The molecular portrait of triple-negative breast cancer: the LAG3 gene single nucleotide polymorphism rs2365094 has no impact on the clinical picture

Katarzyna Boguszewska-Byczkiewicz¹, Thomas Wow², Bożena Szymańska³, Michał Kośny⁴, Agnieszka Kołacińska-Wow⁵

Introduction. Triple-negative breast cancer (TNBC) is characterized by a lack of oestrogen, progesterone and human epidermal growth factor receptors. It is the one of most heterogeneous and highly-aggressive breast cancers, resulting in fast progression. In humans, the lymphocyte activation gene 3 (LAG3) is located on chromosome 12p13 and encodes an immune-regulatory molecule. The aim of the study was to perform a molecular analysis of LAG3 gene polymorphisms.

Material and method. The presence of single-nucleotide polymorphisms (SNPs) at rs2365094 was determined in 30 TNBC patients and 30 healthy controls using a polymerase chain reaction (PCR) and commercially-available TaqMan SNP Genotyping Assays. SNP status was then compared with the clinical outcome.

Results. The allelic alterations in LAG3 gene SNP in rs2365094 appear to have no influence on the clinicopathological picture among TNBC patients. The carriage rate for a single allele did not differ significantly between patients and controls.

Conclusions. No significant relationship was observed between the rs2365094 SNP status and clinicopathological determinants.

Key words: LAG3, triple negative breast cancer, immune checkpoints, immunotherapy
LAG3, CD223, known since 1990 [11], is a non-cellular component of the tumor microenvironment, a transmembrane protein consisting of 489 amino acids and a member of the IgG group. The LAG3 protein is integrally expressed by tumor cells and immune cells and has been associated with a clinical response [12]. The ligand of LAG3 is FGL1 [13]. Although it is expressed on the surface of breast cancer cells, LAG3 can also be found in the cytoplasm in non-small cell lung cancer (NSCLC) [13, 14]. LAG3, together with PD-1, inhibits anti-tumor immunity by interacting with MHC-II on activated T cells [9], LAG3 inhibits CD8+ and CD4+ T cell proliferation [15]. LAG3 signaling blockade restores anti-tumor activity. Although LAG3 has been introduced for immunotherapy in TNBC, insufficient response has been noted [16].

TNBC and hormone-receptor-positive breast cancer cells have been found to co-express LAG3 and PD-1 or PD ligand 1 (PD-L1), known as double-positive expression [17, 18]. In addition, approximately half of PD-L1+ TNBC cells have been found to demonstrate LAG3 and PD-1 co-expression [18, 19].

Such co-expression reinforces cytokine production by LAG3/FGL1 ligand conjugation [13]. Resistance to immune check-
lymph node status, Ki-67 (%) level and histological subtype are presented in table I.

Genomic DNA was isolated from 200 µL of frozen blood using the GeneMATRIX Quick Blood DNA Purification Kit (EURex) according to the manufacturer's protocol. DNA was quantified using the PicoDrop spectrophotometer (Picodrop Limited) and immediately used for PCR reaction or stored at −20°C.

The status of the rs2365094 SNP in the \textit{LAG3} gene was determined using a polymerase chain reaction (PCR) and commercially-available TaqMan SNP Genotyping Assays (Applied Biosystems): Context Sequence GGAGAAGACAAGTCTAAAGC-CAGGT\([\text{C/G}]\)CCTGTTTCCAGGAGCTTCCGGCTTG (table II). PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems) in a 20 µl reaction volume containing 10 ng DNA, 10 µl TaqMan® Universal PCR Master Mix and 0.5 µL (40x) appropriate TaqMan® SNP Genotyping Assay. The following PCR cycle was performed: initial denaturing at 95°C for 10 min; 40 cycles of 92°C for 15 s and 60°C for 1 min. Each 96-well plate contained the test samples and three reaction mixtures without DNA template (no-template control). End-point fluorescent intensities of each probe were monitored using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The genotypes were determined automatically and verified visually using Sequence Detection System 2.3 Software.

**Statistical analysis**

Patient data and the SNP status of the gene coding for the LAG3 gene were analyzed using the \textit{chi}² test with Fisher’s exact test (taking into consideration the small sample size); the aim was to determine the significance of the co-occurrence between the minor allele and the clinicopathological picture. The addition of Fisher’s exact test provides more reliable results with the smaller studied group. Furthermore, logistic regression was performed to determine the impact of \textit{minor allele load} (i.e. the number of minor allele variants – 0, 1 or 2) on the risk of cancer development and certain clinical aspects. The odds ratio (OR) with 95% confidence interval (CI) was also calculated to evaluate the risk associated with allele frequency of rs2365094 (C/G). Statistical significance was assumed for \( p = 0.05 \). The analysis was performed using the Statistica v.13 TIBCO Software Inc.

**Results**

The study examines the status of potential polymorphic changes in \textit{LAG3} at rs2365094 (C/G) in 30 TNBC patients and 30 healthy controls. The rs2365094 reference allele is G, present in the population at a level of 0.71091 (G = 0.71091) [29]. In the TNBC group, 18 (60%) were found to be rs2365094 GG carriers, 11 were CG carriers (36.7%), and one (3.3%) was a CC carrier. Regarding the healthy controls, 13 (43.3%) were GG carriers, 14 (47.7%) CG carriers, and three (10.0%) CC carriers. Additionally, most genotypes were homozygous GG; these were found at a slightly higher frequency in the patients than the controls (60% vs. 43.3%), but this was not significant (\( p = 0.3634 \)).

Our findings do not indicate any association between the status of the rs2365094 polymorphism and the risk of cancer progression. Also, no correlation was observed between rs2365094 minor allele distribution and the risk of TNBC (OR 0.5319; CI 95%; 0.2257–1.2535; \( p = 0.1489 \)). In addition, the rs2365094 SNP did not appear to have any significant relationship with the TNBC phenotype, nor with the tested clinicopathological parameters (tumor size, lymph node invasion, and Ki-67 status). Evaluation was included for 60 samples (table III, fig. 1 and 2).

**Discussion**

Immune checkpoints are immunotherapeutic targets and have often yielded remarkable outcomes in treating advanced malignancies. The LAG3 protein is involved in the activation of T cells and in maintaining immune homeostasis. LAG3 activation is used by tumor cells to evade the host immune system.

**Table I.** The clinicopathological characteristics of breast cancer patients participating in the study (N = 30)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>age, years median (range)</td>
<td>67.5 years (38–84 years)</td>
</tr>
<tr>
<td>side of involved breast</td>
<td>left – 14 right – 17</td>
</tr>
<tr>
<td>tumor size (T in TNM classification 2021)</td>
<td>T1a – 1 T1b – 9 T2 – 12 T3 – 1 T4 – 6</td>
</tr>
<tr>
<td>node status (N in TNM classification 2021)</td>
<td>Nx – 1 N0 – 15 N1 – 3 N2 – 5 N3 – 6</td>
</tr>
<tr>
<td>Ki-67(%)</td>
<td>&lt;20% – 3 ≥20% – 27</td>
</tr>
<tr>
<td>histological grade</td>
<td>grade 1 – 1 grade 2 – 14 grade 3 – 15</td>
</tr>
</tbody>
</table>

**Table II.** Characteristics of lymphocyte activating 3 gene (LAG3) rs2365094 sequence, primer and chromosomal location

<table>
<thead>
<tr>
<th>Gene name</th>
<th>SNP (rs) number</th>
<th>Chromosomal location</th>
<th>Primer sequence</th>
<th>Polymorphism</th>
<th>Minor allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAG3</td>
<td>rs2365094</td>
<td>chr.12.6774504 on build GRCh38</td>
<td>context sequence [VIC/FAM] GGAGAAGACAAGTCTAAAGAGGAGGT([C/G])CCTGTTTCCAGGAGCTTCCGGCTTG</td>
<td>C/G, transversion substitution</td>
<td>G = 0.71091</td>
</tr>
</tbody>
</table>

113
Administration (FDA), no reliable or precise LAG3 marker is available to guide the clinical use of anti-LAG3 therapy. It is noteworthy that the incorporation of immunocheckpoint expression into a basic diagnostic panel can yield significant benefits for TNBC patients. LAG3 transmembrane protein expression has been found to demonstrate prognostic value in a large series of breast cancer patients and LAG3 expression correlated with crucial biomarkers. It has been found that the level of infiltration of LAG3-positive basal-like breast cancer cells in the tumor microenvironment appears to be significantly associated with increased survival, and that LAG3 and PD-1 are co-expressed on tumor infiltrating lymphocytes (TIL) [2]. For tumor therapy, Shi et al. [12] note that LAG-3 protein expression appears to influence anti-PD-1, EGFR-TKI and gefitinib therapy resistance.

Tumor-associated stromal cells support and increase tumor metastatic potential. Studies on metastatic TNBC immunotherapeutics suggest that the formation of the tumor microenvironment may influence drug resistance: out of all breast cancers, TNBC has been found to have the highest amounts of tumor-infiltrating lymphocytes [31]. However, no data exists on the functional significance of any LAG3 gene SNPs in the immune-environmental network of various macromolecules.

Recently, a number of studies have examined the potential of anti-LAG3, either alone or in combination with PD-1/PD-L1 blockade, for treating cancer. So far, three LAG3-targeted immunotherapeutics have been identified:

- a phase I clinical trial examined the use of a first-in-class biospecific molecule binding LAG3 and PD-1 (MDG013 [20]) together with NCT03219268, FS118 [21] in TNBC,
- soluble LAG3Ig (IMP321, clinically tested in metastatic breast carcinoma [30]),
- antagonistic LAG3 antibodies (immunotherapeutics drug named: LAG525, BMS-986016, REGN3767, TSR-033).

Among known molecular biomarkers, the TNBC subtype is considered to be one of the most immunogenic. LAG3 and PD-1/PD-L1 are mutually expressed within TNBC tumor-infiltrating cells and tumor cells. As a result, LAG3-targeted immunotherapeutics designed to coordinately block PD-1 and LAG3 are almost perfect for treatment. A study of tumor-infiltrating lymphocytes, co-expressing PD-L1 and LAG3 in TNBC patients, found all LAG3-positive cases to be PD-L1-positive, but not vice versa [18]. A combined blockade of PD-1 and LAG3 could yield survival benefits exclusively in PD-L1 and LAG3-positive TNBC patients. Although immunohistochemical testing for PD-1 expression has been approved by the US Food and Drug Administration (FDA), no reliable or precise LAG3 marker is available to guide the clinical use of anti-LAG3 therapy.

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Table III. Summary of statistical analysis for association between minor allele presentation in study group (χ2 test with Fisher’s exact test)

<table>
<thead>
<tr>
<th>Minor allele presentation</th>
<th>OR (odds ratio)</th>
<th>Lower limit of 95% confidence interval for OR</th>
<th>Upper limit of 95% confidence interval for OR</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>study group (triple negative breast cancer presentation)</td>
<td>0.5319</td>
<td>0.2257</td>
<td>1.2535</td>
<td>0.1489</td>
</tr>
<tr>
<td>clinicopathological determinants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>0.9718</td>
<td>0.2690</td>
<td>3.5104</td>
<td>0.9652</td>
</tr>
<tr>
<td>N</td>
<td>0.5647</td>
<td>0.1476</td>
<td>2.1607</td>
<td>0.4039</td>
</tr>
<tr>
<td>Ki-67 (%) &gt;20</td>
<td>0.3484</td>
<td>0.0354</td>
<td>3.4281</td>
<td>0.3660</td>
</tr>
<tr>
<td>T ≤ 3</td>
<td>0.9803</td>
<td>0.2148</td>
<td>4.4728</td>
<td>0.9795</td>
</tr>
<tr>
<td>T = 4</td>
<td>0.6509</td>
<td>0.1157</td>
<td>36614</td>
<td>0.6261</td>
</tr>
</tbody>
</table>

Figure 1. Genotype frequencies of LAG3 rs2365094 in the study group

Figure 2. Genotype frequencies of LAG3 rs2365094 in the control group
To our knowledge, there are no studies that investigate the LAG3 gene polymorphism in breast cancer. In whole genome sequencing (WGS) of the LAG3 gene, Manichaikul et al. examined the polymorphism of the LAG3 locus to identify those associated with plasma LAG3 protein concentrations and clinical outcomes [22]. Finally, they reported that a common SNP in the intone region of the LAG3 gene (rs3782735, allele G) is positively associated with plasma LAG3 protein levels [22].

The LAG3 in intrinsic regions was previously examined in women with multiple myeloma by Lee et al. The two SNPs in the LAG3 gene (rs2365094 and rs3782735) were significantly associated with a risk of multiple myeloma [28]. However, there are no available data on the functional significance of any LAG3 SNPs in any subtype of breast cancer. Further study is warranted to elucidate the molecular mechanisms of the LAG3 gene polymorphism with regard to TNBC characteristics.

Conclusions
TNBC displays poor prognosis. However, the observation that both LAG3 and PD-1 inhibit anti-tumor activity has led to a significant growth in therapeutic strategies aimed at the tumor microenvironment. Than immunostopcheck-based therapeut regimens require a better understanding of the underlying mechanisms of LAG3 presentation, which LAG2 gene sequencing. The sequence analysis of the LAG3 gene found rs2365094 status may be a predictor of TNBC patient outcome. Our present findings based on a group of Polish patients with the TNBC LAG3 gene, genotyped for the first time, identify no significant difference in allelic distribution between TNBC patients and group of healthy controls in rs2365094. In addition, SNP status does not appear to be significantly associated with clinicopathological determinants. Although this work was intended as a pilot study towards a future randomized trial with a larger group, its findings provide a better understanding of the genetic basis of TNBC.

Conflict of interest: none declared

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Received: 17 Jan 2023
Accepted: 17 May 2023

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