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

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**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

## Introduction

Triple-negative breast cancer (TNBC) is an aggressive condition that is negative for hormone-receptor and human epidermal growth factor receptor 2. TNBC accounts for 20% of overall breast cancers [1]. A key prognostic factor for this type of breast cancer comprises a complete pathological response to first-line neoadjuvant therapy and primary chemosensitivity [2] but high risk of recurrence [3]. Even so, unlike other breast cancers, TNBC demonstrates a much higher likelihood of metastasis to the brain or lung rather than bone.

The 2016 Lehmann et al. classification [4] divides TNBC into four subgroups based on genomic analysis of *BRCA1/2, STAT4, TP53, APC, BRAF, MAP2, MAPK13, MDC1, PTEN, RB1, CDKN2A, UTX,*

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*CTNNA, DDX18, HUWE1, NFKBIA, PIK3CA, KRAS, HRAS*: basal-like immunosuppressed, basal-like immune-activated, luminal androgen receptor and mesenchymal types [3]. The classification of TNBC decides on the treatment strategy [4, 5]. Individual patients have distinctive molecular portraits for breast cancer, and a better understanding of the gene expression patterns of triple negative breast cancer is likely to improve therapeutic strategies with targeted agents. Jurj et al. [6] published a comprehensive study of SNPs in TNBC that may be used as potential prognostic biomarkers for early-stage TNBC, predicting the presence of TNBC and prognosis. These include rs3817198(LSP1), rs1436904(CHST9), rs1219648(FGFR2), rs4415084(locus 5p12), rs799917(BRCA1), rs8100241(ANKLE1), rs201360779(PDE4D), rs201654150/ rs149590841(FBXL22).

Of all breast cancers, TNBC is considered to be one of the most immunogenic. Briefly, the expression of immune checkpoint members in the tumor microenvironment boosts tumor growth, making the tumor invisible to the defense mechanisms of the innate immune reaction. Immune checkpoint inhibitors reverse this mechanism, thus inducing removal of tumor tissue by the immune system. The components of the peritumoral and intratumoral microenvironment may serve as surrogate biomarkers for treatment qualification, and such aberrations may facilitate the action of chemotherapy [7]. Anti-immune checkpoint treatments are the backbone for many clinical trials in breast cancer, including TNBC. These drugs are often combined with chemotherapeutics [8], angiogenesis inhibitors and recently, with other immune checkpoint inhibitors [9]. The individual schedule of the bispecific antibodies CTLA4, PD-1/PD-L1 and LAG3 plays a crucial role in tumor immunospecification [10].

Lymphocyte-activation gene 3 (*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 checkpoints can be mutually mediated and co-targeting of PD-L1 and LAG3 could provide a new therapeutic approach [9, 20, 21].

In bispecific antibodies, the presence of a single nucleotide polymorphism (SNP) could result in variation in their biological properties and effector functions [22]. The *LAG3* gene is a key predictive factor associated with the LAG3 protein network. The gene is located on chromosome 12 (12p13.32) [23]. Current findings indicate significant correlations between the status of eight SNPs, *viz.* rs1922452, rs951818, rs870849, rs188255, rs11227, rs2365094, rs3782735 and rs2365095 and multiple myeloma, sepsis, Parkinson's disease, HDL-cholesterol and multiple sclerosis as origin-of-inflammation cascades [22, 24–28].

The status of the *LAG3* gene SNP rs1922452 was found to be associated with multiple sclerosis co-morbidity [24]. In addition, the T allele of rs2365095 in the *LAG3* gene was found to be less frequent in multiple sclerosis cases, and the C allele could be considered a risk factor [27]. Furthermore, the T allele of *LAG3* rs870849 was found to be a protective factor against primary immune thrombocytopenia [22], and SNP rs951818 may be involved in the neuroinflammatory mechanisms of disease pathogenesis in Parkinson's disease [25].

In patients with sepsis, those with the *LAG3* rs951818 AA-homozygote showed significantly increased 28-day mortality (17.3%) compared to carriers of the C-allele (23.7%) [26]. A study of *LAG3* gene rs2365094 and rs3782735 found that rs2365094 has an association with multiple myeloma risk [28]. Also, a positive association was identified between the *LAG3* rs3782735 variant allele G and plasma LAG3 protein level in a study of HDL-C, coronary heart disease and all-cause mortality [23]. However, no data exists on the functional significance of any *LAG3* gene SNPs in TNBC. Hence, the present paper examines the influence of *LAG3* gene SNP rs2365094 on triple negative breast cancer (TNBC) and its potential interactions with clinical features that can be used to stratify cancer patients.

## Material and methods

A total of 30 TNBC breast cancer cases were analyzed for *LAG3* rs2365094 SNP. The description of the clinical features of TNBC patients is shown in table I. The study included 30 TNBC patients (n = 30) and 30 healthy controls (n = 30) treated in the Department of Surgical Oncology, Copernicus Provincial Multidisciplinary Centre of Oncology and Traumatology in Lodz, Poland. The study was conducted with the approval of the Independent Ethics Commission of the Medical University of Lodz (study number RNN/298/19/KE) and all participants gave their written consent to take part in the study.

Sixty samples of whole blood in ethylenediaminetetraacetic acid (EDTA) were obtained from peripheral veins according to standard procedures and stored at –20°C. The cancer patients did not receive preoperative chemotherapy or radiotherapy before blood collection. The medical records of the patients, including age of diagnosis, grading, tumor size,

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

| Characteristics | Parameter |
|---|---|
| age, years median (range) | 67.5 years (38–84 years) |
| side of involved breast | left – 14<br>right – 17 |
| tumor size (T in TNM classification 2021) | Tx – 1<br>T1a – 1<br>T1b – 1<br>T1c – 9<br>T2 – 12<br>T3 – 1<br>T4 – 6 |
| node status (N in TNM classification 2021) | Nx – 1<br>N0 – 15<br>N1 – 3<br>N2 – 5<br>N3 – 6 |
| Ki-67(%) | <20% – 3<br>≥20% – 27 |
| histological grade | grade 1 – 1<br>grade 2 – 14<br>grade 3 – 15 |

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 *LAG3* gene was determined using a polymerase chain reaction (PCR) and commercially-available TaqMan SNP Genotyping Assays (Applied Biosystems): Context Sequence GGAGAAGACAAGTCTAAAGCCAGGT [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 TagMan® 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 chi2 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 **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 *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%), 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, grade, Ki-67 status or age of diagnosis). 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 II.** Characteristics of lymphocyte activating 3 gene (LAG3) rs2365094 sequence, primer and chromosomal location

| Gene name | SNP (rs) number | Chromosomal location | Primer sequence | Polymorphism | Minor allele |
|---|---|---|---|---|---|
| *LAG3* | rs2365094 | chr.12:6774504 on build GRCh38 | context sequence [VIC/FAM] GGAGAAGACAAGTCTAAAGCCAGGT_ [C/G]CCTGTTTCCAGGAGCTTCCGGCTTG | C/G, transversion substitution | G = 0.71091 |

**Table III.** Summary of statistical analysis for association between minor allele presentation in study group (chi2 test with Fisher's exact test)

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

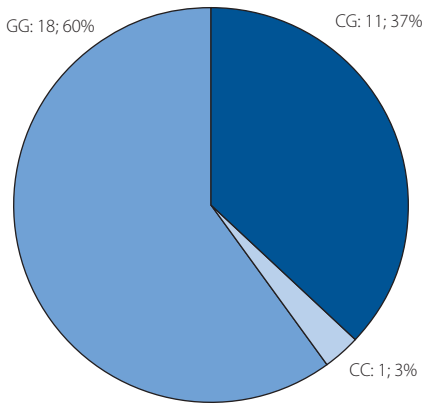


**Figure 1.** Genotype frequencies of *LAG3* rs2365094 in the study group

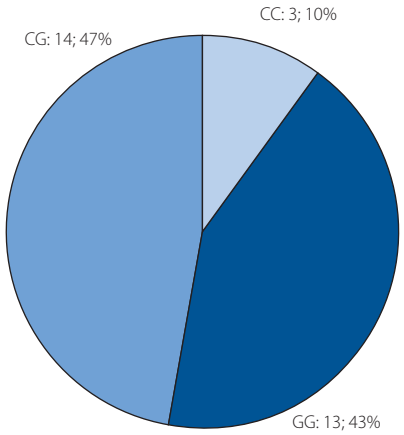


**Figure 2.** Genotype frequencies of *LAG3* rs2365094 in the control group

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.

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 immunotherapy 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.

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, allel G) is positively associated with plasma LAG3 protein levels [22].

The *LAG3* in intronic 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 immunocheckpoint-based therapeutic regimens require a better understanding of the underlying mechanisms of LAG3 presentation, which *LAG3* 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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