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ORIGINAL RESEARCH ARTICLE

Characterization of *GATA3* mutational status in early-stage breast cancer patients from Slovenia

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Abstract

Introduction: GATA3 is a zinc-finger transcription factor essential for luminal cell fate specification in the mammary gland and a well-established marker of estrogen receptor (ER)-positive breast cancer (BC). High GATA3 expression characterizes the luminal A subtype and is associated with a favorable prognosis, while reduced expression and somatic mutations in its zinc-finger and C-terminal domains have been linked to aggressive tumor behavior. However, the molecular determinants of GATA3 loss in early-stage BC remain poorly understood.

Objective: To characterize GATA3 mutational status in early-stage breast cancer patients.

Methods: We characterized GATA3 protein expression by immunohistochemistry and assessed somatic mutations in exons 2–6 in a prospective cohort of 60 early-stage BC patients from Slovenia. We additionally performed a comprehensive analysis of somatic variations across the GATA3 gene using COSMIC data and evaluated the structural impact of single-nucleotide variants (SNVs) on GATA3 protein stability using in silico tools.

Results: Most tumors (90%) exhibited high GATA3 immunoreactivity, which correlated significantly with ERα expression ($p = 0.006$). No somatic mutations were identified in exons 2–6 in tumors with absent or low GATA3 expression, suggesting that epigenetic mechanisms such as promoter hypermethylation drive expression loss in early-stage disease. Among 469 COSMIC-cataloged somatic variations, frameshift mutations predominated, and exon 6 harbored the highest mutational burden (55.88%). In silico analysis revealed that 51.46% of SNVs exerted destabilizing effects on GATA3 structure, predominantly within the zinc-finger 2 domain. Two variants, P42L and S93F, exhibited stabilizing effects warranting further functional investigation.

Conclusion: Our findings provide characterization of GATA3 in early-stage BC from Slovenia and implicate epigenetic silencing as an alternative mechanism of GATA3 inactivation.

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1. Introduction

GATA3 belongs to the evolutionarily conserved family of zinc-finger transcription factors that play a crucial role in the gene regulatory networks governing cell specification and fate determination. It has been shown that GATA3 is necessary for both the specification and the long-term maintenance of the luminal cell fate in the mammary gland, where its expression is indispensable for normal ductal morphogenesis.[1-3] The importance of GATA3 in maintaining luminal identity is underscored by the broad consequences of its loss in breast tissue. Beyond the breast, GATA3 has been implicated in the development and differentiation of cells and cell structures in a wide range of tissues and organs throughout the body, including blood,[4-6] heart, placenta and uterus, kidney,[7] prostate, adrenal and parathyroid gland, inner ear,[8] lens, nervous system,[9-12] adipose tissue and skin.[13] This wide tissue distribution reflects the fundamental and non-redundant roles that GATA3 plays across multiple developmental programs.

In line with its developmental function in breast tissue, it has been well established that GATA3 plays a critical role in shaping malignant phenotypes in BC. Initial transcriptome studies on primary breast cancer (BC) using RNA microarrays revealed a strong and reproducible correlation between GATA3 expression and estrogen receptor alpha (ERα)-positive status.[14-16] Furthermore, RNA microarray analyses showed that GATA3 is a molecular biomarker for the luminal subtype of BC, which showed the best prognosis among the subtypes.[17] RNA expression data were also confirmed on a protein level using tissue microarray analysis. High GATA3 expression was shown to be associated with a lower grade, smaller tumor size, and increased ER and progesterone receptor (PR) expression.[18-21] Previous studies have also demonstrated that high GATA3 levels are associated with significantly longer disease-free and overall survival in BC patients, underscoring its potential value as a prognostic biomarker.[18] Conversely, low GATA3 protein expression has been consistently associated with an aggressive BC phenotype.[18,22-24] Moreover, low GATA3 expression was a significant predictor for shorter overall survival in subgroups of ER-positive and low-grade BC patients.[20] Taken together, the highest GATA3 expression is characteristic of the luminal A BC subtype. Patients with luminal A subtype exhibit a favorable prognosis characterized by lower relapse rates and less frequent development of metastases in the liver, lung, and central nervous system,[25] and treatment of these tumors is primarily based on hormonal therapy.

However, the influence of GATA3 expression levels on BC tumor development, prognosis, treatment response, and therapy resistance is further modulated by other etiological factors, most notably somatic *GATA3* genetic mutations. GATA3 is widely regarded as one of the most frequently mutated genes in BC, with mutation frequencies varying across molecular subtypes. Mutations within the *GATA3* gene can be categorized into one of five distinct classes based on position and/or functional consequence: zinc finger 2 region, splice site, truncation, extension, and missense mutations.[26,27] The majority of mutations cluster within the zinc finger 2 region and the C-terminus of the GATA3 protein.[26,27] Whereas mutations in the ZNF2 region are usually truncating, C-terminus mutations are usually categorized as extension mutations as they are associated with the addition of a neo-peptide, resulting in a protein with a higher molecular weight, whose function remains to be determined.[28,29] Nevertheless, previous studies have shown that such mutations can exert a profound effect on stability, interactions with target molecules, and subcellular localization of GATA.3[30] Altered interactions with its target promoters and protein cofactors in the transcription complexes are of particular significance for the GATA3 function as they lead to cellular reprogramming, which is a prerequisite for the development of cancer-associated cell features.[31] Moreover, the effect of *GATA3* mutations may be hormone context-dependent, as suggested by the X308_ Splice mutation. X308_Splice mutation results in a novel GATA3 protein associated with excellent prognosis in patients. *In vitro* experiments showed that overexpression of the novel GATA3 protein induced cell proliferation when both estrogen and progesterone levels were high, while it showed a growth disadvantage when estrogen prevailed.[32] Finally, the cellular function of GATA3 is also highly context-dependent, varying according to tumor cell phenotype. Specifically, GATA3 has been shown to induce proliferation in cancer stem cells while, in contrast, restraining proliferation, promoting differentiation, and suppressing the dissemination of tumors in late-stage BC carcinomas.[3,31]

Despite the accumulating literature on the function of GATA3 in BC, its specific role in early-stage BC tumors remains insufficiently characterized. *In vitro* studies demonstrated that decreased GATA3 expression triggered apoptosis in both normal breast epithelial cells and in

early tumors, providing evidence in support of a tumor suppressor function for this transcription factor.[3] This was further corroborated by clinical studies showing that early-stage tumors with high GATA3 expression consistently exhibit a favorable prognosis. These findings in normal tissue and early tumors contrast sharply with the potential oncogenic effects of GATA3 in more developed tumors. Most importantly, specific GATA3 mutations can induce cell proliferation and tumor growth, therefore representing a potential tumor driver underlying the initial molecular and cellular events that lead to BC tumor development. To delineate the contribution of GATA3 somatic mutations to early BC tumorigenesis, we evaluated GATA3 protein expression by immunohistochemistry in early-stage BC tumors from female patients in Slovenia. Tumors with absent or diminished GATA3 immunoreactivity were subsequently analyzed for somatic protein-coding mutations in regions of the *GATA3* gene encompassing the zinc-finger domains and the C-terminal region. In addition, we performed a comprehensive distribution analysis of somatic variations throughout the entire *GATA3* gene and assessed the effect of single-nucleotide substitutions on the GATA3 protein structure.

2. Materials and methods

2.1. Patients

In the present study, 60 female patients aged ≥ 18 years with early-stage BC (T1+T2) were recruited prospectively between 2019 and 2021 at the University Medical Centre Maribor, Slovenia. Exclusion criteria were age at diagnosis < 18 years, presence of neoplastic lesions outside of the breast tissue, and presence of breast metastases derived from malignant neoplasms outside of the breast tissue. General clinical data, including age, menopausal status, number of births, the use of oral hormone contraceptives or hormone replacement therapy, prior cancer treatment, and family cancer occurrence, were gathered for every patient. Each patient underwent mammography, ultrasound examination, and ultrasound-guided core needle biopsy at the Division of Gynecology and Perinatology, University Medical Centre Maribor.

The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (approval ID: KME RS 0120-403/2019/7) and conducted according to the Declaration of Helsinki. All patients provided written informed consent to participate in the study.

2.2. Tissue collection and DNA extraction

For each patient, two sets of tumor biopsies containing adjacent non-lesion tissue were taken preoperatively. For molecular analysis, biopsies were immediately immersed in the RNAlater preservative (Thermo Fisher Scientific Inc., USA) and stored at −80 °C until further processing. Biopsies that served for pathological examination were embedded in paraffin blocks and were later used for immunocytochemical analysis.

DNA was extracted from fresh frozen biopsies using a DNA Mini Extraction Kit (51304, QIAGEN N.V., Netherlands) according to manufacturer's instructions. Briefly, up to 25 mg of tissue was lysed at 56 °C for 3 h using Proteinase K, followed by incubation at 70 °C for 10 min. DNA was precipitated with absolute ethanol and washed with a washing buffer 1 at 6,000 × g for 1 min and washing buffer 2 at 20,000 × g for 3 min on a silica membrane. Samples were additionally centrifuged at 20,000 × g for 1 min to remove traces of ethanol from the washing steps. Finally, DNA was eluted from the silica column with 200 µL of nuclease-free distilled water at 6,000 × g for 1 min. DNA concentration and purity were assessed using a Synergy 2 spectrophotometer (BioTek, United States of America [USA]). DNA samples were stored at −20 °C.

2.3. Sanger sequencing

Genetic variations in exons 2–6 of *GATA3* and in the first 500 bp of the *GATA3* promoter region were assessed by Sanger sequencing. Target *GATA3* regions were amplified in a 10-µL polymerase chain reaction (PCR) mixture containing 1× DreamTaq Buffer, 2 mM dNTP mix, 0.25 U of DreamTaq polymerase (Thermo Fisher Scientific Inc., USA), and 0.1 µM each of forward and reverse primers. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s; and final extension at 72 °C for 7 min. Primer sequences are shown in Table 1.

To prepare PCR amplicons for sequencing, the PCR products were separated on a 1.5% agarose gel and purified using the Minielute Gel Extraction Kit (28004, QIAGEN N.V., Netherlands) following manufacturer's instructions. Briefly, DNA fragments were excised from the gel and incubated in 300 µL of Buffer QG at 50 °C for 10 min to completely dissolve the gel slices. DNA was precipitated with isopropanol and bound to a silica membrane for washing. First, DNA was washed with the washing Buffer QG at 10,000 × g for 1 min, followed by washing with Buffer PE at 10,000 × g for 1 min. Samples were centrifuged at 10,000 × g for an additional 1 min to remove traces of ethanol. Finally, DNA was dissolved in 10 µL of nuclease-free distilled water and washed from the column at 10,000 × g for 1 min. DNA was stored at −20 °C until sequencing.

Table 1. Primer sequences

GATA3 region	Primer sequence	Annealing temperature
Promoter region 1	F: 5'-ATTTAACGCGCACGTTTCCC-3' R: 5'-TCACCAGTACCAACCTGGG-3'	62 °C
Promotor region 2	F: 5'-AGTGGCTCAAAGGACCGAG-3' R: 5'-AAATAGGCACGCATGGATCA-3'	62 °C
Exon 2	F: 5'-CCCCCACCGAAAGCAAATCA-3' R: 5'-GATGGGGGCTTTCGCTTGAC-3'	62 °C
Exon 3	F: 5'-AGAGATTCCCCAGGTGTCCC-3' R: 5'-GGAGAAAAGGCTCCAGGGAAG-3'	62 °C
Exon 4	F: 5'-CCTAAGTGGCTTATCTGTGCT-3' R: 5'-GCTGACACGATTGGAGGCTA-3'	62 °C
Exon 5	F: 5'-ATCCTGACATGCTCCAGTGG-3' R: 5'-CGGATTGCTGCATGGTATGT-3'	62 °C
Exon 6	F: 5'-CCTTCTTGGTGTGCGAGAG-3' R: 5'-AGGCTTCATGATACTGCTCCT-3'	62 °C

Sanger sequencing was performed at the commercial provider Eurofins Genomics (Germany).

Genetic variations in the *GATA3* and its proximal promoter region were determined by aligning nucleotide sequences from BC patients to the reference genome hg19 using Clustal W.[33] All determined genetic variations were further characterized according to the Catalogue of Somatic Mutations in Cancer[34] and the Single Nucleotide Polymorphism Database.[35]

2.4. Immunohistochemistry and quantification of GATA3 immunoreactivity

GATA3 expression was evaluated by immunohistochemistry (IHC) according to the standardized method using an automatic stainer (VENTANA HE 600, Roche Diagnostics, USA) at the Department of Pathology, UKC Maribor. GATA3 was visualized using validated primary mouse anti-human GATA3 antibodies (1:250; sc-269, Santa Cruz Biotechnology, USA) and secondary anti-mouse antibodies conjugated with horseradish peroxidase (sc-2005, Santa Cruz Biotechnology, USA).

GATA3 immunoreactivity was quantified by a certified pathologist blind to the experimental and clinicopathological features of the specimens. GATA3 immunoreactivity was assessed according to the standardized protocol using a four-level scale. Both the level of GATA3 immunoreactivity and the number of GATA3 immunopositive cells were used to assess the final level of tissue GATA3 expression.

2.5. Characterization of *GATA3* somatic mutations and variations

Somatic mutations in the *GATA3* were obtained from the COSMIC v102 (QIAGEN N.V., Netherlands), a curated database of somatic mutations in human cancer.[36] The impact of single-nucleotide variants (SNVs) on the GATA3 protein stability was assessed by the INPS-MD bioinformatic tool (Version 2.0, Bologna Biocomputing Group, Italy).[37,38]

2.6. Statistical analysis

Statistical analysis was performed using SPSS statistical suite (SPSS Statistics for Windows, Version 21.0, IBM Corp, USA). Normal distribution of data sets was examined by Shapiro–Wilk's test. The association between GATA3 or GATA3/ERα immunoreactivity and other clinicopathological parameters was assessed by Fisher's exact test with Freeman–Halton extension or Mann–Whitney test. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Expression of GATA3 and ERα was positively associated in breast cancer tumors

In the present study, we assessed GATA3 expression in a prospective cohort of 60 BC patients from Slovenia. IHC analysis showed immunopositivity for GATA3 in 90% (54/60) of tumors, whereas decreased GATA3

immunoexpression with the overall score of 2 or 1 was found in 5% (3/60) or 3.33% (2/60) of tumors, respectively. GATA3 immunoreactivity was absent in 1.67% (1/60) of cases. Next, we investigated the relationship between GATA3 immunoreactivity and histopathological and molecular features of BC tumors. GATA3 immunoreactivity showed a positive correlation ($p = 0.006$) with the ERα expression, whereas no significant correlation was found between GATA3 immunoreactivity and tumor type, tumor grade, mitotic count, lymphovascular invasion, PR expression, HER2 expression, or sentinel node biopsy status (Table 2). To get a better insight into the clinicopathological features of the tumors, we further categorized the BC tumors according to the level of GATA3 and ERα expression into four groups: GATA3 low/ERα low, GATA3 low/ERα high, GATA3 high/ERα low, and GATA3 high/ERα high. Comparison of the four groups of tumors showed a significant ($p < 0.001$) association between the GATA3/ERα and HER2 expression, with GATA3 high/ERα high tumors being associated with decreased HER2 expression (Table 3). In addition, GATA3 high/ ERα high tumors exhibited significantly ($p < 0.05$) higher PR expression compared to the other three groups of tumors (Table 3). Other clinicopathological features were not significantly associated with the GATA3 and ERα expression in BC tumors (Table 3).

3.2. Absent or reduced GATA3 protein expression showed no association with somatic mutations in GATA3 exons 2–6

Tumors exhibiting absent or low (score 1) GATA3 immunoexpression were analyzed for somatic mutations in *GATA3* exons 2–6. Mutational analysis revealed no somatic mutations within this region.

3.3. Characterization of somatic variations in *GATA3*

A total of 469 unique somatic variations have been characterized in *GATA3* in BC patients to date. According to their type, mutations belong to frameshift insertions (39.84%), frameshift deletions (20.68%), missense substitutions (17.91%), nonsense substitutions (2.35%), synonymous substitutions (1.71%), in-frame insertions (1.28%), in-frame deletions (1.28%), and complex frameshift mutations (1.28%). A total of 13.86% of genetic variations were uncharacterized. The highest number of somatic variations (233; 55.88%) was found in the exon 6, followed by the exon 5 with 109 (26.14%) different somatic variations. Exons 2, 3, and 4 harbored 21 (5.04%), 39 (9.35%), and 15 (3.60%) somatic variations, respectively.

Frameshift variations were the most abundant among all variation types, with the most frequent being frameshift insertions P409Afs*99 (exon 6), D336Gfs*17 (exon 5), and N332Efs*21 (exon 5), and frameshift deletion S237Afs*29 (exon 3) found in 149, 63, 23, and 16 tumor samples, respectively. The most prone to frameshift variations were exons 6 and 5, harboring a total of 189 (65.40%) and 84 (29.07%) different variations, respectively. The number of frameshift variations in total found in exons 2, 3, and 4 were 4 (1.38%), 10 (3.46%), and 2 (0.69%), respectively. The exons with the most different SNVs were exons 6 and 3, harboring 32 (31.07%) and 28 (27.18%) different SNVs, respectively. The total number of SNVs in exons 2 (16; 15.53%), 4 (11; 10.68%), and 5 (16; 15.53%) was in the same range. The most frequent SNV was a missense substitution M294K in exon 4, found in 42 breast tumors. Two additional sites in *GATA3* susceptible to SNVs were at the position p.367 and p.396 (both in the exon 6), with the R367* nonsense and A396T missense substitution being present in 18 and 11 cases, respectively.

Qualitatively, among the SNVs in 189 mutated BC samples, the most frequent were substitutions at position C (34.20%) and T (30.57%), followed by G (21.24%) and A (13.99%) (Figure 1A). The most frequent C substitutions were C>T (68.18%), followed by C>A (16.67%) and C>G (15.15%) substitutions (Figure 1B). T>A substitution was the most frequent (67.80%) at the T site, followed by the T>G (25.42%) and T>C 6.78%) substitutions. The most frequent substitution at the G site was G>A substitution (60.98%), followed by the G>T (31.71%) and G>C (7.32%) substitutions. Frequencies of A>G and A>T were 62.96% and 37.04%, respectively. Substitutions A>C were not found (Figure 1B).

Next, we assessed the impact of single-nucleotide substitutions on the stability of the GATA3 protein structure. Among the 103 SNVs studied, 51.46% were predicted to have destabilizing or weakly destabilizing effects on the protein structure (Table S1). Destabilizing SNVs were present throughout the GATA3 protein; however, the most susceptible portion of the GATA3 protein was from amino acid positions 294 to 367, encoded by exons 4 to 6. In addition, the most frequent missense substitutions M294K and A396T exhibit a destabilizing effect on GATA3. Interestingly, two rarely reported missense substitutions, P42L and S93F, with a stabilizing effect on protein structure, were characterized, which may be of significance in terms of GATA3 function in BC.

4. Discussion

In the present study, we characterized *GATA3* mutational status and protein expression in a prospective cohort of early-stage BC patients from Slovenia. The vast majority of tumors examined (90%) exhibited high GATA3 immunoreactivity, a finding that is entirely consistent

Table 2. Correlation between GATA3 immunoreactivity and histopathological and molecular characteristics of breast cancer tumors

Clinicopathological characteristic	GATA3 immunoreactivity			*p*-value
	Low	Medium	High	
Tumor type				
IDC	3/60	2/60	41/60	0.8129
ILC	0/60	1/60	8/60	
Mixed	0/60	0/60	1/60	
Other	0/60	0/60	4/60	
Tumor grade				
I	0/60	0/60	8/60	1
II	2/60	2/60	31/60	
III	1/60	1/60	15/60	
Mitotic count				
1	0/60	1/60	29/60	0.1716
2	2/60	2/60	15/60	
3	1/60	0/60	10/60	
LVI				
No	1/60	1/60	39/60	0.09418
Yes	2/60	2/60	15/60	
ERα expression				
≤5%	2/60	1/60	7/60	0.006*
100%	1/60	2/60	47/60	
PR expression				
0%	2/60	1/60	14/60	0.622
1–50%	0/60	0/60	8/60	
51–99%	0/60	1/60	7/60	
100%	1/60	1/60	25/60	
HER2 expression				
Negative	1/60	2/60	50/60	0.07585
Positive	1/60	1/60	4/60	
SNB				
Negative	3/60	1/60	41/60	0.2838
Positive	0/60	2/60	13/60	

Note: *$p \leq 0.05$.

Abbreviations: ERα: Estrogen receptor alpha; IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; LVI: Lymphovascular invasion; PR: Progesterone receptor; SNB: Sentinel node biopsy.

Table 3. Tumor characteristics based on GATA3 and ERα expression

Characteristics	GATA3-high/ERα-high	GATA3-high/ERα-low	GATA3 low/ERα-high	GATA3-low/ERα-low	*p*-value
Tumor type					
IDC	35/60	6/60	2/60	3/60	0.9635
ILC	7/60	1/60	1/60	0/60	
Mixed	1/60	0/60	0/60	0/60	
Other	4/60	0/60	0/60	0/60	
Tumor grade					
I	7/60	1/60	0/60	0/60	0.2436
II	29/60	2/60	3/60	1/60	
III	11/60	4/60	0/60	2/60	
Mitotic count					
1	27/60	2/60	1/60	0/60	0.103
2	13/60	2/60	2/60	2/60	
3	7/60	3/60	0/60	1/60	
LVI					
No	34/60	5/60	1/60	1/60	0.2141
Yes	13/60	2/60	2/60	2/60	
PR expression					
0%	8/60	5/60	0/60	2/60	
1–50%	7/60	2/60	0/60	1/60	<0.05*
51–99%	10/60	0/60	1/60	0/60	
100%	22/60	0/60	2/60	0/60	
HER2 expression					
Negative	46/60	4/60	3/60	1/60	<0.001*
Positive	1/60	3/60	0/60	2/60	
SNB					
Negative	35/60	6/60	2/60	2/60	1
Positive	12/60	1/60	1/60	1/60	

Note: *$p \leq 0.05$.

Abbreviations: IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; LVI: Lymphovascular invasion; PR: Progesterone receptor; SNB: Sentinel node biopsy.

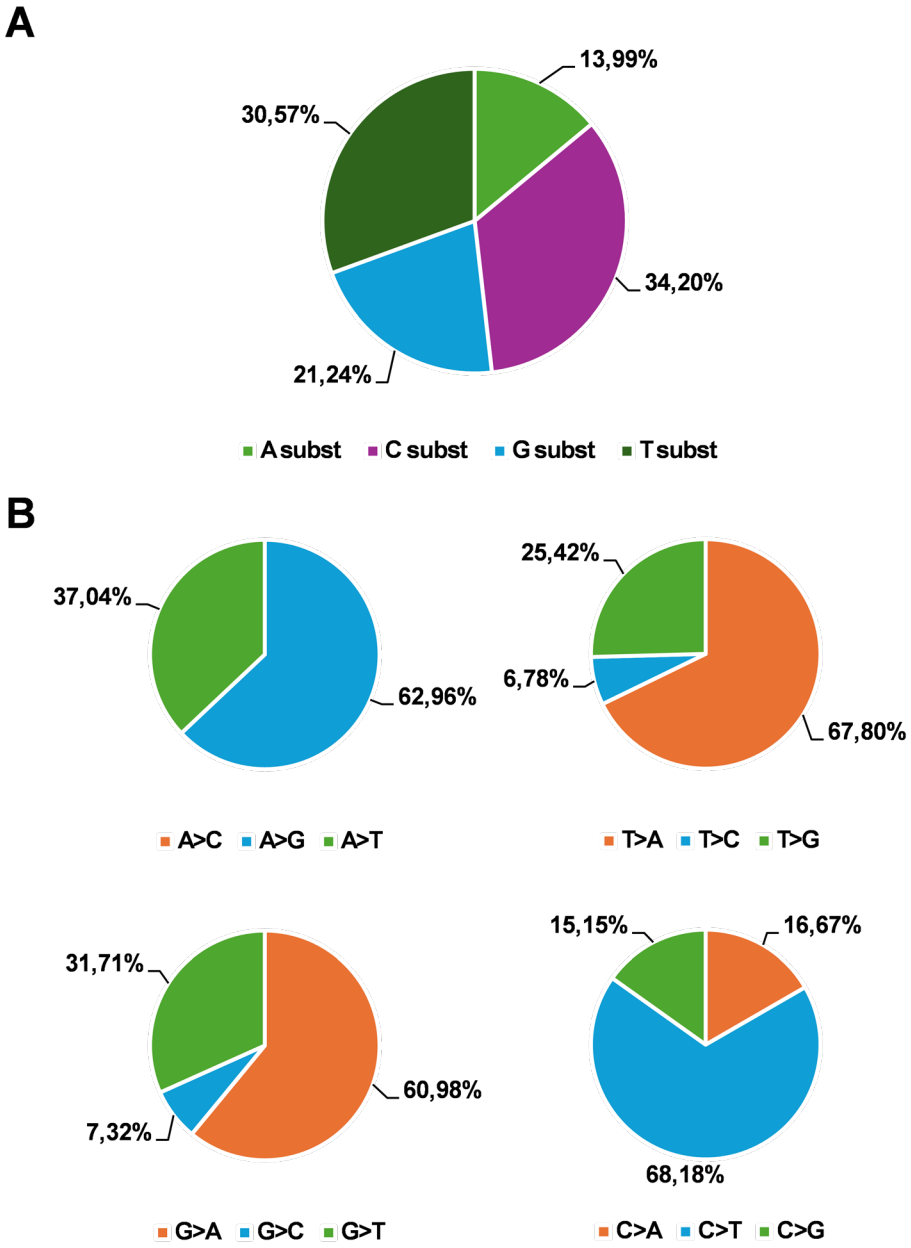

Figure 1. Single-nucleotide variations in breast cancer patients. (A) The total frequency of A, G, C, and T substitutions. (B) Frequencies of nucleotide substitutions at A, T, G, and C bases.

with the well-established role of GATA3 as a defining marker of the luminal BC subtype and its extensively documented association with ER-positive tumor status.[17-19] The statistically significant positive correlation between GATA3 and ERα immunoreactivity observed in our cohort (p = 0.006) is in agreement with a substantial body of prior evidence reporting that GATA3 expression is tightly coupled with ER signaling and is highly characteristic of the luminal A molecular BC subtype, which generally carries the most favorable prognosis among all recognized BC subtypes.[1,20] This correlation is further consistent with the findings of functional studies, particularly the bidirectional regulatory feedback loop operating between GATA3 and ER signaling pathways, which was first formally described nearly two decades ago.[39] Specifically, GATA3 is required for the expression of the gene encoding ERα itself,[39] the most likely mechanism being GATA3 binding to distal cis-regulatory enhancers within the gene, which is essential for

recruiting RNA polymerase II to ERα promoters. Beyond maintaining receptor levels, GATA-3 is also indispensable for the hormone-mediated stimulation of cell cycle progression and overall growth in ERα-positive BC cells.[39] Thus, it is possible to surmise that GATA3 is one of the key regulators of this cell-type–specific transcriptional network that mediates hormone sensitivity of these tumors.[39]

Our combined analysis of GATA3 and ERα expression further revealed that tumors co-expressing both high GATA3 and high ERα were significantly associated with lower HER2 expression ($p < 0.001$) and higher PR expression ($p < 0.05$). These findings align well with the previously reported molecular characteristics of luminal A tumors, which are typically defined by ER-positivity, HER2-negativity, and an overall association with favorable clinical outcomes.[25] The predominance of such tumors in our cohort most likely reflects the specific demographic and clinical features of the recruited patient population, in which early-stage, low-to-intermediate grade invasive ductal carcinomas represented the most frequently encountered tumor type. Notably, no statistically significant correlations were identified between GATA3 immunoreactivity and a range of other clinicopathological parameters examined, including lymphovascular invasion, tumor grade, mitotic index, and sentinel node biopsy status. This pattern of associations—significant for ER and PR but not for grade or invasion markers—suggests that, aside from its observed co-expression with ER, GATA3 expression may not be a reliable independent prognostic factor in early-stage BC. Nevertheless, special care should be taken when considering rare tumors with high GATA3 but low ERα, which have biomarker profiles and clinicopathological characteristics similar to triple-negative BC.[40,41]

A key aim of the present study was to determine whether absent or diminished GATA3 protein expression in early-stage BC tumors was attributable to somatic mutations within the zinc-finger and C-terminal coding regions of GATA3 (exons 4–6). Mutational analysis of tumors with absent or low (score 1) GATA3 immunoreactivity revealed no somatic mutations in the limited analyzed regions of our modest tumor subset. Given the restricted scope of sequencing and the modest sample size, this observation does not exclude the possibility of low-frequency protein-coding mutations that remain undetected due to the limitations of Sanger sequencing. Rather, it indicates that mutations in these specific regions were not detected in the tumors examined, in contrast to patterns more commonly reported in larger, population-level BC cohorts.[26,27] We hypothesize that this discrepancy also arises from our tumors being sampled from patients with early-stage BC detected at an early point in tumor progression. Alternative mechanisms, including epigenetic silencing (e.g., promoter methylation) or post-translational regulation of GATA3, may also underlie the loss of expression in this subset of early-stage tumors and warrant further investigation. Among alternate mechanisms of GATA3 loss, hypermethylation is thought to be the most likely reason for decreased GATA3 expression, as the *GATA3* gene is reported to be often hypermethylated in luminal BC.[42,43] Recently, a study of aggressive BC has reported DNMT3B to cause CpG-rich DNA sequences in the promoters of many onco-suppressor genes to become hypermethylated, with GATA3 emerging as a particularly important target of this silencing mechanism.[44] Moreover, the authors report distinct molecular signatures of *DNMT3B*$^+$ tumors, where GATA3 is silenced via hypermethylation, and tumors with *GATA3* mutations.[44] This suggests that loss of GATA3 activity due to protein-coding mutations or due to hypermethylation of promoter regions may lead to very different tumor behavior on the molecular level. Beyond BC, epigenetic silencing of GATA3 has been reported in clear cell renal cell carcinoma (ccRCC),[45] where loss of GATA3 expression is associated with the loss of TβRIII expression, a key event in renal carcinoma development, as it acts as a critical cell proliferation and growth regulator within the kidneys.[45] For instance, miR-455-3p has been shown to mediate GATA3 tumor suppression in mammary epithelial cells,[46] thus loss of miR-455-3p expression may contribute to diminished GATA3 protein expression. Similarly, a GATA3-miR-29b axis has been identified that inhibits metastasis, and loss of miR-29b causes decreased GATA3 expression.[47]

To contextualize the mutational landscape observed in our cohort within the broader COSMIC database, we performed a comprehensive characterization of somatic variations across the entire *GATA3* gene in our BC samples. Among the 469 unique somatic variations identified, frameshift insertions and deletions constituted the largest proportion of genetic changes, a distribution that is consistent with the well-established hotspot nature of the *GATA3* gene in BC and the susceptibility of its coding sequences to replication slippage errors.[28,29] Overall, exon 6 harbored the highest mutational burden of somatic variations (55.88%), with the frameshift insertion P409Afs*99 being the single most prevalent mutation (identified in 149 tumor samples), thereby corroborating its status as the most recurrent *GATA3* mutation in BC as reported in international databases. Similarly, D336Gfs*17 and N332Efs*21 in exon 5, and S237Afs*29 in exon 3, were among the most frequently observed frameshift variations in our cohort, findings that are also in agreement with existing COSMIC data. Taken together, these observations

reinforce the notion that *GATA3* mutations predominantly cluster in regions encoding the zinc-finger 2 domain and the C-terminus, where they are most likely to disrupt protein function and stability.[30,31]

Our analysis of single-nucleotide substitution patterns has shown that C and T substitutions were the most common nucleotide changes observed, with C>T transitions predominating among all C-site substitutions. This mutational profile is consistent with age-associated mutational processes well characterized in BC and is indicative of the spontaneous deamination of methylated cytosines, a process that accumulates progressively over time.[48] Collectively, these data suggest that *GATA3* point mutations in BC may be mostly attributed to endogenous mutational processes rather than extrinsic mutagenic exposures, as indicated by the prevalence of C>T substitutions at key *GATA3* loci.

In silico analysis of the functional impact of SNVs on GATA3 protein stability demonstrated that over half (51.46%) of SNVs exerted destabilizing or weakly destabilizing effects on GATA3 protein structure. Destabilizing variants clustered predominantly within the region spanning amino acids 294–367. These amino acids are encoded by exons 4–6, which encompass the zinc-finger 2 domain and C-terminal sequences critical for DNA binding and protein–protein interactions.[30] Notably, the two most frequent missense substitutions in our data, M294K and A396T, both displayed destabilizing effects according to *in silico* prediction, suggesting that these hotspot mutations could compromise GATA3 structural integrity and may thereby impair its transcriptional regulatory function.

Conversely, two novel missense substitutions, P42L and S93F, were found to exert stabilizing effects on GATA3 protein structure. These predictions should be taken with caution, as they are based on *in silico* algorithms trained on protein orthology, and the predicted stabilizing effects may not reflect the true impact the mutations have on the overall protein structure. To our best knowledge, these variants have not been previously reported to have molecular significance within the context of BC and may have potential to cause gain-of-function changes that could alter GATA3 activity, making them noteworthy candidates for characterization in future functional studies. P42L has been detected in a single proband in a BC study of the safety and efficacy of combined use of lumretuzumab, pertuzumab, and paclitaxel,[49] but was not reported as relevant. Meanwhile, S93F has been reported once in the supplementary material of a prognostic study of somatic mutations in ER-positive BC, but S93F was not singled out in the study as particularly significant.[50] The predicted stabilizing effect in both variants may cause GATA3 to display greater resistance to ubiquitin-mediated degradation, alter cofactor recruitment, or affect protein interactions such as T-bet binding.[51] Beyond changes on the protein level, it might also be worthwhile to employ more advanced computational methods to study the effects of P42L, S93F, and other GATA3 variants on mRNA stability and ncRNA binding sites. Specifically, such a computational approach would require targeted modeling of mutation impact outcomes on the specific protein structure and/or protein expression levels. For example, a study on bone metastasis in BC employed such a targeted scoring approach to model their lipid metabolism hypothesis and analyzed it with support vector machine recursive feature elimination.[52] Finally, mutations are also subject to non-genetic effects, which may influence pathogenesis and cancer development. How non-genetic factors and cancer genetics interact may be quite complex; for example, molybdenum blood levels are associated with ovarian but not BC in BRCA1 carriers.[53]

The present study has several limitations that should be acknowledged. The cohort size of 60 patients, while sufficient to provide a meaningful preliminary characterization of GATA3 expression and mutational status in the Slovenian population, inevitably limits the statistical power of subgroup analyses and reduces the likelihood of detecting rare mutational events. The restriction of Sanger sequencing to exons 2–6 and the proximal promoter, while justified by the well-documented distribution of mutational hotspots in the *GATA3* locus, does not exclude potential contributions from mutations located elsewhere in the gene, including intronic variants capable of affecting pre-mRNA splicing, in tumors with decreased GATA3 expression. In addition, Sanger sequencing has limited sensitivity for low-frequency signals, which may cause detection issues for low-frequency variants or highly heterogeneous tumor samples. Furthermore, functional studies designed to validate the predicted effects of the identified SNVs on GATA3 protein stability, DNA binding affinity, and transcriptional activity were beyond the scope of the present work; such experiments are nevertheless essential and will be needed to confirm the biological relevance of our *in silico* predictions.

5. Conclusion

This study provides the first characterization of GATA3 protein expression and somatic mutational status in a prospective cohort of early-stage BC patients from Slovenia. The predominance of high GATA3 immunoreactivity and its significant positive correlation with ERα expression confirm that the molecular profile of this population is consistent with the luminal A subtype, which carries

the most favorable prognosis among BC subtypes and is primarily managed with hormonal therapy.

A central finding of this work is that somatic protein-coding mutations in the zinc-finger and C-terminal coding regions of *GATA3* (exons 4–6) were absent in the limited analyzed regions of our modest set of tumors with low or absent GATA3 expression. While this finding shows that protein-coding mutations in these regions do not account for GATA3 expression loss in the examined tumors in early BC, mechanical conclusions drawn from these results should be taken with caution. Alternative mechanisms—most plausibly epigenetic silencing via promoter hypermethylation or post-transcriptional regulation through non-coding RNAs—remain a plausible basis of new hypotheses as the possible determinants of expression loss, representing new priorities for future investigation.

Analysis of the broader GATA3 mutational landscape confirmed that frameshift mutations predominate, with exon 6 constituting the principal hotspot and P409Afs*99 the single most recurrent variant. These findings are consistent with international databases and reinforce the susceptibility of the zinc-finger 2 and C-terminal regions to functionally disruptive mutations. *In silico* stability modeling further revealed that the majority of SNVs exert destabilizing effects within the zinc-finger 2 domain and C-terminus, while two novel variants, P42L and S93F, displayed stabilizing effects that may represent gain-of-function alterations warranting dedicated functional characterization.

Taken together, these findings advance our understanding of GATA3 biology in early BC, underscore the importance of looking beyond coding mutations when investigating GATA3 inactivation, and lay the groundwork for larger studies and functional experiments needed to fully elucidate the role of GATA3 in early breast tumorigenesis.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (approval ID: KME RS 0120-403/2019/7) and conducted according to the Declaration of Helsinki. All patients provided written informed consent to participate in the study.

Consent for publication

This article does not contain any identifiable information.

Availability of data

Data within this article are available upon request to the corresponding author.

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