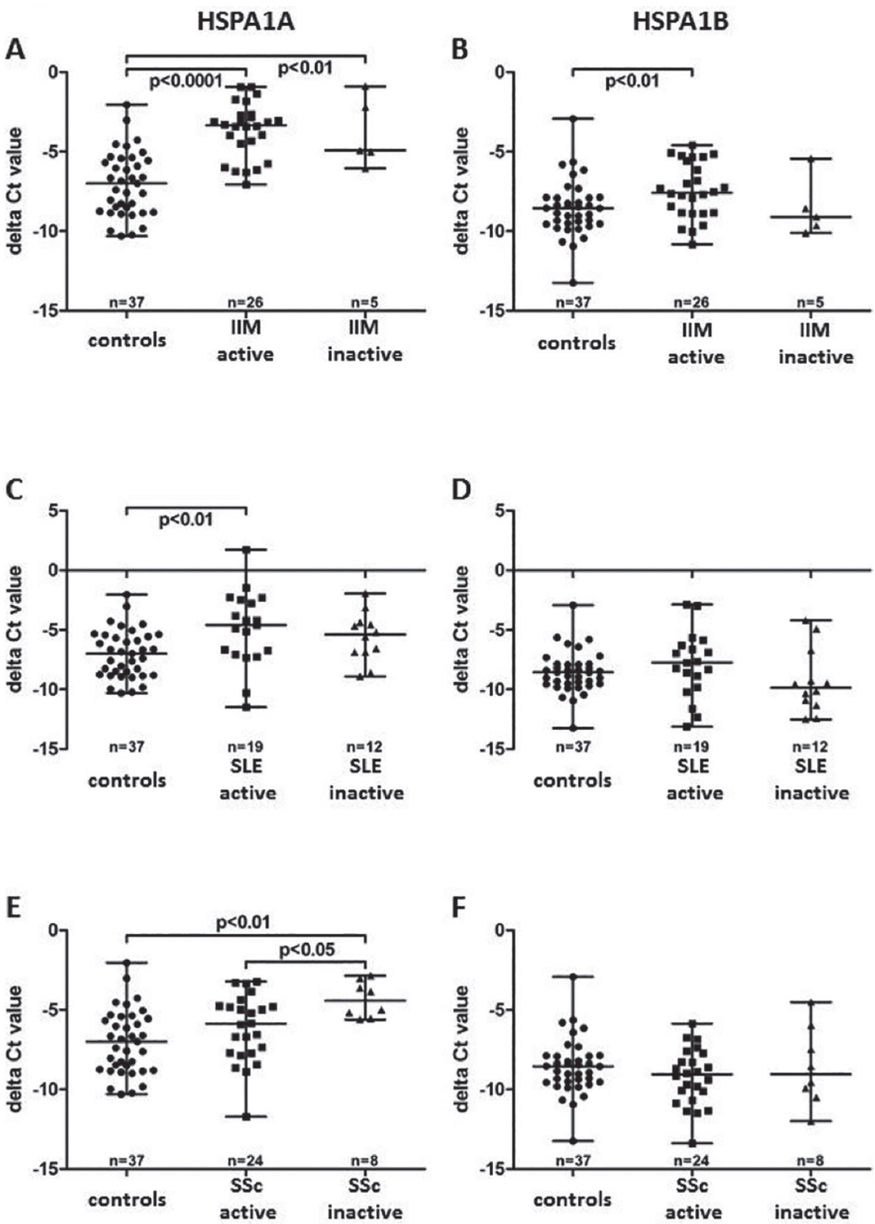

Fig. 2. The expression of HSPA1A and HSPA1B gene in patients with IIM (**A**, **B**), SLE (**C**, **D**) and SSc (**E**, **F**) in active and inactive disease. Based on the distribution of activity among patients and on the clinical experience with patients with IIM we have selected the cutoff for disease activity at 40. Patients below this cut-off were considered to be low activity or inactive. SLE patients with SLEDAI above 4 were regarded as active. In patients with systemic sclerosis, those with the total score ≥3 represented an active disease.

pression ($p<0.0001$) in comparison to healthy controls, but not of the HSPA1B gene (Fig. 1A-B). When the diagnosis of patient was taken into consideration, we have found significant up-regulation of HSPA1A gene in patients with SLE ($p<0.01$), scleroderma ($p<0.01$) and myositis ($p<0.0001$). The expression level of HSPA1B was higher only in patients with IIM compared to healthy individuals ($p<0.05$) (Fig. 1C-D).

Further, every group of patients was divided into two subgroups, according to the disease activity: to patients with active and low or inactive disease. In patients with IIM, the expression of HSPA1A gene in both active as well as in inactive was significantly higher compared to healthy controls ($p<0.0001$, $p<0.01$; Fig. 2A). In SLE patients, the expression was significantly increased only in active patients in comparison to controls ($p<0.01$; Fig. 2C). The expression of the HSPA1A gene was found to be significantly higher only in SSc patients in inactive compared to SSc patients in active or healthy controls ($p<0.05$, $p<0.01$; Fig. 2E). The expression of HSPA1B gene was significantly higher only in patients with myositis in active compared to controls ($p<0.01$; Fig. 2B). No significant difference in expression of HSPA1B gene was found in SLE and SSc patients (Fig. 2D-F).

We also divided patients into two other subgroups: treated patients, *i.e.* receiving therapy for at least 1 month at the time of blood collection and untreated patients, *i.e.* patients prior to therapy initiation. No significant differences in the expression of the inducible HSP70 genes were found when treated and untreated patients were compared (data not shown).

The expression of HSPA1B mRNA is related to the presence of HLA risk allele

The HSPA1A and HSPA1B genes are located within the MHC complex, very close to the HLA-DRB1 gene, and in general they are expected to be a part of the MHC haplotype. Therefore the expression of MHC genes, including the HSP70 genes is expected to be regulated in dependence on the MHC haplotype polymorphisms, or at least on the presence of the disease associated risk HLA allele. For this reason, we have analysed HLA-DQB1 and HLA-DRB1 allelic polymorphisms in all of our patients and controls and investigated this possible relation.

To date, the HLA-DRB1*03 allele has been identified as the major risk factor in IIM (18). The relation between expression level of HSPA1A and the presence of DRB1*03 allele is shown in Figure 3A. IIM patients had statistically significant difference in expression of HSPA1A between patients and controls, both for DRB1*03 non-carriers ($p<0.05$) and DRB1*03 carriers ($p<0.001$). HSPA1B expression level was increased in patients with myositis carrying DRB1*03 allele (Fig. 3B) in comparison with myositis patients not carrying the DRB1*03 allele ($p<0.05$). It suggests that the increased expression of HSPA1B in DRB1*03 carriers is related to the presence of HLA-DRB1*03

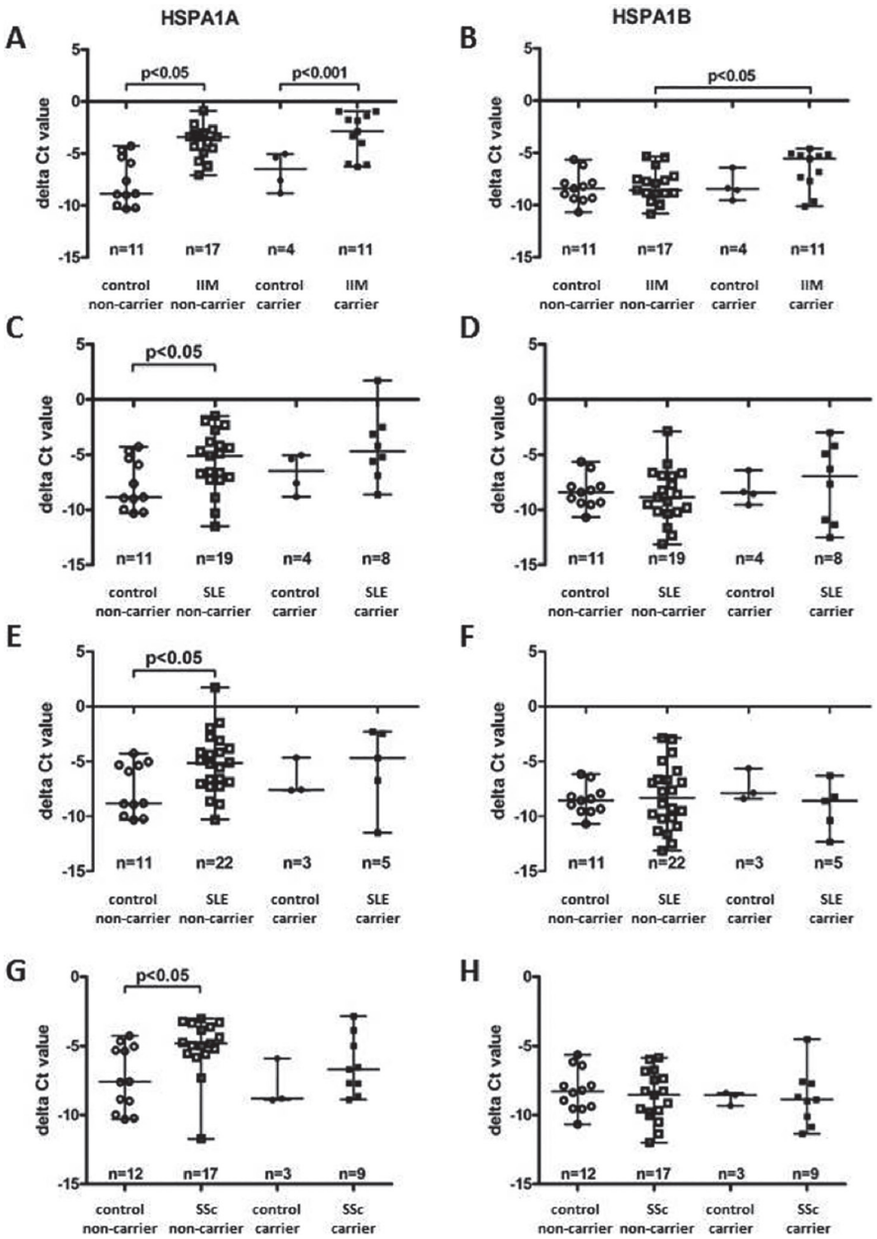

Fig. 3. Dependence of the mRNA expression level of the two tested HSP70 genes on the presence of HLA-DRB1 risk alleles in three studied diagnoses (IIM, SLE and SSc) (in figures displayed as carriers or non-carriers). Allelic polymorphism of HLA-DRB1 and HLA-DQB1 genes was analysed by DNA based typing using commercial sets according to manufacturer's instructions (OneLambda, Los Angeles, USA).

The relation between expression level of HSPA1A and HSPA1B and the presence of DRB1*03 allele in patients with IIM is shown in Figure 3A and 3B. In Figure 3C and 3E, the relation between expression level of HSPA1A and the presence of DRB1*03 and DRB1*15 alleles in patients suffering from SLE is shown. The relation between HSPA1B and the presence of DRB1*03 and DRB1*15 alleles in patients with SLE is shown in Figure 3D and 3F. In patients with scleroderma, the expression level of HSPA1A and HSPA1B genes was analysed according to the presence of DRB1*11 allele. The results are shown in Figures 3G and 3H.

allele. In SLE, majority of studies have identified strong association of classical class II alleles, in particular HLA-DRB1*03:01 and HLA-DRB1*15:01 with this disease (19-21). We have found significantly increased expression of HSPA1A in DRB1*03 negative and also in DRB1*15 negative group of patients compared to DRB1*03 (p<0.05; Fig. 3C) or DRB1*15 (p<0.05; Fig. 3E) negative group of healthy controls. Also in SSc, disease subtypes and auto-antibody profiles are strongly associated with HLA-DRB1 and DQB1 alleles. The most frequently reported associations with SSc are the HLA-DRB1*11 and HLA-DQB1*03 alleles (22-25). In patients with scleroderma, the expression level of HSPA1A gene was found to be significantly higher in HLA-DRB1*11 non-carrier group of patients compared to HLA-DRB1*11 non-carrier group of controls (Fig. 3G). The expression level of HSPA1B gene was not changed in relation to the presence of HLA risk allele in SLE and SSc patients (Fig. 3D, 3F, 3H).

The mRNA expression of HSPA1B correlates with the presence of autoantibodies in patients with myositis and SLE

The presence of disease-specific autoantibodies is a significant marker for the specific disease phenotype. In patients suffering from myositis we have found, that the level of HSPA1A is significantly increased (p<0.05) in patients positive for autoantibodies compared to patients negative for autoantibodies (Fig. 4A). Similar difference was observed when IIM patients positive and negative for autoantibodies were compared with healthy controls (p<0.0001) (Fig. 4A). In this analysis, patients were positive for at least one of the MSA or MAA antibody. When we divided myositis patients into two groups according to the presence of MSA and MAA, we have found that the expression of the HSPA1A gene is significantly higher in patients positive for MSAs and MAAs (p<0.05) when compared to autoantibody negative patients (Fig. 4C, 4E). Similar difference was observed when myositis patients positive and negative for MSA or MAA autoantibodies were compared with healthy controls (p<0.0001) (Fig. 4C, 4E). The expression of the HSPA1B gene was significantly increased in the presence of MSAs compared to MSAs negative patients (p<0.05) and also in the presence of autoantibodies in general, compared to healthy controls (p<0.01) (Fig. 4B, 4D). The expression of HSPA1B was also increased in individuals positive for MAAs, when compared to healthy controls (p<0.01) (Fig. 4F).

In patients suffering from SLE we found that the expression of HSPA1A

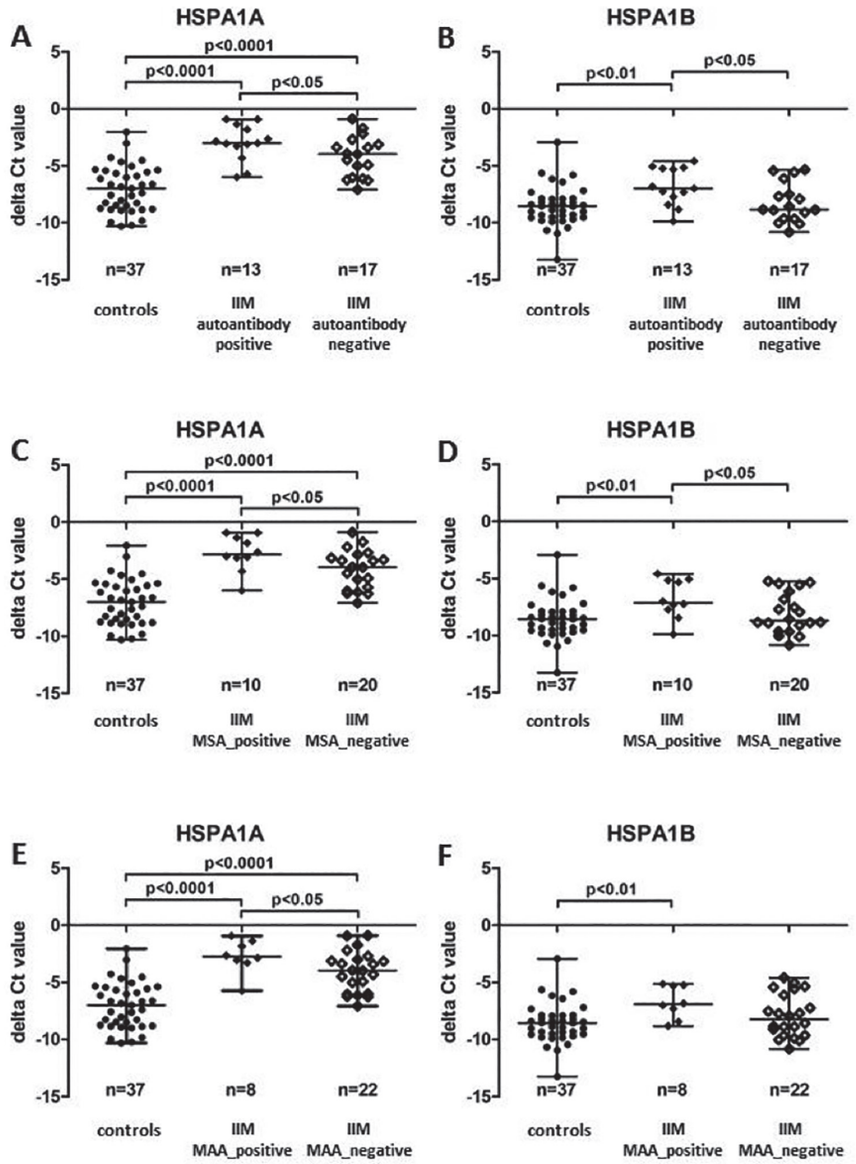

Fig. 4. Expression level of HSP70 mRNA in patients with myositis studied in relation to the presence of MSA/MAA autoantibodies. Autoantibody profiles of IIM patients were determined during routine diagnostic workout using indirect immunofluorescence for antinuclear antibodies (Immuno concepts, USA), line immuno assay (Imtec Human, Germany) and myositis-westernblot (Euroimmun, Germany) for the detection of individual autoantibodies. Main autoantibodies were grouped on the basis of diagnostic specificity: Myositis-specific autoantibodies (MSAs) including anti-tRNA synthetase (anti-Jo-1), anti-Mi-2 and anti-signal recognition particle (anti-SRP), and myositis-associated antibodies (MAAs) such as anti-Ro/SSA, anti-U1RNP, anti-PM/Scl and anti-Ku as the prominent ones.

gene was significantly increased in autoantibody positive group of patients compared to healthy controls ($p<0.01$; Fig. 5A). In patients suffering from SLE we found that the expression of the HSPA1A gene was significantly increased in anti-Sm as well as in anti-dsDNA positive patients compared to healthy controls ($p<0.05$, $p<0.01$; Fig. 5C, 5E). No differences in regulation of the HSPA1B gene were found (Fig. 5B, 5D, 5F). We did not find any relation between the presence of autoantibodies and the regulation of HSPA1A or HSPA1B in patients with SSc (data not shown).

The level of extracellular HSP70 protein is increased in patients; the level of intracellular HSP70 is comparable between patients and healthy controls

Gene expression at the mRNA level is usually closely related to the expression at the protein level. The two inducible HSP70 genes are coding for two isoforms of the HSP70 protein. Therefore, products of HSPA1A and HSPA1B are undistinguishable at the protein level. Since we have found an increased expression of HSP70 genes at the mRNA level, we have also investigated the HSP70 expression of both of the protein forms: the intracellular (in the PBMC samples) and the extracellular (in samples of plasma).

The analysis of expression of the intracellular HSP70 in our patients did not show any significant change in comparison with healthy controls. However, as expected, the highest expression of HSP70 protein was found in $CD14^+$ monocytes in all PBMCs samples from patient as well as from controls (data not shown).

The levels of HSP70 in plasma was increased in whole patient cohorts compared to controls in general ($p<0.01$; Fig. 6A). In detail, the HSP70 levels were higher in patients with myositis (median: 0.153 ng/ml, range [0.010–1.693]) and scleroderma (0.086 ng/ml [0.010–1.667]) compared to healthy controls (0.010 ng/ml [0.010–1.008]; $p<0.01$ and $p<0.05$) (Fig. 6B). However, no significant differences in HSP70 levels were found in patients suffering from SLE compared with controls.

Discussion

Heat shock proteins have been implicated in the pathogenesis of number of autoimmune diseases and inflammatory conditions, such as type-1 diabetes (26, 27) Crohn's disease (28), atherosclerosis (29, 30), or juvenile idiopathic arthritis (31, 32). Additionally, various stressful stimuli, such as hypoxia, toxic chemicals, and inflammation, can induce overexpression of HSPs (33, 34). In this study, we have demonstrated that the expression of two stress-inducible MHC-linked HSP70 genes (HSPA1A and HSPA1B) was lower in healthy controls compared to patients with three various systemic autoimmune diseases - SLE, SSc and IIM. Our results show that the HSPA1B expression level was closely related to IIM since we observed a significant correlation between the increase of HSPA1B with active

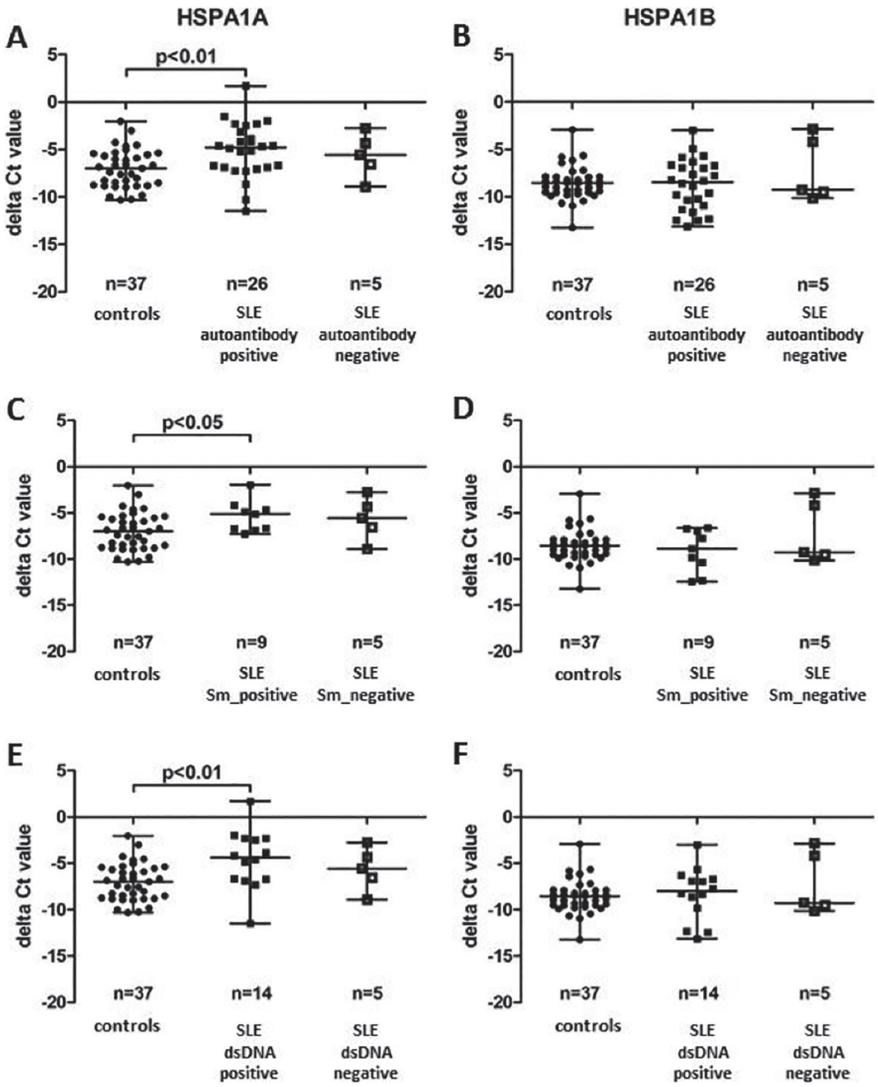

Fig. 5. Comparison of SLE patients positive and negative for disease-specific autoantibodies with each other and with controls. In these patients anti-nuclear (ANA) antibodies were detected using indirect immunofluorescence. Anti-Sm were detected using a line-immuno assay (Euroimmun, Germany) and anti-dsDNA using immunofluorescence (Immuno Concepts, USA) or ELISA (Orgentec, Germany).

IIM, HLA-DR risk allele and autoantibodies. On the other hand, the increase of HSPA1A - in contrast to HSPA1B - is not associated with activity of the diseases, HLA-DR risk allele or either autoantibodies. Differences in expression profile of HSPA1A and HSPA1B gene among patient groups may occur due to the different genetic background of these three autoimmune diseases, but also due to the presence of different alleles of functional polymorphisms in the region of HSP70 genes and their association with these diseases (35).

Many studies have implicated HSP70 gene polymorphism in susceptibility to various autoimmune diseases (36-38). However, these associations are more often caused by the linkage disequilibrium between alleles within the MHC and formation of distinct MHC haplotypes. Since the association between MHC-linked HSP70 genes and HLA class II genes could shed light on the understanding of the genetic contribution to a disease, genes mapping to the MHC class II region have been considered as additional candidates for the onset of the autoimmune diseases (39).

Over the past several years, a number of studies have provided considerable insight regarding genetic contributions in autoimmune diseases. For example, the occurrence of IIM in monozygotic twins and first-degree relatives supports a hypothesis of genetic predisposition in some families (40, 41). The best known genetic risk factors are certain HLA alleles, but the HLA genes that are associated with inflammatory myopathies vary in different population around the world. HLA-DRB1*03:01 is the strongest known risk factor for all major forms of sporadic and familiar forms of myositis in white adults and children in both, the United States and Europe (42, 43). Other associations have been reported for other ethnic groups. In contrast to European American patients with IIM, African American patients with IIM, in particular those with polymyositis, had no strong disease associations with HLA alleles of the 8.1 ancestral haplotype. However, African Americans with dermatomyositis or with anti-Jo-1 autoantibodies shared the risk factor HLA-DRB1*0301 with European Americans. Furthermore, a novel HLA risk factors was detected in African American patients with myositis overlap DRB1*08. In Japanese patients with IIM, the frequency of DRB1*0803 allele was increased compared with controls (44, 45). The typical HLA class II allele predisposing for SLE development is the DRB1*03 and DRB1*15 (46). The relative contribution of genetic background to SSc development is uncertain to date. Early studies showed relatively weak associations with DRB1*11 and DRB1*03:01 (47).

It is generally known, that the disease-related HLA alleles are associated with the presence of disease specific or disease associated autoantibodies. Chinoy *et al.,* observed a strong association between HLA-DRB1*03 and anti-Jo-1 status (48). In African American patients, the frequency of DQA1*0102 allele was strongly associated with anti-signal recognition particle (anti-SRP) and DRB1*0302 allele with anti-Mi-2 autoantibodies (45). In Japanese patients, DRB1*0405 was increased in patients with anti-ARS autoantibodies compared with controls (44). We have also found an increased expression of HSPA1A in the presence of MSA, and MAA in myositis patients.

Based on the existing results we can assume that the increase of HSPA1A gene is not associated with activity of the disease, HLADR risk allele or au-

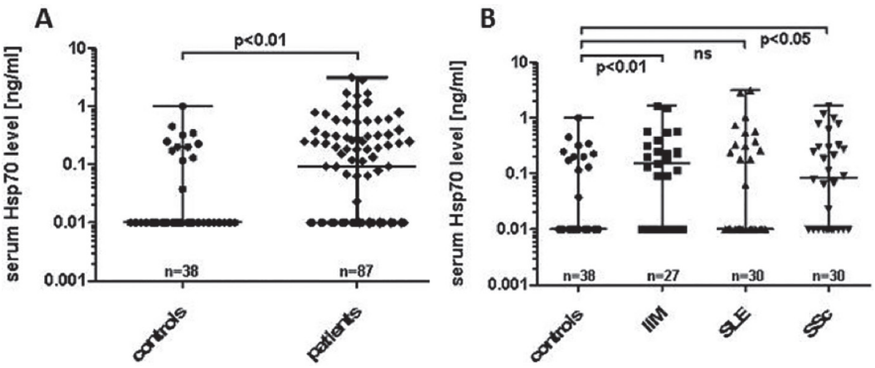

Fig. 6. The expression level of extracellular HSP70 protein in plasma samples of all patients (**A**) with studied systemic autoimmune diseases compared to healthy controls and in patients with three different diagnoses (**B**), respectively. The extracellular HSP70 protein levels were measured in plasma samples using a commercially available high-sensitivity sandwich enzyme-linked immunosorbent assay (ELISA) for use with serum and plasma samples (Assay Designs EKS-715, USA).

toantibodies. Its increase in all studied diagnosis has a biological meaning, which needs to be cleared. It might be for example a consequence of inflammatory processes that take place in autoimmune diseases. On the other hand, the increase of HSPA1B gene is in IIM patients correlated with activity of the disease, HLADR risk allele and autoantibodies.

The expression of HSPs can be induced by various stress conditions. Located inside the cell, they have strong cell-protective effects and behave as molecular chaperones by assisting the folding of newly synthesised polypeptides, the assembly of multiprotein complexes, and the transport of proteins across cellular membranes (49, 50). Apart from their protective roles in the cytosol, HSPs have been found to play key roles in the stimulation of the immune system when located in the extracellular space or on the plasma membrane. HSP70 group, through its peptide-carrier function, can also have cytokine-inducing effect and can demonstrate its immunogenic function (50, 51). Furthermore, stress also induces immune dysregulation partly through alterations in the production of pro-inflammatory cytokines. Both physical and psychological stressors can provoke transient increases in pro-inflammatory cytokines.

It has been suggested that HSPs provide the link between innate and adaptive immune systems (52) and that their presence in the circulation serves as danger signals for the immune system (53, 54). In relation to these findings, our observations that the level of extracellular HSP70 protein is increased in patients suffering from autoimmunity show, that the production of HSP70 in autoimmune process has more immunogenic function than anti-apoptotic function.

In conclusion, our observations point out that the MHC-linked HSP70 genes may be involved in the pathogenesis of distinct types of autoimmunity, at least in the pathogenesis of myositis, SLE and SSc and suggest the HSP70 genes as interesting candidate molecules for either further studies of principles and etiology of autoimmunity, or for possible targeted suppression of distinct types of autoimmunity.

Acknowledgements

We are grateful to Mrs Jitka Smékalová and Markéta Kubálková for their excellent technical assistance.

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