Intratumoral morphological heterogeneity of breast cancer: neoadjuvant chemotherapy efficiency and multidrug resistance gene expression

Evgeny V. Denisov1,2*, Nikolay V. Litviakov1,2*, Marina V. Zavyalova2,3,4, Vladimir M. Perelmuter3,4, Sergey V. Vtorushin3,4, Matvey M. Tsyganov1,2, Tatiana S. Gerashchenko1,2, Evgeny Yu. Garbukov5, Elena M. Slonimskaya6 & Nadezhda V. Cherdyntseva1,2,6

1Department of Experimental Oncology, Cancer Research Institute, Siberian Branch of the Russian Academy of Medical Sciences, Tomsk, Russian Federation, 2Laboratory of Translational Cell and Molecular Biomedicine, Tomsk State University, Tomsk, Russian Federation, 3Department of Pathological Anatomy and Cytology, Cancer Research Institute, Siberian Branch of the Russian Academy of Medical Sciences, Tomsk, Russian Federation, 4Department of Pathological Anatomy, Siberian State Medical University, Tomsk, Russian Federation, 5Department of General Oncology, Cancer Research Institute, Siberian Branch of the Russian Academy of Medical Sciences, Tomsk, Russian Federation, 6Department of Oncology, Siberian State Medical University, Tomsk, Russian Federation.

In this study, the influence of intratumoral morphological heterogeneity of breast cancer on neoadjuvant chemotherapy (NAC) efficiency was investigated. In particular, we analysed the association of NAC response and pre- and post-NAC expression of the main multidrug resistance (MDR) genes - ABCBI, ABCC1, ABCC5, ABCG1, and ABCG2, with the presence of different morphological structures in breast tumors. In addition, the expression of MDR genes was investigated in different morphological structures and in their microenvironment by comparing probes obtained using laser microdissection. The results of this study showed that tumors with alveolar structures were more frequently NAC-nonresponsive than cases without this structural type (p = 0.0028, Bonferroni-corrected p = 0.014). The presence of trabecular structures in breast tumors was also associated with chemoresistance (p = 0.0272, Bonferroni-corrected p = 0.136). High expression of MDR genes was not found in alveolar structures (including their microenvironment) and in tumors containing this structural type. In contrast, more active MDR genes and expression of the ABCBI gene were found only in trabecular structures. Taken together, our data indicate that breast tumors with alveolar structures possess resistance to NAC, which is not related to high expression of MDR genes, whereas chemoresistance of tumors with trabecular structures can depend on the expression level of ABCBI.

Breast cancer is a complex disease with high inter- and intratumoral heterogeneity1,2. Invasive carcinoma of no special type (IC NST)3, previously classified as invasive ductal carcinoma, not otherwise specified (NOS)4, account for a substantial proportion (up to 75%) of breast cancer cases and display extremely diverse morphological characteristics that make these tumors difficult to classify histologically3. IC NST tumors often contain minor components of special types of histology, including architectural features of the invasive process, which may vary widely both within a single tumor and from case to case6. Previously, we have described five different types of invasive component or morphological structures in IC NST tumors - tubular, trabecular, solid, alveolar structures, and discrete groups of tumor cells7 - and found that such intratumoral morphological heterogeneity was related to cancer metastasis3,8. In addition, our recent data provided evidence that phenotypic drift can be cause of the development of intratumoral morphological heterogeneity in IC NST9.

At present, the study of intratumoral heterogeneity appears to be key for the development of personalized approaches in cancer treatment10,11. In 2008, we reported that intratumoral morphological heterogeneity of IC
NST was associated with the efficiency of neoadjuvant chemotherapy (NAC): the presence of alveolar and trabecular structures in breast tumors resulted in a poor response to NAC\textsuperscript{12}. The mechanisms involved in drug resistance of heterogeneous tumors are not completely established. It is reasonable to assume that tumors contain different clones of tumor cells with different degrees of responsiveness to chemotherapy. Various molecular factors can be involved in tumor drug resistance, mainly ATP-binding cassette (ABC) transporters, which are encoded by a large family of multidrug resistance (MDR) genes and play a major role in mediating drug resistance\textsuperscript{13}. In this study, we focused on the key MDR genes - ABCB1, ABCC1, ABCC5, ABCC11, and ABCG2.

Thus, on a larger scale than in our previous research\textsuperscript{12}, we aimed to study the association between the NAC response and the presence of different types of morphological structures in IC NST tumors. Then, we investigated pre- and post-NAC expression levels of the MDR genes in breast tumors and whether they depend on the presence of various morphological structures. Finally, using laser microdissection we estimated expression levels of the MDR genes directly in different morphological structures and in their microenvironment.

**Results**

**Analysis of correlation of intratumoral morphological heterogeneity of IC NST and NAC response.** Using hematoxylin & eosin staining and morphological analysis, we identified different morphological structures in breast tumors (n = 382; patient characteristics are listed in Table 1). Statistical analysis of the association between NAC response and the presence/absence of different morphological structures in breast tumors was conducted using Pearson’s chi-square test with Bonferroni correction (Table 2). Patients with alveolar structures were more frequently NAC-nonresponsive than were cases without this structural type (61.9\% vs. 46.4\%; p = 0.0028, Bonferroni-corrected p = 0.014). In addition, breast tumors with trabecular structures more often demonstrated chemotherapy resistance as compared with tumors without these structures (58.8\% vs. 45.3\%; p = 0.0272). However, the difference did not reach statistically significance after Bonferroni correction (p = 0.136).

**Analysis of correlation of intratumoral morphological heterogeneity of IC NST and expression of MDR genes.** Using RT-PCR, we have compared the expression levels of MDR genes in the pre- and post-NAC tumor samples (n = 69) with the presence/absence of different morphological structures. Statistical analysis of the correlation of gene expression with different morphological structures of tumor was performed using logistic regression with Bonferroni correction. Expression of ABCB1 (p = 0.007) and ABCC5 (p = 0.027) genes was significantly lower in post-NAC tumors than in pre-NAC tumors with trabecular structures compared with tumors without trabecular structures. Patients with solid structures displayed a decreased post-NAC expression of ABCB1 (p = 0.011) and ABCG2 (p = 0.002) genes in comparison with patients whose tumors did not contain the solid structures. However, the differences in the expression of ABCB1 and ABCG2 were not significant after Bonferroni correction (p > 0.05; Table 3). In Tables 4 and 5, we showed the results of analysis of MDR gene expression in the distinct morphological

### Table 1 | The clinicopathological parameters of BC patients, n = 382

| Clinicopathological parameter | N (%) |
|-------------------------------|-------|
| Age (year) ≤50                  | 221 (57.9) |
|                              >50         | 161 (42.1) |
| Menstrual status Pre           | 188 (49.2) |
|                              Post        | 194 (50.8) |
| Tumor size T1                  | 102 (26.7) |
|                              T2         | 239 (62.5) |
|                              T3         | 35 (9.2)  |
|                              T4         | 6 (1.6)   |
| Lymph node status N0           | 176 (46.1) |
|                              N1         | 122 (31.9) |
|                              N2         | 78 (20.4) |
|                              N3         | 6 (1.6)   |
| Estrogen receptor Positive     | 146 (38.2) |
|                              Negative    | 136 (35.6) |
|                              No data     | 100 (26.2) |
| Progesterone receptor Positive | 150 (39.3) |
|                              Negative    | 132 (34.5) |
|                              No data     | 100 (26.2) |
| HER2 0                        | 219 (57.3) |
|                              1+         | 36 (9.4)  |
|                              2+         | 16 (4.3)  |
|                              3+         | 36 (9.4)  |
|                              No data     | 75 (19.6) |
| Histological form Unicentric   | 322 (84.3) |
|                              Multicentric | 60 (15.7) |
| NAC regimen FAC                | 114 (29.9) |
|                              FAC         | 206 (53.9) |
|                              Taxotere    | 62 (16.2) |
| NAC response Complete response | 0 (0)     |
|                              Partial response | 169 (44.2) |
|                              Stable disease | 202 (52.9) |
|                              Progressive disease | 11 (2.9) |

All patients had invasive carcinoma of no special type

Abbreviations: NAC, neoadjuvant chemotherapy; CAX, Cyclophosphamide-Adriamycin-Cyclophosphamide; FAC, 5-Fluorouracil-Adriamycin-Cyclophosphamide; HER2 testing is performed in accordance with American Society of Clinical Oncology/College of American Pathologists Guideline 2007

**Recommendation**\textsuperscript{48}.

Analysis of correlation of intratumoral morphological heterogeneity of IC NST and expression of MDR genes. Using RT-PCR, we have compared the expression levels of MDR genes in the pre- and post-NAC tumor samples (n = 69) with the presence/absence of different morphological structures. Statistical analysis of the correlation of gene expression with different morphological structures of tumor was performed using logistic regression with Bonferroni correction. Expression of ABCB1 (p = 0.007) and ABCC5 (p = 0.027) genes was significantly lower in post-NAC tumors than in pre-NAC tumors with trabecular structures compared with tumors without trabecular structures. Patients with solid structures displayed a decreased post-NAC expression of ABCB1 (p = 0.011) and ABCG2 (p = 0.002) genes in comparison with patients whose tumors did not contain the solid structures. However, the differences in the expression of ABCB1 and ABCG2 were not significant after Bonferroni correction (p > 0.05; Table 3). In Tables 4 and 5, we showed the results of analysis of MDR gene expression in the distinct morphological

### Table 2 | The response to neoadjuvant chemotherapy depending on the presence of different types of morphological structures in breast tumors

| | n | PR N (%) | SD + PD N (%) | Uncorrected p value | Corrected p value* |
|---|---|----------|---------------|---------------------|--------------------|
| Alveolar structures no | 151 | 81 (53.6) | 70 (46.4) | 0.0028 | 0.014 |
| yes | 231 | 88 (38.1) | 143 (61.9) | 0.0272 | 0.136 |
| Trabecular structures no | 86 | 47 (54.7) | 39 (45.3) | 0.7819 | NA |
| yes | 296 | 122 (41.2) | 174 (58.8) | 0.6068 | NA |
| Tubular structures no | 248 | 111 (44.8) | 137 (55.2) | 0.9043 | NA |
| yes | 134 | 58 (43.3) | 76 (56.7) | NA |
| Solid structures no | 225 | 102 (45.3) | 123 (54.7) | 0.5014 | 0.206 |
| yes | 157 | 67 (42.7) | 90 (57.3) | 0.5127 | 0.211 |
| Discrete groups of tumor cells no | 155 | 68 (43.9) | 87 (56.1) | 0.0001 | 0.0001 |
| yes | 227 | 101 (44.5) | 126 (55.5) | 0.0001 | 0.0001 |

Statistical analysis: p value, Pearson’s chi-squared test;

*Bonferroni-corrected p value was calculated as the each p value multiplied by the number of tests (n = 5).

Abbreviations: n, number of patients with presence (yes) or absence (no) of any morphological structures; N, number of patients with presence/absence of any morphological structures possessing response/non-response to neoadjuvant chemotherapy; PR, partial response; SD, stable disease; PD, progressive disease; NA, not applied.
alveolar structures
trabecular structures
tubular structures
solid structures
discrete groups of tumor cells

gene expression

| Gene expression | pre-NAC | post-NAC |
|-----------------|---------|---------|
| ABCB1           | 0.18    | 1.13    |
| ABCC5           | 0.91    | 0.84    |
| ABCG1           | 1.39    | 1.23    |
| ABCG2           | 6.70    | 4.78    |
| ABCC1           | 4.63    | 2.77    |
| ABCC2           | 3.00    | 1.30    |
| ABCC3           | 1.07    | 0.79    |
| ABCC4           | 3.72    | 2.30    |
| ABCC6           | 1.52    | 1.23    |
| ABCG2           | 2.45    | 1.20    |

Table 3: The link of pre- and post-NAC expression levels of MDR genes with the presence/absence of different types of morphological structures in breast tumors

Discussion

In this study, we demonstrated that intratumoral morphological heterogeneity of breast cancer, which was previously described in the most common histological type – invasive carcinoma of no special type, influences the efficiency of neoadjuvant chemotherapy. On a larger scale than our previous study, we confirmed, with greater statistical significance, the association between the presence of alveolar structures in breast tumors and poor NAC response, which was significant after Bonferroni correction. In addition, trabecular structures were also related to chemotherapy resistance as previously suggested; however, the Bonferroni correction did not show the significance of this association.

Chemotherapy efficiency is composed of many host factors and tumor alterations. The most common reason for cancer drug resistance is the expression of one or more ATP-binding cassette transporters that detect and eject anticancer drugs from tumor cells. The data of our previous study suggest that changes in expression of MDR genes during the chemotherapy process or the development of adaptive MDR, but not the mRNA levels of these genes per se, are associated with NAC efficiency. In particular, reduction in MDR gene expression in post-NAC samples in comparison with pre-NAC tumors was linked with good response to chemotherapy, whereas patients displaying MDR gene upregulation exhibited resistance to therapy. In addition, recent data indicate that chemotherapy-induced upregulation of MDR genes can result in decreased distant metastasis and disease-free survival.

The data obtained in this study suggested that chemoresistance of breast tumors with trabecular structures is associated with high levels of expression of MDR genes. Trabecular structures showed more active MDR genes. In addition, only trabecular structures were found to express the ABCB1 gene encoding P-glycoprotein, which is a broad-spectrum drug efflux pump and plays a central role in MDR. This is in accordance with our published study performed on the basis of FFPE tumor material. Interestingly, an increased expression of MDR genes both before and after NAC, and their upregulation during chemotherapy were not found in breast tumors with trabecular structures.
In contrast, poor response of breast tumors with alveolar structures to NAC seems not to correlate to MDR gene activity. In particular, tumors with alveolar structures did not show high expression of MDR genes both before and after NAC. Moreover, MDR gene upregulation was also absent in these tumors. In addition, laser microdissection-based expression analysis showed a low activity of MDR genes in alveolar structures. Previously, MDR genes and ABC transporters were found to be expressed in different cells of the tumor microenvironment, but no upregulation was also absent in these tumors. In contrast, immunohistochemical analysis of two IC NST cases used in laser microdissection of the present study showed that in alveolar structures expression of MUC-1 (EMA) is observed in the whole cytoplasmic membrane and/or cytoplasm. In addition, in alveolar structures the change of cell polarization is not found, although the loss of interaction with stroma sometimes occurs (Fig. 2). It should be also pointed out that in IC NST, the frequency of alveolar structures comprises 60–83% (our unpublished data), whereas the proportion of micropapillary foci constitutes only 7.0%29.

It is interesting to note that tumor spheroids (microemboli) were previously detected in peripheral blood of patients with lung24–26, prostate26–27, renal cell26, colorectal26,40, and breast26 carcinoma. In comparison with circulating single tumor cells, such tumor clusters were shown to have anoikis suppression and the highest metastatic potential26. In addition, it has been suggested that the lack of apoptosis and perhaps proliferation make tumor clusters more resistant to chemotherapy than solitary tumor cells41.

Overall, our data confirm that intratumoral morphological heterogeneity of invasive carcinoma of no special type is related to NAC efficiency. Breast tumors containing alveolar structures demonstrate a poor response to NAC, and such observation is not explained by initial and adaptive MDR or upregulation of MDR genes during chemotherapy. In addition, the presence of trabecular structures in breast tumors is also associated with chemoresistance probably via ABCB1 expression (P-glycoprotein). Further studies are needed to investigate what factors/mechanisms are involved in chemoresistance of breast tumors with trabecular and alveolar structures.

### Methods

**Patients, tumors, and treatments.** Patients (n = 382) with clinical stage IIA to IIIIC (T1-4 N0-3 M0) IC NST, between 25 and 71 years of age (mean age: 51.9 ± 5.0), and treated in the Cancer Research Institute (Tomsk, Russia) between 2006 and 2012 were included (Table 1). The procedures followed in this study were in accordance with the Helsinki Declaration (1964, amended in 1975 and 1983). This study was approved by the institutional review board, and all patients signed an informed consent for voluntary participation. All patients received two to four preoperative cycles of FAC (5-Fluorouraill, Adriamycin, and Cyclophosphamide), CAX (Cyclophosphamide, Adriamycin, Xeloda) regimen, or Taxotere. Physical examination was performed

### Table 4 | Expression levels of MDR genes in different types of morphological structures and in their microenvironment of the first breast tumor

| Genes | Alveolar structures | ME | Trabecular structures | ME | Solid structures | ME | Discrete groups of tumor cells | ME |
|-------|---------------------|----|-----------------------|----|------------------|----|-----------------------------|----|
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |

Abbreviations: ME, microenvironment

### Table 5 | Expression levels of MDR genes in different types of morphological structures and in their microenvironment of the second breast tumor

| Genes | Alveolar structures | ME | Trabecular structures | ME | Solid structures | ME | Discrete groups of tumor cells | ME |
|-------|---------------------|----|-----------------------|----|------------------|----|-----------------------------|----|
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |
| ABCB1 | 0.002               | 0.0004 | 5.351            | 301.593 | 0.001 | 0.739 | 0.053 | 6.386 | 0 |

Abbreviations: ME, microenvironment
before NAC and was repeated after 2 cycles of NAC and before surgery to determine clinical response. Imaging of the primary breast lesion was performed with mammography and/or ultrasonography. Clinical and imaging responses to NAC were categorized into the following groups according to the International Union Against Cancer criteria: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Patients were grouped into clinical responders (PR) and non-responders (SD and PD). Fresh tumor tissues (n = 382) obtained after NAC were fixed in 10% neutral formalin (Karbolit, Russia) for 24 hours, rinsed with an isopropanol solution (Biovitrum, Russia), and embedded in paraffin (Biovitrum, Russia). In addition, out of 382 fresh tumor samples, 69 (randomly selected) samples and their biopsies (before NAC) were placed in RNA-later solution (Ambion, USA) and were stored at –80°C until RNA isolation. The operative samples from two patients without NAC were collected after surgery in nitrogen and were stored at –80°C until laser microdissection. Cases with CR were excluded from this study because of a loss of post-NAC tumor samples for expression analysis.

Morphological analysis. Morphological analysis included identification of different morphological structures in breast tumors and was performed by light microscope (Axio Scope, Carl Zeiss, Germany). The presence of tubular, trabecular, solid, and alveolar structures, and discrete groups of tumor cells was evaluated in breast tumors (n = 382) using 5 µm-thick tumor sections stained by hematoxylin (Dako, Denmark) and eosin (Dako, Denmark). All tumor slides (five from each sample) were reviewed by three experienced pathologists. Tubular structures used in tumor grading were identified as rows of tiny tube-shaped cell aggregations. Trabecular structures were formed by two or more rows of cells. Solid structures represented groups of hundreds of cells with different sizes and shapes. Discrete groups of tumor cells were detected as single cells or as groups of up to five cells. Alveolar structures with rounded shapes contained 10–30 cells. It is important to note that tumors from different patients may have different types of morphological structures. Detailed descriptions and images of different types of morphological structures were presented in our previous paper.

RNA isolation and cDNA synthesis. Total RNA was extracted from 69 samples of pre- and post-NAC tumor tissues using the RNeasy Mini Kit Plus DNase I digestion (Qiagen, Germany). Ribolock RNase inhibitor (Fermentas, Lithuania) was added to the isolated RNA. To assess RNA integrity, RIN was measured using 2200 TapeStation Instrument and R6K ScreenTape (Agilent Technologies, Inc., Santa Clara, USA). RNA with RIN > 6 was reverse transcribed to cDNA using the

Table 6 | The relationship between changes in MDR gene expression and presence/absence of different types of morphological structures in breast tumors

|          | ABCB1 | ABCB1 | ABCCC1 | ABCG1 | ABCG2 |
|----------|-------|-------|--------|-------|-------|
| Alveolar structures | 7/12 (37/63) | 9/10 (47/53) | 10/9 (53/47) | 5/14 (26/74) | 7/12 (37/63) |
| yes | 9/8 (53/47) | 10/7 (59/41) | 10/7 (59/41) | 7/9 (44/56) | 10/7 (59/41) |
| yes | 25/27 (48/52) | 26/26 (50/50) | 25/25 (50/50) | 19/32 (37/63) | 21/31 (40/60) |
| Tubular structures | 15/13 (54/46) | 15/13 (54/46) | 13/13 (50/50) | 11/16 (41/59) | 12/16 (43/57) |
| yes | 19/22 (46/54) | 21/20 (51/49) | 22/19 (54/46) | 15/25 (38/62) | 19/22 (46/54) |
| Solid structures | 20/11 (54/46) | 17/14 (55/45) | 18/12 (50/50) | 10/1 (35/65) | 11/14 (35/65) |
| yes | 14/24 (37/63) | 19/19 (50/50) | 17/20 (46/54) | 15/21 (42/58) | 14/24 (37/63) |
| Discrete groups of tumor cells | 14/8 (64/36) | 10/12 (45/55) | 13/9 (59/41) | 10/11 (48/52) | 15/7 (68/32) |
| yes | 20/27 (43/57) | 26/21 (55/45) | 22/23 (49/51) | 16/30 (35/65) | 16/31 (34/66) |

Statistical analysis: Pearson’s chi-squared test was used to detect significance of relationship between changes in MDR gene expression and the presence/absence of different types of morphological structures.\(^1\) \(p = 0.022\) (Bonferroni-corrected \(p = 0.55\)); \(^2\) \(p = 0.008\) (Bonferroni-corrected \(p = 0.2\)). The Bonferroni-correction was calculated as the each \(p\) value multiplied by the number of tests (n = 25).

Abbreviations: n1, number of patients with increase in gene expression after neoadjuvant chemotherapy; n2, number of patients with decrease in gene expression after neoadjuvant chemotherapy; no, the absence of any morphological structures; yes, the presence of any morphological structures.
RevertAid Kit with random hexanucleotide primers (Fermentas, Lithuania) following the manufacturer’s instructions.

**Laser microdissection.** Frozen tumor samples from two untreated patients with IC NSU were isolated for PALM MicroBeam laser capture microdissection (Carl Zeiss, Germany). Alveolar, trabecular, solid structures, and discrete groups of tumor cells were isolated from 5 μm-thick sections stained by hematoxylin (Dako, Denmark) and eosin (Dako). In Fig. 11, the microenvironment of these structures was also isolated. Note that tumors of these patients did not contain tubular structures. The microdissected material was collected in RLT lysis buffer (RNasey Plus Micro Kit, Qiagen, USA), and RNA was extracted according to the manufacturer’s instructions. Ribolock RNase inhibitor (Fermentas, Lithuania) was added to the isolated RNA. RIN was measured using 2200 TapeStation Instrument and High Sensitivity R6K ScreenTape (Agilent Technologies, Inc., Santa Clara, USA). cDNA was synthesized, ligated, and amplified using a QuantiTect Whole Transcriptome Kit (Qiagen, USA) according to the manufacturer’s instructions.

**Expression analysis.** The expression levels of the MDR genes were measured by quantitative real-time PCR (qRT-PCR) based on TaqMan technology using a Rotor-Gene-6000 instrument (Corbett Research, Australia). qRT-PCR was performed in triplicate reactions in a volume of 15 μl containing 250 μM dNTPs (Sibenzyme, Russia), 300 nM forward and reverse primers, 200 nM probe, 2.5 μM MgCl₂, 1× SS buffer (67 mM Tris-HCl pH 8.8 at 25 °C, 16.6 mM (NH₄)₂SO₄, 0.1% Tween-20), 2.5 U Hot Start Taq polymerase (Sibenzyme, Russia), and 50 ng of template cDNA. Samples were heated for 10 min at 95 °C followed by 40 cycles of amplification for 10 s at 95 °C and 20 s at 60 °C. The primer and probe sequences of ABCB1, ABCG1, ABCG2, and ABCG2 were obtained from a previous study. Two internal genes - GAPDH (in case of RNA from tumor bulk) and ACTB (RNA from the microdissected material) were used to normalize expression levels of the studied genes. The average C₅ (cycle threshold) was estimated for both the gene of interest, GAPDH and ACTB. Relative expression was evaluated using the Pfaff method, and the formula was used to determine the expression ratio between the sample and the calibrator. The relative expression level was also normalized to a calibrator consisting of a pool of normal breast tissue specimens. For this purpose, specimens of adjacent normal breast tissue from 10 breast cancer patients (NAC-free) were used as a source of normal RNA. In case of the microdissected samples, we normalized the expression levels relative to normal breast tissue of the same patient. The results were presented as a fold differences in MDR gene expression relative to GAPDH/ACTB and normal breast tissue.

**Statistics.** Statistical analyses were performed using STATISTICA 8.0 software (StatSoft, Tulsa, OK, USA). The arithmetic mean value and standard error were calculated for each sample group. Logistic regression was applied to identify the link between pre-/post-NAC expression levels of MDR genes and the presence/absence of different types of morphological structures in breast tumors. Pearson’s chi-square test was used to detect the association of NAC response and change (increase or decrease) in expression of MDR genes with the presence/absence of multiple morphological structures in breast tumors. The Bonferroni correction was applied to address the problem of multiple comparisons and was calculated as the calculated p value multiplied by the number of comparisons. The necessary to apply Bonferroni correction to the comparisons made was dictated by the confirmatory nature of this study with low key question concerning several hypotheses analysed by multiple significance tests. P-values that were corrected to values more than 1 were truncated to 1. Differences were significant if the corrected p value was less than 0.05. All p values were two-sided.

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Author contributions
N.V.L., E.V.D., V.M.P. and N.V.C. designed the study. M.V.Z., S.V.V., E.Yu.G. and E.M.S. collected the tumor samples and the patient records. N.V.L., E.V.D., M.V.Z., S.V.V., M.M.T. and T.S.G. performed the experiments. N.V.L., E.V.D., M.V.Z. and T.S.G. analysed and statistically processed the data. N.V.L. and E.V.D. wrote the paper. E.M.S., V.M.P. and N.V.C. assisted in critiquing, editing, and refining the paper. All authors reviewed the manuscript.

Additional information
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