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Genetic and epigenetic factors in etiopathology of AITD: molecular and clinical implications

Czynniki genetyczne i epigenetyczne w etiopatogenezie AITD: znaczenie molekularne oraz kliniczne

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S u m m a r y

Autoimmune thyroid diseases (AITDs) are caused by interactions between many genes, environmental factors as well as epigenetic modifications. In present review we focus on genetic background of AITD, mainly on genetic predisposition in hyperthyroid Graves' disease (GD) and Hashimoto's (goitrous) thyroiditis, including genes affecting the immune response, mainly genes coding human leukocyte antigens (HLA), cytotoxic T-lymphocyte antigen (CTLA-4), CD40, FOXP3, protein tyrosine phosphatase (PTPN-22), as well as STAT proteins. We point out the possible role of epigenetic regulation mechanism via gene promoter methylation in Xq21-22 and Xp11 regions in AITD development. The importance of studies focused on functional polymorphism variants of immunoregulatory genes, genotype-phenotype correlations, as well as not completely yet recognized epigenetic regulation have also been underlined.

Key words: AITD, genetic factors, epigenetic gene regulation

S t r e s z c z e n i e

W rozwoju autoimmunologicznych chorób tarczycy znaczącą rolę odgrywają interakcje pomiędzy genami, czynnikami środowiskowymi a zmianami epigenetycznymi. W naszej pracy przeglądowej skupiamy się przede wszystkim na czynnikach genetycznych predysponujących do rozwoju choroby Hashimoto oraz choroby Gravesa-Basedowa, zwłaszcza genów zaangażowanych w procesy odpowiedzi immunologicznej: HLA, CTLA-4, CD40, FOXP3, PTPN-22 oraz genów STAT. Epigenetyczna regulacja regionu Xq21-22 oraz Xp11, poprzez metylację promotora może być istotnym mechanizmem zaangażowanym w rozwój AITD. Badania genów immunoregulatorowych udowodniły znaczącą rolę polimorfizmów genów oraz korelacji genotyp-fenotyp w AITD, natomiast regulacja epigenetyczna AITD nie jest dokładnie poznana.

Słowa kluczowe: autoimmunologiczne choroby tarczycy, czynniki genetyczne, epigenetyczna regulacja genów

INTRODUCTION

Autoimmune thyroid diseases (AITDs), which include hyperthyroid Graves' disease (GD), Hashimoto's (goitrous) thyroiditis, atrophic autoimmune hypothyroidism, postpartum thyroiditis (PPT), thyroid-associated orbitopathy (TAO), drug-induced thyroiditis, such as interferon-induced thyroiditis, thyroiditis associated with polyglandural autoimmune syndromes and the presence of thyroid antibodies (Tabs) with no apparent clinical disease, are recognized as multifactorial diseases with vital genetic background. The interaction between environmental and genetic factors cause the impairment of self-tolerance to thyroid autoantigens – cellular and humoral immune responses – directed against the thyroid gland due to AITD developing in genetically predisposed individuals. The twin studies and familial aggregation studies have shown the polygenic basis in Graves' disease (GD) and Hashimoto's thyroiditis (HT), the most common endocrine disorders in childhood and adolescence. Both diseases are characterized by the presence of thyroid-reactive T cells and infiltration of the thyroid gland. In GD, the majority of T cells undergo a Th2 differentiation and activate B cells to produce TSH receptor (TSHR) antibodies which stimulate the thyroid resulting in clinical hyperthyroidism. In contrast, HT involves Th1 switching of the thyroid infiltrating T cells which induces apoptosis of thyroid follicular cells and clinical hypothyroidism (1).

In recent years the genetic factors as well as epigenetic background of AITD, particularly GD and HT, have

been significantly recognized. Especially, immune regulatory genes affecting the immune response, such as genes for human leukocyte antigens (HLA), cytotoxic T-lymphocyte antigen (CTLA-4), CD40, FOXP3, protein tyrosine phosphatase (PTPN-22), TSHR as well as genes for signal transducer and activator of transcription proteins (STATs) are acknowledged as predisposing to AITD (fig. 1) (2).

Moreover, the epigenetic mechanism is also observed in pathogenesis of autoimmune diseases. The recent study has shown that X chromosome inactivation (XCI) via DNA methylation, may be involved in AITD development, thus explaining the female-predominant tendency (3).

THE GENETIC FACTORS INVOLVED IN THE ONSET OF AITD

Genes for human major histocompatibility complex (MHC)

The MHC region is recognized as a large genomic area that encodes MHC molecules which play an important role in the immune system and autoimmunity. MHC genomic region consists of a complex of genes located on chromosome 6p21. The genes for MHC molecules are divided into 3 regions: (1) Class I genes encode the HLA antigens A, B, and C, (2) Class II genes encode the heterodimeric HLA-DR, DP, and DQ molecules and (3) Class III genes include genes for: complement components (e.g. *C4*), tumor necrosis factor alpha (TNFα), heat shock protein 70 (Hsp70), and several other genes. The MHC class II molecules are responsible for the initiation of adaptive immune responses. It has been documented that T-cells recognize and respond to antigens when they are attached to the binding groove of an HLA class II molecule (mostly *DR* and *DQ*) on the surface of antigen presenting cell. HLA region is highly polymorphic and contains several other immunoregulatory genes, therefore it has been acknowledged as a pivotal candidate locus for AITD as well as for other autoimmune diseases. Additionally, HLA region is recognized as an important chain of immunological synapse involving peptide antigen bound to HLA molecule, T cell receptor, co-stimulatory molecules, receptors on APCs, as well as integrins (4).

Association of HLA with Graves' disease

Initial studies based on transmission disequilibrium test (TDT) approach, conducted in a large cohort of GD families, have demonstrated strong linkage between GD and HLA. In another studies focused on the role of HMC area in AITD, tough GD association with *HLA-DR3*(*HLA-DRB1*03*) has been found. However, in a study from UK only weak evidence for linkage between GD and the HLA region has been recognized, and another study has reported linkage only when conditioning on *DR3*. Moreover, it has been documented that *HLA-B8* and *HLA-DQA1*0501* seems to be crucial in GD in Caucasians (5), Additionally, there are reports demonstrating the role of *HLA* polymorphisms in the clinical manifestation of GD. Interestingly, some studies have documented an association between the likelihood of relapse of GD and *HLA-DR3* (5). The increased frequency of *HLA-DR3* in patients with Graves' ophthalmopathy has been observed, but it has not been confirmed by others.

Association of HLA with Hashimoto's thyroiditis

The association between HLA haplotypes and HT is less definitive than in GD. Early studies failed to find

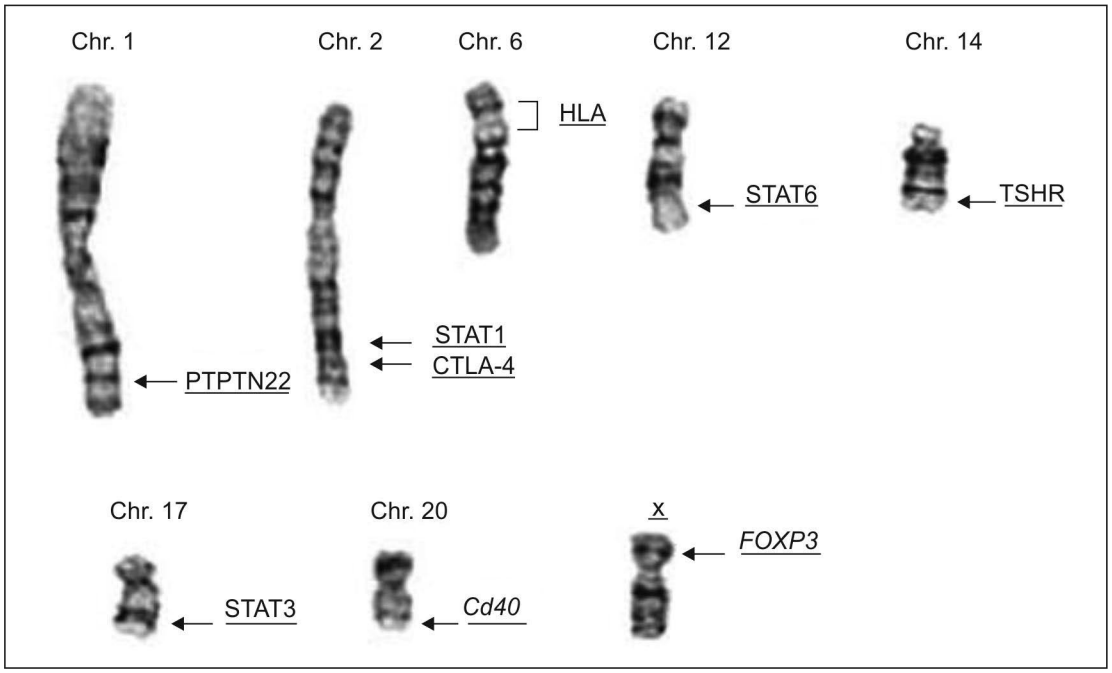

Figure 1. The chromosome localization of main identified AITD susceptibility genes.

an association between goitrous HT and HLA A- B- or C-antigens. Subsequently, the association of goitrous HT with *HLA-DR5* and of atrophic HT with *DR3* has been shown (6). The weak association of HT with *HLA-DR4* (6) and *DR3* (7) in Caucasians was reported in later studies. This observation has been earlier suggested by the studies on animal model in transgenic mice. Finally, *HLA-DQW7* (*DQB1*0301*) was also reported to be associated with HT in Caucasians (8).

Detailed analysis of the HLA class II locus demonstrated that the major HLA haplotype contributing to the shared susceptibility to T1D and AITD was *DR3-DQB1*0201*, with DR3 conferring most of the shared risk (9).

The effect of ethnicity on the association of HLA with AITD

Many studies have documented that susceptibility of HLA loci to AITD varies among populations, and no consensus has been obtained. In non-Caucasian population HLA alleles were different from those observed in Caucasian groups. Strong association of HLA-DR3 with AITD was observed in Caucasians while in Asian population (for example in Japanese) the associations between *HLA-B35* and GD have been found. One study has reported that *HLA-DR3*, the primary HLA class II allele, predisposes to the joint susceptibility for T1D and AITDs in families in which both diseases cluster. Moreover, a significant association between *DRB1*03* and early onset of polyglandular failure has been found, therefore a cooperative susceptibility genes for AITD and other autoimmune diseases have been confirmed (10).

Moreover, an increased frequency of *HLA-BW46* has been reported in Chinese population. However, other class I and II HLA alleles have also been reported to be increased in Asian GD patients. The increased frequency of *HLA-DRB3*0202* has been observed in African-Americans (11). Interestingly, one study of a mixed population in Brazil demonstrated an association with *HLA-DR3*, frequent in European group, indicating that this allele may predispose to AITD in different ethnic groups (12). Alternatively, this Brazilian population may have been comprised mostly of European ancestry.

However, two recent genomewide scans in AITD have failed to detect linkage at 6p21 markers (13). On the other hand, whole-genome linkage screening performed by testing a panel of linkage markers of HLA in AITD, has identified the AITD susceptibility gene on: 2q, 6p (HLA), 8q, 10q, 12q,14q and 20q (4).

Reassuming, the available data suggest that *HLA* involves immune regulatory genes modulating the gene for AITD but not a primary susceptibility gene.

Functional effects of HLA polymorphism

HLA class II molecules are heterodimeric molecules consisting of α and β chain, which form functional highly polymorphic peptide binding pocket (14). In several autoimmune diseases, including AITD, this specific pocket amino acid sequence is recognized as associated with diseases. Moreover, the specific *HLA-DR* pocket variants have recently been identified as vital for the development of Graves' disease (GD) (15) and Hashimoto's thyroiditis (HT).

Sequencing of *HLA-DQ* genes have documented that arginine at position 74 of the DRβ1 chain is a critical shared amino acid for the development of both GD and HT (15). It has been claimed that a molecular marker of the *HLA-DR* pocket, determined by specific amino acids, confers a significant risk for the development of AITD as well as T1D – higher than the risk involving the susceptibility genes. The haplotype consisting of *HLA-DR* pocket amino acids Tyr-26, Leu-67, Lys-71, and Arg-74 has appeared to be strongly associated with AITD and T1D, while amino acids: Leu-26, Phe-26, Ile-67, Asp-70, Glu-71, Ala-71, and Gln-74 have been recognized as protective. Especially, arginine at position 74 of *HLA-DRB1* (*DRB1-Arg-74*), has been shown to be the pivotal in the development of HT and GD (15). Further analysis has shown that the presence of glutamine at position 74 is protective for GD (15). According to functional mechanism of *HLA* polymorphism, it is hypothesized that the presence of *HLA-DR* allele with the appropriate amino acids peptide binding pocket, determines the binding of an autoantigenic thyroidal peptide (16). It is recognized that in pocket 4 (P4) of the DR peptide there is a cleft with position 74 of the DRb1chain. Thus, there is a real possibility that an arginine at position 74 changes the structure of the pocket and therefore has an influence on peptide binding and presentation to T-cells. Indeed, structural modeling analysis has shown that the alteration at position 74, from the common neutral amino acids (Ala or Gln) to a positively charged basic amino acid (Arg), significantly converts the three dimensional structure of the P4 peptide binding pocket. This could modify the peptide binding properties of the DR pocket during presentation to T-cells and favor antigenic peptides that induce GD (15). However, this hypothesis awaits confirmation as very few studies have examined the process of binding and presentation of thyroidal autoantigens to T-cells by different *HLA-DR* subtypes. Interestingly, the results of Hodge et al. (18) study have suggested the interaction at the genetic level between thyroglobulin gene variant and *DRB1-Arg74* predisposing to GD. This observation indicates that the thyroglobulin/*DRB1-Arg74* genetic interaction is revealed in biochemical relations, in which Arg74 implicates the presentation of thyroglobulin peptides in the initiation phase of GD. Similarly, the recent studies have identified a pocket *HLA-DR* amino acid signature that presents strong risk for HT.

Cytotoxic T-lymphocyte-associated protein 4 gene (*CTLA-4*)

The *CTLA-4* gene, located on chromosome 2q33, encodes immunoregulatory molecule, expressed on the surface of activated T cells, which via interaction

with B7 molecule downregulates immune functions mediated by T-cells activation (19). It is known that T-cells are activated by APCs that present to the T-cell receptor (TCR) an antigenic peptide bound to an HLA class II protein on the cell surface. However, a second signal is needed for full T-cell activation, including co-stimulatory signals provided by the APCs themselves or other local cells. The CTLA-4 molecule following activation of the TCR is able to transmit signal in response to its ligation with either B7-1 or B7-2 through competition with CD28.

Human *CTLA-4* gene is postulated as a major negative regulator of T-cell activity and important genetic factor responsible for susceptibility to variety of autoimmune diseases (20), including: type 1 diabetes mellitus (T1D) (21), asthma (22), Addison's disease, myasthenia gravis, Sjogren's syndrome (23), systemic lupus erythematosus (SLE) (24), systemic sclerosis (25), ulcerative colitis (26) and all forms of AITD (GD, HT, as well as the production of thyroid antibodies TAbs) (27).

CTLA-4 gene is highly polymorphic, that has been confirmed in many linkage and association studies in various autoimmune disorders:

1) A to G SNP substitution (49 A/G) at position 49 in exon 1 *CTLA-4*, resulting in threonine to alanine substitution at codon 17;
2) C to T SNP substitution in the promoter region at position _318 relative to exon 1 start site (_318 C/T);
3) microsatellite polymorphism *CTLA-4(AT)n*, which is a dinucleotide (AT) repeat in the 3'UTR of the *CTLA-4* gene
4) A to G SNP located downstream and outside of the 3'UTR of the *CTLA-4* gene (designated CT60).

Recently, it has been claimed that T-effectors activity could be determined by *CTLA-4* SNPs. Especially, A49G dimorphism (Thr/Ala exchange in a peptide) leads to the expression of defective receptor, resulting in the inhibitory effect of CTLA-4 molecule on lymphocyte T-cell (28).

The association between AITDs (HT and/or GD) and *CTLA-4* polymorphisms (A49G, 1822 C/T and CT60 A/G) and some other polymorphic sites has been confirmed in several studies. Microsatellite polymorphism *CTLA-4(AT)n* in 3'UTR region has been reported as a first one associated with autoimmune conditions and consistent in different populations (27). In some studies it has been underlined that *CTLA-4(AT)n* in 3'UTR region is the most powerful polymorphism associated with GD (27). However, particularly *CTLA-4* A49G and CT60 polymorphisms have been correlated with susceptibility to AITD development (29). These associations have been consistent irrespective of ethnic backgrounds, and have been found characteristic in many European as well as Asian populations (30).

Functional effects of CTLA-4 polymorphism

Among the all known polymorphism, *A/G49 SNP* that substitutes threonine for alanine in the signal peptide, leads to misprocessing of *CTLA-4* in the ER, resulting in less efficient glycosylation and diminished surface expression of CTLA-4 protein (31). Other reports bring evidence for association between G allele and reduced control of T-cell proliferation (27). It is claimed that this association may be involved in direct effect of the *A/G49* SNP or another polymorphism in linkage disequilibrium with the *A/G49* SNP. However, the functional studies have revealed that there are no difference in *CTLA-4* expression and/or function in case of transiently transfected T-cell line with endogenous CTLA-4 (Jurkat cells), containing a CTLA-4 construct harboring either G or A allele of the A/G49 SNP (32). This observation has suggested that *A/G49* is not the causative SNP, but rather remains in linkage disequlibrium with the causative variant.

The results of association studies, focused on *C/T_318* SNP *of CTLA-4*, are also controversial. The study of have confirmed the association of C/T_318 haplotype with *CTLA-4* activity, while the results of have been contrary. Recently performed analysis of *C/T_318* SNP have established that *T* allele is related with higher promoter activity in comparison to *C* allele (33). It has been accepted that the presence of *T* allele is associated with significantly enhanced expression of *CTLA-4* on the surface of stimulated cells, and significantly increased *CTLA-4* mRNA level in resting cells (34). Thus, mechanistically, the *C/T_318 SNP* may influence *CTLA-4* levels by changing the binding of a transcription factor *LEF*-1 (ang. *lymphoid enhancing factor 1*), via changing TT(C/T)AAG site, which contains the *C/T* polymorphism (33).

Moreover, it has been shown that *3'UTR (AT)n* is involved in affecting the functions of *CTLA*. It has been demonstrated that individuals who are carriers of the longer repeated (35). Moreover, the long (AT)n repeats are associated with meaningfully shorter half life of *CTLA-4* mRNA in comparison with the short repeats. In addition, the region of *CTLA-4 3'UTR* in which the (AT)n repeats are located contains three AUUUA motifs which may influence the mRNA stability. Similarly, functional analysis of *CT60 SNP* in a small group of patients has suggested that the *GG* genotype (disease susceptible) is associated with the reduced mRNA expression of the soluble form of *CTLA-4*. In contrast, an association between *CT60* genotypes and soluble *CTLA-4* mRNA expression levels has not been found in a recently performed large study (36). Regarding the clinical implication of *CTLA-4 A/G49* SNP polymorphism, it has been documented that *A/G49* in Graves' disease may be associated with the severity of the initial thyrotoxicosis, mirrored in higher levels of free T4. Moreover, the *G* allele of this polymorphism has also been found to be associated with the childhood onset of the disease (37). Patients with diagnosed GD and *GG* genotype of the *CTLA-4 A/G49* SNP more often failed to go into remission after five years on anti-thyroid medications (38). In addition, several studies has shown that the development of Graves ophthalmopathy (GO) – usually oc-

curing in a close temporal relationship with hyperthyroidism – may be associated with CTLA-4 A/G49 SNP. On the other hand, the contrary observations have also been reported (39).

It should be stressed that *CTLA-4* gene region has been found to be linked with the production of thyroid autoantibodies (TAbs) without clinical manifestations. In another report, an association between *G* allele of the *CTLA-4 A*/*G49* SNP and thyroid autoantibody diathesis has been documented (40). The G allele of the *A*/*G49* SNP has been also shown to be responsible for higher levels of both thyroglobulin (TgAbs) and thyroid peroxidase autoantibodies (TPOAbs) (41). However, the subsequent studies have recognized that the functional role of *CTLA-4* is much more complex than previously suggested. It is foolproof that *CTLA-4* may predispose to high levels of Tabs, and at the same time it may eventually lead to the development of clinical AITD. Thus, it has been presumed that *CTLA-4* nonspecifically plays a role in the susceptibility to thyroid autoimmunity, but the interaction with other loci (e.g., *CD40)* and/or environmental factors (e.g., iodine) is necessary to the development of a specific AITD phenotype, such as GD.

B cell-associated molecule gene, (*CD40*)

The *CD40* gene, an important regulator of B cell function, is located within the linked region on chromosome 20q11 and, therefore, it is a possible positional candidate gene for GD. CD40 is a 45-50 kDa transmembrane glycoprotein, which is a cell surface receptor expressed on the surface of all mature B cells and most mature B-cell malignancies. The CD40 molecule has been recognized to be expressed predominantly on B cells, and also on monocytes, dendritic cells, epithelial cells and others (42). It is a member of the tumor necrosis factor receptor family of molecules which bind to a ligand (CD40L or CD154), and are expressed mainly on activated T cells. Binding of CD40L to CD40 induces B cells to proliferate and undergo immunoglobulin isotype switching. CD40 posesses a short cytoplasmic tail with no intrinsic enzymatic activity and directly binds the TNFR *Associated Factors* (TRAFs: TRAF2, TRAF3, TRAF5, and TRAF6). These interactions result in activation of mitogen and stress-activated protein kinase (MAPK/SAPK) cascades, transcription factor activation, cytokine secretion, proliferation, differentiation of B cells into Ig-secreting plasma cells, and the formation of humoral memory (43).

Linkage and association studies have identified *CD40* as a susceptibility gene for GD. Interestingly, linkage studies have shown that the *CD40 locus* was linked and associated with Graves' disease (44), but not with HT. Sequencing analysis of *CD40* gene have documented the presence of *C*/*T* polymorphism at 5'UTR, located in the Kozak sequence of *CD40*, consisted of nucleotides flanking the start ATG codon in vertebrate genes, that is basic to the initiation of translation. Case-control association studies have reported an association between *CC* genotype and GD (44). This finding has been confirmed in several studies, carried out in different populations, including Caucasian (44), Korean (45), and Japanese (46). On the contrary, the results of some other studies have not confirmed the association between *C* allele and GD (47). Recently, based on metaanalysis, an association between the *CC* genotype and GD have been finally demonstrated (48). In addition, persistently high levels of thyroid antibodies after treatment in patients carrying *CC* genotype have been documented (48).

Functional effects of CD40 polymorphism

It has been demonstrated that the *CD40 Kozak SNP* is important in *CD40* expression regulation. The *C* allele of *C*/*T CD40* SNP increases the translational efficiency of nascent *CD40* mRNA transcripts, as compared with T allele. It has been suggested that CC genotype may alter CD40 protein translation and enhance *CD40* expression contributing to Graves' disease development (49).

It has been recognized that the presence of C allele may reveal serious consequences at B-cell level. Even the little changes in *CD40* expression levels may significantly increase the amount of CD40 on the surface of B-cells. The B-cells expressing more *CD40* on the surface are expected to have a lower threshold for activation, and therefore may lead to the autoimmunity development. Indeed, there are some independent study, in which *CD40* upregulation in the context of Graves' disease has been documented. It is claimed that there are two possible mechanism leading to the predominance of peripheral, autoreactive B-cells: 1) changes in B-cell longevity and 2) changes in cellular activation threshold (50).

It is expected, that *C Kozak allele* would also increase the efficiency of translation in various tissues expressing the *CD40*. Thyroid gland has been recognized as a candidate for supporting this statement, regarding the fact that *CD40* is expressed on B-cell as well as on thyroid follicular cells. It is possible that the increased CD40 expression on B-cells and/or thyroid follicular cells predisposes to GD. Moreover, in some functional situations of thyroid, the thyrocyte can express MHC class II molecules and therefore act as a facultative APC.

To sum up, it should be pointed that there are two, non-mutually exclusive mechanism associated with *CD40* overexpression on thyrocytes and genetic susceptibility to GD: an intrinsic mechanism, and an extrinsic mechanism. The last one is related to $CD4^+$T-cell expression and *CD40* signaling pathway activation in the thyrocytes. This pathway, required for dynamic immune responses, also plays a role in autoimmune disease pathogenesis via overexpression of IL-6 that may favor thyroid inflammation. Thus, the extrinsic mechanism, however, concerns an increased costimulation of T-cells by overexpression of *CD40* on thyro-

cytes resulting in the CD4+ T-cell polarization toward a Th2 response. Finally, it may enhance the cytokine production and activate B-cells leading to the increase of TSHR-stimulating antibodies (48). So far, it has been shown that *CD40* Kozak polymorphism may also correlate with rheumatoid arthritis, multiple sclerosis but not with Hashimoto's thyroiditis (44). Recently, it has been also shown that the C-allele of the CD40 Kozak SNP is strongly associated with high IgE levels in asthma (51).

Forkhead box P3 gene; (*FOXP3*)

FOXP3 gene is located in Xp11.23, within the area of autoimmune disease linkage, and therefore is an excellent positional candidate gene for autoimmunity at this locus. *FOXP3* is an essential transcriptional regulatory protein, known as a regulatory T-cell molecule. The level of FOXP3 immunoexpression commits native T cells to become Treg cells, that is critical for normal immune homeostasis (52). *FOXP3* cooperates and associates with a group of other transcriptional factors, co-repressors and co-activators for Treg cells to form one or more dynamic regulatory ensembles (53). These complexes cooperate with multiple key signaling pathways to either upregulate or downregulate the expression of downstream target genes, such as cytokines and cell surface receptors, which are essential to keep normal immune responses under control (53). Two whole genome scans for linkage in GD and two localized linkage scans of the X-chromosome have demonstrated the linkage at X chromosome: in Xq21 *locus* and Xp11 *locus* (54). Additionally, Xp11 has also been linked to other autoimmune diseases, such as type 1 diabetes (T1D), multiple sclerosis, and rheumatoid arthritis, implying that there are common polymorphism(s) leading to the autoimmunity.

According to the recent study performed in the UK population, several *FOXP3* polymorphisms have been recognized as associated with GD. Subsequently, Ban et al. (55) have also found the association between *FOXP3* gene and AITD. They have analyzed the microsatellite polymorphisms *[(GT)n; (TC)n; DXS573; DXS1208]* in some loci flanking the *FOXP3* gene (55). This study have revealed a significant association of *(TC)n* polymorphism with AITD in the Caucasian male AITD patients. Similarly, significant association between the *DXS573 microsatellite* and AITD in the Caucasian female AITD patients has been documented. However, in the Japanese cohort, the association between *FOXP3* gene regions and AITD have not been confirmed. Thus, it has been claimed that *FOXP3* gene contributes to the development of AITD only in Caucasians but not in Japanese population, similarly to the *PTPN22* gene (56). Alternatively, it is possible that different, not recognized so far polymorphisms in the *FOXP3* gene, are associated with AITD in Japanese. Reassuming, it has been assumed that the inherited abnormalities of Treg function may play a major role in the pathoetiology of AITD.

Protein tyrosine phosphatase, non-receptor type 22 gene; (*PTPTN22*)

The lymphoid tyrosine phosphatase (LYP) encoded by the *protein tyrosine phosphatase 22* (*PTPN22*) gene, located on chromosome 1 (1p13), is recognized as a strong suppressor of T-cell activation. It has been confirmed that LYP is expressed in lymphocytes where it interacts with the protein kinase (Csk), an inhibitor of the lymphocyte-specific protein tyrosine kinase (Lck) and the protein tyrosine kinase p59fyn (Fyn). This interaction is involved in T-cell receptor (TCR) signaling pathway.

Some of the functional single nucleotide polymorphisms (SNPs) are recognized as AITD associated gain-of-function variants. A functional *C*/*T* SNP at nucleotide position 1858 leads to an arginine (CGG) to tryptophan (TGG) amino acid substitution at codon 620 (R620W) of the LYP protein. The minor *T* allele of this polymorphism has recently been found to be associated with type 1 diabetes mellitus (T1D) (57) and finally Graves' disease (58), as well as Hashimoto's thyroiditis (59). This SNP has been found to provoke a functional alteration in LYP protein. As the result of this substitution, the LYP protein is unable to associate with C-terminal Src kinase (Csk), a partner molecule in an inhibitory group that is responsible for the regulation of key T-cell receptor signaling kinases (Lck, Fyn, ZAP-70) (57). Interestingly, this mutation may contribute to stronger than gain-of-function inhibition of T-cells (60).

Recently, it has been claimed – but this thesis needs further experimental confirmation – that a lower T-cell signaling causes that self-reactive T-cells escape thymic deletion more and thus remain in the periphery. Studies performed in different geographic regions have shown significant ethnic differences in association between immunological disease and (*PTPN22*) gene polymorphic variants. This is, most likely, due to the founder effects and/or due to the absence of certain variants in some ethnic groups. This also results in genetic heterogeneity. Especially, in the Japanese population the tryptophan variant (Trp/Arg at position 620 *PTPN22*) has not been found (56), however other polymorphisms such as *G1123C polymorphism* (*rs2488457*) in the promoter region, *Arg620Trp* (*C1858T*) *polymorphism* (*rs2476601*) in exon 14, *IMS-JST146695 polymorphism* (*rs3789607*) in intron 19, and SNP*37*(*rs3789604*) downstream of the *PTPN22* gene, have been reported to confer the susceptibility to GD (61). The results of another study have revealed that PTPN22 gene is a joint susceptibility locus for AITD (especially HT) and T1D (62). The mentioned study have confirmed that the frequency of minor *T* allele is meaningfully increased in both (HT and GD) groups of diseases, as compared with patients with T1D, controls or HT and GD independently (62).

Thyroid specific genes: thyroid stimulating hormone receptor gene; (*TSHR*) and thyroglobulin gene (*TG*)

The thyroid stimulating hormone receptor (TSHR) and its ligand, thyroid stimulating hormone (TSH), are

key regulators of thyroid activity. In GD, the stimulating autoantibodies target the TSHR, thus mimicking the action of TSH, and resulting in the characteristic hyperthyroid state. Therefore, not surprisingly that many studies have provided strong evidences that *TSHR* exacerbates the autoimmune process, which may be a key factor, specifically in relation to the onset of GD.

The early studies focused on three nonsynonymous SNPs: *D36H, P52T, D727E* and the intronic SNP C/GIVS1, have provided the ambiguous results. The nsSNP P52T, located in the extracellular domain, has been reported as the first regarding the association with GD in a case-control cohort study. It has been documented that mainly the frequency of 52T allele in GD patients was significantly increased. Similarly, *D727E* located within the intracellular domain, has been shown to be in strong association with GD in a small Russian cohort (63). However, it should be stressed that both *P52T* and *D727E* nsSNP, as well as *D36H*, have failed to be confirmed as associated with GD in some subsequent case-control study of Caucasians or other ethnic populations (64). Regarding intron 1 C/G + IVS1 SNP in *TSHR* gene, the association of G allele with GD have been confirmed in the combined cohort, in Chinese and Indian GD patients separately, but not in the Malay population (64). Therefore, this controversial results support the conclusion that constant searching of explicit evidences for *TSHR* association with GD have been inconclusive. On the other hand, it has been recognized that *TSHR* intron 1 polymorphisms (SNPs rs179247 and rs12101255), located strictly in the proximity of *TSHR* start codon and promoter region, may influence the TSHR expression, by disrupting transcription factor, enhancer/repressor or miRNA binding sites. Indeed, rs179247 and rs12101255 SNPs are functional via interference with transcription factor binding sites, however this observation requires functional validation (65).

Thyroglobulin gene (TG) is recognized as the second pivotal factor which targets the immune response in AITD. The whole-genome linkage study has confirmed the *TG* participation in AITD susceptibility in different populations (5). Moreover, sequencing analysis of *TG* has recognized three amino acid variants that are associated with AITD: *A734S, V1027M*, and *W1999R* (5). It has been proposed that functionally *TG* variants may susceptible to disease by altering *TG* degradation in endosomes, resulting in a pathogenic TG peptide selection. Additionally, a genetic interaction between *TG* variant – W1999R and *HLA-DR*β*-Arg74* – in GD patients has been documented to influence the peptide interactions (66). Further, it has been shown that only a small set of exclusive TG peptides can bind to the *HLA-DR*β*-Arg74* pockets and one of these peptides represents a major T-cell epitope (67). Recently, a 1623A/G SNP in *TG* promoter has been observed and associated with AITD in Caucasian population. It has been recognized that this type of SNP causes the modification of binding site for interferon regulatory factor-1 (IRF-1), in which the G allele associated with disease leads to the increased level of *TG* promoter activity by *IRF-1* interaction (68).

Genes encoded Signal Transducers and Activators of Transcription proteins; STATs

The family of STAT proteins (STAT1-STAT4, STAT5A, STAT5B and STAT6) has been documented to participate in normal cellular process, such as: differentiation, proliferation and immune responses. Constitutively activated STATs are involved in an aberrant signaling pathway which may be the cause of autoimmune disorders and cancer development. STAT proteins are confirmed as managers of Th cells activity (mainly Th1, secreting: IL-2, IL-3, IFN-γ, GM-CSF; Th2, secreting: IL-4, IL-5, IL-10, IL-13), influencing as positive and negative factors on Th cells. A large number of cytokines, hormonal factors and growth factors may activate JAK and/or STAT proteins and modulate immunity and inflammation. Cytokines can induce thyrocyte apoptosis via activation of different signaling pathways and transcription factors and therefore cause thyroid injury.

It has been recognized that in immunological processes of thyroid gland STAT1 may become activated after thyrocytes stimulation by IFN-γ. STAT1 encoded by *STAT1* gene mapped to 2q32 can be activated by IFN-γ, which influences JAK1 and JAK2. STAT1 protein is probably responsible for intercellular adhesion molecule 1 (ICAM-1) expression on the cells secreting T3 and T. In case of thyroid autoimmunological disease development (mainly GD) the decreased immunoexpression of ICAM-1 has been observed. Simultaneously, the increased level of ICAM-1 after exposure to INF-γ in GD has been documented. TSH may block STAT1, JAK1 and α receptor subunit for INF-γ and therefore cause the inhibition of ICAM-1 syntesis. Additionally, TSH may inhibit the induced by INF-γ class II transactivator (CIITA) protein production – responsible for MHC I and II antigens expression.

In the course of Grave's disease, the transcription of the following genes increases: LMP2, TAP, HLA-DM, STAT and NFkB proteins, and therefore the thyrocyte is transformed into antigen presenting cell (APC). In case of lack of STAT1 activation in the development of autoimmunological diseases, the activation of STAT3 is observed. The *STAT3* gene – located on chromosome 17q21 – is regulated by IL-6 signaling pathway *via* inducing the JAK/STAT3 and Ras/Erk/C/EBP pathways (69). In experimental models as well as *in vivo*, it has been confirmed that IL-6 signaling pathway is a major regulator of Treg/Th17 cells and may promote an acute inflammation (69). Additionally, it has been documented that IL6 activates Th2 cytokine production in $CD4^+$ T lymphocytes via CREB transcription factor. In Marazuela et al. study (70) the increased level of $CD4^+$ T lymphocytes in mononuclear cells from human peripheral blood of patients with AITD as well as the disturbed expression of *IL-10, TGF-*β*, FOXP3* and *STAT1* and *STAT3* genes have been observed. More-

over, in chronic inflammation, STAT3 activation may be conducted by IL-10 IL-17, IL-20, IL-21, IL-22, IL-27, TNF, and G-CSF, (71).

STAT6 gene – mapped to 12q13 (161) – regulates Th2 immunity and is recognized as an essential factor for the development of Graves' hyperthyroidism (3). STAT6 is activated mainly by IL-4 and IL-13 and together with GATA3 transcription factor promotes Th2 differentiation.

In addition, *STAT4* gene (locus 2q32.2-q32.3), coding STAT4 protein, is claimed as a pivotal factor in autoimmune diseases and chronic inflammation development. It has been documented in knockout mice that lack of *STAT4* gene leads to Th1-dependent autoimmune diseases and chronic inflammation development. Functionally, it has been recognized that some cytokines (mainly IL-12 and IL23) may activate STAT4, acting as regulator of STAT4-dependent Th0/Th1 balance via IL-2, IL-12, IFN-γ, TNF-β interaction (72).

The suppressor of cytokine signaling (SOCS) is claimed to be essential in negative regulation of JAK/STAT signaling *via* binding inhibitors of JAKs.

In vivo study, focused on the family of CIS/SOCS/JAB/SSI negative regulators, has documented the vital role of SOCS and CIS inhibitor in inflammation pathway (73). SOCS3 is recognized as activated by IL-1 as well as by TNF, whereas CIS3 is induced by different pro- and anti-inflammatory cytokines (e.g., IL-6, IFN-γ and IL-10). The above mentioned cytokines and STATs may be negatively regulated by strong activation of CIS3. During the inflammatory process, in negative feedback regulation, the increased activation of SOCS3 can inhibit IL-6-mediated STAT3 activation. On the other hand, it has been shown that SOCS3 is not the only one negative regulator of inflammatory reaction and it is possible that SOCS3 expression is not able to reach an enough level to decrease STAT3 activation (74).

THE EPIGENETIC MECHANISM INVOLVED IN THE ONSET OF AITD

The epigenetic gene regulation is found in autoimmunity, inflammation-associated cancer, chronic inflammation, as well as in AITD. Epigenetic gene regulation involves different mechanisms: DNA methylation, chromatin remodeling and post-translational modifications of N-terminal tails of histones (75). The mechanism of DNA promoter gene methylation involving covalent attachment of a methyl group at position 5' in the cytosine ring chain is fundamental in gene silencing (75). Additionally, apart from DNA methylation, histone modifications, including acetylation, phosphorylation, ubiquitination, SUMOylation, methylation and ADP ribosylation of core histones H2A, H2B, H3 and H4 have been recognized as a mechanism of chromatin remodeling. This process is regarded as dynamic competition between transcription factors and histone for cis-regulatory sequences in gene promoters (76). In thyroid gland the epigenetic modulation involved in cancer development has been demonstrated in some genes, e.g., *TSHR, NIS, TPO* and pendrin gene (SLC26A4) (77). Unfortunately, little is known about epigenetic modifications in AITD development. So far, the silencing of *TSHR* gene *via* promoter hypermethylation has been documented in patients with diagnosed AITD. The studies focused on thyroid epigenetic modification in patients with AITD have identified some silenced genes in X chromosome (region Xq21-22), e.g. *BTK, AGMXI, XLA* (78). These genes are known as significant factors in immune processes regulation. It has been recognized that BTK is an important regulator of B-cell development (78). *AGMXI* has been recognized as a primary factor of a human immunodeficiency, which is characterized by profoundly low level or absent serum antibodies or B cells circulating in blood due to an early stage of blocking of B-cell development. *XLA* has also been documented as an immunodeficiency factor involved in disturb producing of mature B lymphocytes and has been recognized in association with rearrangement failure of Ig heavy chain.

It has been claimed that Gal-1 protein may exert pleiotropic effects in the pathogenesis of *MLL* rearranged leukemias by modulation of angiogenesis and adhesion in tumor models (79). Moreover, the increased *Gal-1* expression in *MLL*-rearranged B-ALL may be possible determined by MLL-mediated chromatin modification (80). This study has suggested that epigenetic modification of immunoregulatory gene can be also involved in the development of AITD, but this thesis should be supported byfuture studies.

CONCLUSION AND FUTURE DIRECTIONS

The application of molecular biology to the study of AITD has undoubtedly made the significant progress in determining the complex factors that lead to the development of AITD. Another important aspect of these findings is the analysis of the functional consequences of AITD susceptibility genes variants and the search for genotype-phenotype correlations. It is clear that in the near future new susceptibility genes for AITD will be identified, and the mechanisms through which they contribute to the disease development will be explained. This will enable the rapid identification of those individuals, who are at higher risk for AITD before the clinical symptoms. Hopefully, these new discoveries will also be reflected in improved therapeutic targets and novel treatments in the near future. Treatment options will be presumably directed to more preventive strategies, as opposed to the palliative methods of the present management. The therapy of patients with clinical symptoms of the disease will be personalized by targeting the specific pathways that lead to the development an individual's subtype of AITD.

Gene therapy is a promising treatment option for a number of diseases and is gaining more and more importance regarding treatment of autoimmune disorders. However, the technique remains risky, cost intensive and sometimes is associated with side effects,

thus further studies are required to be sure that it will be safe and effective. In AITD vital interaction has been documented between thyroid specific genes, susceptibility genes, environmental factors and immunological synapse genes. It has been proven that these relationships are involved in the genetic susceptibility to AITD and are helpful in the differentiation of AITD phenotypes and response to the therapy.

Epigenetic mechanisms of interaction between environmental (IFNα) and genetic (TG) factors trigger the AITD. Future studies on genetic and epigenetic variations will make it possible to quantify the precise effect of specific susceptibility genes and/or epigenetic variation in estimating the heritability. The relationship between susceptibility genes, environmental factors and epigenetic modulation results in breakdown the self-tolerance leading to AITD. New data have been developed that have shed light on the nature of the susceptibility genes for and the pathophysiology of AITD.

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otrzymano/received: 12.09.2011
zaakceptowano/accepted: 17.10.2011

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