Summary

Cancers remain one of the greatest health challenges of the modern world and represent the second leading cause of death in Poland. Despite significant advances in diagnostics and therapy, the prognosis for many malignancies, including non-small cell lung cancer (NSCLC), bladder cancer (BC), and chronic lymphocytic leukemia (CLL), remains poor. One of the key mechanisms underlying tumor development and progression is the ability of cancer cells to evade immune surveillance, partly through the dysregulation of immune checkpoint (IC) molecule expression. These molecules regulate the delicate balance between immune activation and tolerance, and their altered expression can lead to immunosuppression within the tumor microenvironment.

Classical immune checkpoints such as PD-1/PD-L1 and CTLA-4 form the cornerstone of modern immunotherapy. However, growing attention has been directed toward less-characterized molecules, like BTLA. BTLA (B and T lymphocyte attenuator) is an inhibitory receptor of the immunoglobulin superfamily, expressed on T, B, and NK cells, among others. Through its interaction with the ligand HVEM, BTLA transmits inhibitory signals that suppress immune responses. The BTLA/HVEM axis plays a crucial role in maintaining proper immune homeostasis, and its dysregulation can shift the balance toward inhibitory signaling, promoting immunosuppression and enabling tumor cells to evade immune recognition.

Genetic variability in genes encoding ICs, particularly single nucleotide polymorphisms (SNPs), represents one of the factors that can modulate protein expression or function, thereby influencing immune responses and cancer susceptibility. Depending on their location, SNPs may affect gene expression, RNA stability, protein structure, ligand binding, or intracellular signaling. Such changes can substantially impact immune system activity, either enhancing or weakening immune surveillance, and consequently influencing tumor initiation, progression, and therapeutic response.

In parallel, soluble forms of immune checkpoint molecules (soluble ICs) are important regulators of immune responses found in plasma or serum. These soluble molecules retain the ability to bind their respective ligands or receptors, functioning as circulating modulators of immune activity.

Another critical mechanism controlling the expression of IC molecules is epigenetic regulation mediated by microRNAs (miRNAs). miRNAs are endogenous RNA molecules that regulate gene expression at the post-transcriptional level by inhibiting protein translation. Aberrant miRNA expression profiles have been linked to a wide range of human diseases, including cancer. Dysregulated miRNAs play an important role in tumor pathogenesis by modulating the expression of genes involved in immune regulation, cell proliferation, and apoptosis.

Given its pivotal role in immune regulation across both solid and hematological malignancies, BTLA has emerged as a molecule of increasing interest, as its altered expression may facilitate tumor immune escape and impact disease progression.

The aim of my doctoral dissertation was to evaluate the significance of BTLA gene polymorphisms, epigenetic mechanisms of its protein expression, and the soluble form of this molecule in the pathogenesis of cancer, which may contribute to the identification of new diagnostic, prognostic, or predictive biomarkers.

The introductory part of this dissertation is a review article (Andrzejczak et al., Biomarker Research, 2024), which provides a comprehensive overview of BTLA biology, including its signaling mechanisms, role in immune regulation, and therapeutic potential in oncology.

The dissertation consists of two main research parts. The first part includes three original papers. Two of them focus on the analysis of IC gene polymorphisms in the context of cancer pathogenesis and susceptibility to NSCLC and BC. The third study investigates serum levels of soluble forms of IC molecules, whose concentrations may serve as potential diagnostic or prognostic biomarkers in BC. For the purposes of this dissertation, which primarily focuses on BTLA, only the results directly related to the BTLA gene or its soluble form are summarized below, while findings concerning other IC molecules are not discussed.

The first study (Andrzejczak et al., Frontiers in Immunology, 2023) investigated the presence of seven BTLA gene polymorphisms and analyzed their associations with disease risk, progression, and overall survival (OS) in 383 patients and 474 healthy controls (HC).

In the overall cohort, carriers of the G allele of rs1982809 (AG+GG) were more frequent among NSCLC patients compared to HC (45.29% vs. 38.82%), suggesting that the G allele increases NSCLC susceptibility (OR 1.3). Allele frequency analysis further confirmed a significant association between the G allele of rs1982809 and increased NSCLC risk (OR 1.25). No significant associations were observed for the remaining SNPs.

When smoking status was taken into account, significant differences in rs1982809 genotype distribution were observed among never-smokers compared with the control group. The presence of the A allele (AA+AG) was associated with approximately a 3-fold reduction in NSCLC risk, suggesting a potential protective role. In contrast, no significant associations between rs1982809 genotypes and NSCLC risk were found among smokers.

Sex-stratified analysis revealed significant differences in rs9288953 genotype distribution between female patients and healthy women. The presence of the T allele (CT+TT) increased the risk of NSCLC in women by more than 2-fold. Similarly, carriers of the G allele of rs1982809 (AG+GG) showed a trend toward higher NSCLC risk, consistent with the overall findings. No significant associations were observed among male patients.

Histological subtype analysis did not reveal statistically significant differences, however, adenocarcinoma patients exhibited a non-significant trend toward higher frequencies of the G allele of rs1982809 (AG+GG) and the C allele of rs2705511 (AC+CC) compared to HC (46.85% vs. 38.82% and 50.45% vs. 42.19%, respectively). Stage-specific analysis indicated an overrepresentation of the G allele of rs1982809 and the C allele of rs2705511 in stage II, and carriers of G allel rs1982809 (AG+GG), C rs2705511 (AC+CC), and T rs9288953 (CT+TT) in stage III, while no significant associations were found in stage IV.

Analysis of OS revealed that two variants significantly influenced patient outcomes. Carriers of the C allele of rs2705511 (CC+CA) and the G allele of rs1982809 (GG+AG) lived approximately six months longer than homozygotes for the AA genotype of both SNPs. For rs9288952, a trend toward shorter OS was observed among G allele carriers, whereas homozygosity for CC in rs11921669 was associated with longer OS compared to T allele carriers.

In an unpublished manuscript submitted to the Polish Archives of Internal Medicine, four BTLA polymorphisms were analyzed in 314 patients and over 520 healthy individuals to assess their association with BC susceptibility and clinical course.

Some of the analyzed BTLA polymorphisms appeared to influence BC susceptibility. For rs2705511, genotype distribution differed significantly between patients and HC, with heterozygotes (AC) markedly overrepresented (43.31% vs. 36.87%), suggesting an increased risk for carriers of this genotype (OR 2.5). A similar pattern was observed for rs1982809, with heterozygotes (AG) more frequent among patients (42.04% vs. 34.83%), while rs9288953 showed a non-significant trend toward a higher frequency of the CT genotype in BC patients (53.72% vs. 46.61%).

Sex-stratified analysis demonstrated that rs1982809 was associated with BC susceptibility among female patients. Significant differences in rs1982809 genotype distribution were observed between female patients and healthy women, with the presence of the G allele and AG genotype associated with a 2-fold increase in BC risk.

Multivariate analysis that included all investigated polymorphisms, age, sex, and clinical factors revealed that rs1844089 was significantly associated with tumor grade, with the A allele correlating with a 5-fold higher risk of high-grade tumors.

In another study, accepted for review in Biomarker Research, serum levels of soluble BTLA (sBTLA) were analyzed in 48 BC patients and 27 HC. sBTLA concentrations were more than twice as high in BC patients compared to HC. Elevated sBTLA levels were observed across all clinical stages and tumor grades, with no significant differences between subgroups. Correlation analysis showed a moderate positive correlation between sBTLA and sGal-9, as well as a weaker but

statistically significant correlation with sTIM-3, suggesting that sBTLA may be functionally linked to other key mediators of immune regulation.

The second part of this dissertation comprises two original research papers devoted to the role of BTLA in CLL. The first focused on the epigenetic regulation of BTLA expression and its impact on B-cell proliferation and IL-4 production, while the second explored the potential of targeting the BTLA mRNA–miR-155-5p interaction as a novel immunotherapeutic strategy. The present dissertation discusses findings specifically related to the epigenetic regulation of BTLA expression mediated by miR-155-5p in the context of CLL pathogenesis. The effects of BTLA dysregulation on T-cell proliferation and function are not addressed here, as these studies were conducted by a different research group.

In the study by Karabon et al. (Cells, 2021), the hypothesis proposed by prof. Karabon's group, based on their previous findings, regarding the role of miR-155-5p in the epigenetic regulation of BTLA expression in human leukemic B cells was investigated. Expression analyses revealed that miR-155-5p levels were, on average, 3-fold higher, and BTLA mRNA levels 6-fold higher in CLL patients compared with HC. A similar pattern of BTLA mRNA and miR-155-5p overexpression was observed in the MEC-1 cell line, a well-established CLL model.

To verify the hypothesis that miR-155-5p regulates BTLA expression, PBMCs from CLL patients and HC, as well as MEC-1 cells, were transfected with a miR-155-5p inhibitor (siRNA). After 24 hours, the levels of miR-155-5p, BTLA mRNA, and BTLA protein were assessed. Transfection with the inhibitor resulted in a 10-fold reduction in miR-155-5p levels without significant changes in BTLA mRNA abundance. However, a slight but statistically significant increase in BTLA protein was observed, approximately 5% in CLL B cells and 10% in MEC-1 cells, with no change in HC cells. These results indirectly demonstrated that BTLA expression is regulated by miR-155-5p.

This line of investigation was further extended in Kosmaczewska et al. (Biomolecules, 2025), which confirmed a direct interaction between miR-155-5p and BTLA mRNA. In silico analysis identified a canonical 7mer-A1 binding motif for miR-155-5p within the 3′UTR region of the BTLA gene. This interaction was validated using a luciferase reporter assay. Functional assays employed constructs containing either the full-length or truncated BTLA 3′UTR sequence in wild-type (WT) and mutant (MUT) forms. Co-transfection of HEK293 cells with miR-155-5p and WT constructs resulted in a 20% decrease in luciferase activity for the full-length 3′UTR and an approximately 40% decrease for the truncated form, whereas no effect was observed with MUT constructs or siRNA controls.

The second aspect of the study evaluated the role of miR-155-5p in regulating BTLA levels in T cells from CLL patients to assess the feasibility of miR-155-5p inhibition as an immunotherapeutic approach. Transfection of PBMCs with the miR-155-5p inhibitor did not cause significant alterations in BTLA protein expression in T cells in any of the studied groups.

Collectively, the findings from the five original research papers comprising this dissertation highlight the key role of BTLA gene polymorphisms, sBTLA, and miRNA-mediated epigenetic regulation in modulating immune responses within the tumor microenvironment. Specific BTLA genetic variants were associated with the development and progression of NSCLC and BC, while the soluble form of BTLA emerged as a potential diagnostic biomarker in BC. Furthermore, studies on epigenetic regulation demonstrated that miR-155-5p overexpression in B cells from CLL patients leads to reduced BTLA protein expression, contributing to immune dysregulation in this malignancy. Notably, miR-155-5p inhibition partially restored BTLA expression in leukemic cells without affecting normal T lymphocytes, suggesting the therapeutic potential of this approach as a selective and safe strategy for CLL treatment.