Phyllodes tumor of the breast (PTB) is a rare neoplasm and accounts for 0.2–2.0% of breast cancer in women. Histopathological diagnosis of the tumor is difficult, and histological features do not always predict the course of the disease and the risk of progression. Pathogenesis and molecular biological characteristics as well as PTB prognostic factors are unknown. In search for genetic factors affecting PTB progression, 10 patients were analyzed for whom material from the primary tumor and local recurrence was available. DNA isolated from paraffin blocks was sequenced using the next-generation sequencing method (NGS). In 4 pairs, consisting of primary tumor and local recurrence, probably pathogenic/pathogenic variants were detected, and in three pairs they were observed in the $CDKN2A$ gene, while other variants were found in $PTEN$ and $TP53$ genes. NGS results indicate that the above-mentioned variants are hereditary, which suggests that the $CDKN2A$ gene might be involved in cancerogenesis of PTB. Additionally, the selected pathogenic variant of $EGFR$ gene was exclusively detected in one recurrent tumor, which might suggest the involvement of this gene in the mechanism of progression. In order to determine if this variant is associated with progression, the frequency of this mutation should be examined in larger group of malignant and borderline tumors.

Key words: phyllodes tumor, breast, next-generation sequencing, gene variants.

Introduction

Phyllodes tumor of the breast (PTB) is a rare neoplasm (it constitutes from 0.2 to 2.0% of all breast neoplasms in women), composed of elements of glandular epithelium, myoepithelium and stroma, which determines the biology of this cancer [1]. The World Health Organization classification distinguishes three PTB subtypes (benign, borderline and malignant) [2]. These subtypes present a morphological continuum from benign to malignant [1]. The classification is based on a combination of several histological features including: the degree of stromal atypia, stromal overgrowth, mitotic activity and type
of tumor border. For this reason, histopathological
diagnosis of PTB is difficult and based only on histo-
logical features, it is not always possible to assess the
course of the disease and the risk of cancer progres-
sion [3]. Currently, none of the biomarkers tested and
described in the literature have been used in everyday
clinical practice. Pathogenesis and molecular biolog-
ical characteristics of PTB are unknown and no PTB
prognostic factors have been identified. Generally,
accepted treatment approach for patients with all of
PTB types is breast conserving surgery with a mar-
gin >1 mm, which is sufficient to prevent local re-
currence of PTB [4, 5]. There are no indications for
resection of axillary lymph nodes or sentinel lymph
node biopsy, because metastases to the lymph nodes
occur very rarely [4, 6]. There are also no indications
for hormonal therapy in patients with PTB. Adju-
vant radiotherapy is recommended in borderline and
malignant PTB, if the resection margin is less than
1 cm [4, 5, 7].

Most patients with diagnosed PTB have a good
prognosis, with local recurrence risk from 17%
in the benign type, up to 27% in the malignant
type [8]. Distant metastases occur even in 22%
of malignant PTB cases [8, 9]. The histological
subtype of PTB is the only prognostic factor in
these patients [6]. However, in some individuals,
histological features do not allow for adequate as-
sessment of the course of the disease (prediction
of local recurrence, distant metastases or survival).
This research has been undertaken to search for
gene mutations related to the process of local re-
currence in breast phyllodes tumor. If the onset of
recurrence is caused by specific molecular changes,
these changes should be present in most PTB re-
currences and possibly in their respective primary
tumors.

Material and methods

Patients

Between 1990 and 2013, 159 patients diag-
osed with PTB were treated at the Oncology Cen-
ter in Krakow. In this group, 16 patients developed
local recurrence.

In 4 patients, after re-evaluation of pathology
slides recurrent tumors were classified as sarcomas.
Two patients were operated outside the Oncology
Center and obtaining tissue material from the
primary tumor was impossible. This pilot study
was carried out on a group of 10 selected patients.
There were 4 malignant primary PTBs (in one case
there were two clearly separated components: be-
nign and malignant), 2 borderline PTBs, 3 mixed
types (benign/borderline) and 1 benign PTBs (Ta-
ble I). In 4 cases progression was observed (Table I).

The study has been reported to the Ethical
Committee at the Regional Medical Chamber in
Krakow and received positive decisions (number:
L.DZ.OIL/KBL/1/220/71220). This is a retrospec-
tive study using archived preserved tissues. There
were no direct contact with patients, no modifica-
tion of diagnostic or treatment procedures. None
of personal patients’ data were revealed and no
specific patient consent was needed.

Next-generation sequencing

DNA was extracted from formalin-fixed paraf-
fin-embedded tissues. Before extraction the tissue
slides were evaluated by a pathologist to confirm
the diagnosis and to select the paraffin blocks most
suitable for molecular analysis. In the case of pri-
mary tumor with two clearly separated compo-
nents (benign and malignant), for each component
DNA was isolated separately. DNA was obtained
from tissue sections using a semi-automatic meth-
od with Maxwell® RSC Instrument (Promega). The
Maxwell® RSC DNA FFPE Kit (Promega) was
used to isolate genomic DNA using paramag-
etic particles. The amount of DNA was assessed
fluorimetrically using the Qubit™ dsDNA HS As-
say Kit and Qubit 3.0 device (ThermoFisher Sci-
entific). Next-generation sequencing (NGS) was
performed on IonTorrent platform. Two hundred
and seven regions in 50 genes that are most of-
ten mutated in solid tumors were sequenced with
Cancer Hotspot Panel v2. Libraries for sequencing
were prepared automatically on Chef instrument
using Ion Ampliseq™ Cancer Hotspot Panel v2
Chef-Ready Kit. Sequencing was performed using
S5 instrument, Ion 510™ & Ion 520™ & Ion 530™
Kit and Ion 530™ Chip Kit. S5 Torrent Server Tor-
rent Suite 5.10.1 software was applied for coverage
analysis to identify genes variants. Results (vcf
files) were annotated using wANNOVAR (http://
wannovar.wglab.org). Variants for which satis-
factory sequencing conditions were obtained and
which are estimated to occur in the population
with a frequency below 1% were qualified for fur-
ther analysis. Variants occurring in the population
with a frequency above 1% are classified as poly-
morphisms. With the help of the Varsome (https://
nih.gov/clinvar) databases variants were classified
into 5 classes: pathogenic, probably pathogenic,
of unknown significance, probably benign and be-
nign. The workflow of the experiment is presented
in Figure 1.
Results

Assessment of NGS data

Next-generation sequencing (NGS) was performed for 10 pairs consisting of primary and recurrent tumor. The most important parameters of the run are presented in Table I. The age of the blocks ranged from 5.6 to 24.7 years. For all samples there was enough of DNA to perform sequencing. However, in the case of two pairs with the oldest blocks (aged 23-24 years) the data obtained were of low quality and mutation analysis for these samples was not possible (Table I). DNA from these samples was too fragmented, especially from primary tumor.

Table I. Quality of obtained NGS data

<table>
<thead>
<tr>
<th>Pair No.</th>
<th>Sample number and description</th>
<th>Age of block (years)</th>
<th>Mean read length</th>
<th>Mapped reads</th>
<th>Percent reads on target</th>
<th>Average base coverage depth</th>
<th>Uniformity of base coverage</th>
<th>Target base coverage at 100x (%)</th>
<th>Number of detected variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M5 PT benign/borderline</td>
<td>17.0</td>
<td>99 bp</td>
<td>979 151</td>
<td>93.64%</td>
<td>3857</td>
<td>97.52%</td>
<td>100</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>M6 R(1*) benign/borderline</td>
<td>13.8</td>
<td>103 bp</td>
<td>991 806</td>
<td>95.95%</td>
<td>4106</td>
<td>96.89%</td>
<td>100</td>
<td>344</td>
</tr>
<tr>
<td>2</td>
<td>M7 PT borderline</td>
<td>13.1</td>
<td>106 bp</td>
<td>1 288 888</td>
<td>97.57%</td>
<td>5517</td>
<td>99.48%</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>M8 R(3*) borderline</td>
<td>11.6</td>
<td>113 bp</td>
<td>1 383 908</td>
<td>98.68%</td>
<td>6250</td>
<td>99.48%</td>
<td>100</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>M9 PT benign</td>
<td>10.2</td>
<td>107 bp</td>
<td>1 254 256</td>
<td>98.32%</td>
<td>5433</td>
<td>98.25%</td>
<td>100</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td>M10 R(1*) malignant</td>
<td>7.2</td>
<td>115 bp</td>
<td>1 387 224</td>
<td>98.81%</td>
<td>6365</td>
<td>99.38%</td>
<td>100</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>M11 PT benign/borderline</td>
<td>24.3</td>
<td>89 bp</td>
<td>149 326</td>
<td>70.56%</td>
<td>408.8</td>
<td>73.29%</td>
<td>68.88</td>
<td>858</td>
</tr>
<tr>
<td>5</td>
<td>M12 R(2*) malignant, sarcoma like G3</td>
<td>23.7</td>
<td>96 bp</td>
<td>493 945</td>
<td>86.83%</td>
<td>1734</td>
<td>88.23%</td>
<td>97.73</td>
<td>1211</td>
</tr>
<tr>
<td>6</td>
<td>M13 PT benign/borderline</td>
<td>16.8</td>
<td>103 bp</td>
<td>1 008 257</td>
<td>97.05%</td>
<td>4217</td>
<td>97.50%</td>
<td>100</td>
<td>331</td>
</tr>
<tr>
<td>7</td>
<td>M14 R(3*) malignant</td>
<td>13.6</td>
<td>107 bp</td>
<td>1 430 807</td>
<td>98.77%</td>
<td>6260</td>
<td>87.01%</td>
<td>100</td>
<td>41</td>
</tr>
<tr>
<td>8</td>
<td>M15 PT borderline</td>
<td>17.7</td>
<td>95 bp</td>
<td>502 980</td>
<td>85.67%</td>
<td>1748</td>
<td>88.14%</td>
<td>100</td>
<td>987</td>
</tr>
<tr>
<td></td>
<td>M16 R(2*) malignant</td>
<td>7.50</td>
<td>111 bp</td>
<td>1 268 905</td>
<td>98.60%</td>
<td>5679</td>
<td>99.48%</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>M17 PT c. benign**</td>
<td>10.7</td>
<td>112 bp</td>
<td>1 165 877</td>
<td>98.41%</td>
<td>5271</td>
<td>99.48%</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>M18 PT c. malignant</td>
<td>10.7</td>
<td>114 bp</td>
<td>909 468</td>
<td>98.21%</td>
<td>3997</td>
<td>99.48%</td>
<td>100</td>
<td>19</td>
</tr>
<tr>
<td>10</td>
<td>M19 R(1*) malignant</td>
<td>10.5</td>
<td>104 bp</td>
<td>1 088 939</td>
<td>96.62%</td>
<td>4569</td>
<td>99.17%</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>11</td>
<td>M20 PT malignant</td>
<td>6.8</td>
<td>106 bp</td>
<td>1 353 842</td>
<td>98.45%</td>
<td>5859</td>
<td>87.84%</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>12</td>
<td>M21 R(1*) malignant</td>
<td>6.6</td>
<td>109 bp</td>
<td>1 371 358</td>
<td>98.70%</td>
<td>6070</td>
<td>89.48%</td>
<td>100</td>
<td>39</td>
</tr>
<tr>
<td>13</td>
<td>M23 PT malignant</td>
<td>6.6</td>
<td>113 bp</td>
<td>1 385 681</td>
<td>98.67%</td>
<td>6309</td>
<td>98.84%</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>14</td>
<td>M24 R(1*) malignant</td>
<td>5.6</td>
<td>109 bp</td>
<td>1 295 685</td>
<td>98.49%</td>
<td>5714</td>
<td>86.35%</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td>15</td>
<td>M25 PT malignant</td>
<td>24.7</td>
<td>126 bp</td>
<td>5 510</td>
<td>33.47%</td>
<td>7.02</td>
<td>65.13%</td>
<td>0.21</td>
<td>17</td>
</tr>
<tr>
<td>16</td>
<td>M26 R(1*) malignant</td>
<td>24.2</td>
<td>133 bp</td>
<td>1 487 752</td>
<td>65.42%</td>
<td>382.3</td>
<td>75.63%</td>
<td>69.03</td>
<td>904</td>
</tr>
</tbody>
</table>

*– the number of tested recurrence, ** c – component, PT – primary tumor, R – recurrence

Fig. 1. Workflow of the experiment and data analysis
These data were characterized by too low number of reads, low uniformity (65-88%), percent reads on target (33-86%) and low target base coverage at 100× (i.e. the percentage of bases for which 100 readings were obtained, the recommended minimum required for analysis is 98%) (Table I). For all other cases, coverage at 100× was 100%, so even for 17-year-old blocks it was possible to obtain material that allowed analysis. However, it should be noted that for blocks aged 13-17 years a large number of observed changes should be considered as sequencing errors (low sequencing parameters) and changes typical for the formalin-fixed material (change G>A and T>C).

Comparison of primary and recurrent tumor

Eight primary tumor – recurrence pairs (16 samples) were qualified for the analysis of mutation status. In 4 out of 8 pairs, probably pathogenic/pathogenic variants, according to Varsome and/or ClinVar databases, were detected. In three of them mutations were found in CDKN2A gene (Table II). In two pairs, the same variant creating the STOP codon and causing synthesis of the truncated protein was detected. The other variant in CDKN2A gene was a non-synonymous change, resulting in insertion of another amino acid. In the case of the PTEN gene, there was a change of the reading frame, resulting in truncated protein, while in the TP53 gene non-synonymous change was found (Table II). Moreover, in one patient, two clearly separated components: benign and malignant were identified in the primary tumor. Both components were isolated and tested separately. Pathogenic/probably pathogenic variants in the CDKN2A and TP53 genes were detected only in the malignant component. The same mutations were also detected in the recurrent tumor. Only in one pair, a mutation that was not present in the primary tumor (involving the EGFR gene) was detected in the recurrence.

Discussion

Breast phyllodes tumor is a rare breast neoplasm and most patients with diagnosed PTB have a good prognosis. Metastatic disease is observed less frequently than local recurrences, however, it is associated with worse prognosis [10]. Recurrences of malignant PTB are found in 23-30% of patients, while in benign and borderline PTBs recurrences appear in 10-20% and 14-25% of cases, respectively [1]. The frequency of local recurrence is higher in patients with malignant and borderline PTB than in benign cases [9]. Breast conserving therapy is proposed in all cases of local PTB recurrences, if a margin of healthy tissue can be achieved with good cosmetic result [11]. Benign PTB is successfully treated with surgery alone. In borderline and malignant type, with a tumor-free margin < 1 cm (0.3-0.8 cm), adjuvant radiotherapy is recommended [4]. Only in the cases of extensive recurrences, simple mastectomy may be necessary to obtain a negative margin.

In our study, we discovered mutations in primary tumour and also in matched local recurrence in CDKN2A (in 3 pairs), PTEN (1 pair) and TP53 (1 pair) genes. Additionally, in one pair only in the recurrent lesion mutation in the EGFR gene was observed. According to COSMIC database (https://cancer.sanger.ac.uk/cosmic) a frequently mutated gene in

Table II. List of detected pathogenic or likely pathogenic variants according to Varsome and/or ClinVar databases

<table>
<thead>
<tr>
<th>Paired sample numbers</th>
<th>Pathogenic or likely pathogenic variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>M7 PT borderline/</td>
<td>primary tumor and recurrence: CDKN2A, NM_000077.4:c.238C&gt;T (p.Arg80Ter)</td>
</tr>
<tr>
<td>M8 R(3+) borderline</td>
<td></td>
</tr>
<tr>
<td>M9 PT benign/</td>
<td>primary tumor and recurrence: CDKN2A, NM_000077.4:c.251A&gt;G (p.Asp84Gly)</td>
</tr>
<tr>
<td>M10 R(1+) malignant</td>
<td></td>
</tr>
<tr>
<td>M20 PT malignant/</td>
<td>recurrence: EGFR, NM_005228.5:c.2297T&gt;C (p.Met766Thr)</td>
</tr>
<tr>
<td>M21 R(1+) malignant</td>
<td></td>
</tr>
<tr>
<td>M23 PT malignant/</td>
<td>primary tumor and recurrence: PTEN, NM_000314.8:c.170dupT (p.Leu57PhefsTer6)</td>
</tr>
<tr>
<td>M24 R(1+) malignant</td>
<td></td>
</tr>
<tr>
<td>M17 PT c. benign**/</td>
<td>primary tumor benign component – lack of variants</td>
</tr>
<tr>
<td>M18 PT c. malignant/</td>
<td>primary tumor (malignant component) and recurrence:</td>
</tr>
<tr>
<td>M19 R(1+) malignant</td>
<td>CDKN2A, NM_000077.4:c.238C&gt;T (p.Arg80Ter)</td>
</tr>
<tr>
<td></td>
<td>TP53, NM_000546.5:c.711G&gt;A (p.Met237Ile)</td>
</tr>
</tbody>
</table>

* = the number of tested recurrence, ** = component, PT = primary tumor, R = recurrence
phyllodes tumours is MED12 (55%). Unfortunately, Cancer Hotspot Panel v2 does not assess mutation in MED12 gene. However, mutations of MED12 gene were less frequently observed in malignant phyllodes tumours than in fibroadenomas, benign and borderline phyllodes tumours [1]. Moreover, the presence of MED12 mutation was associated with longer disease-free survival, whereas its absence was related to a higher likelihood of recurrence [1].

In our study, we observed pathogenic variants in PTEN, TP53, EGFR and CDKN2A genes. The first three of them are reported by COSMIC in “Top 20 genes” for phyllodes tumours: TP53 (mutation in 12%), EGFR (6%), PTEN (4%). In our study, most frequently mutated gene was CDKN2A, in which mutation were observed both in primary tumours and local recurrences. Mutations or homozygous deletions in CDKN2A gene in phyllodes tumour are also reported by other authors [12, 13, 14]. Tan et al. studied a group of 20 cases of phyllodes tumour, including 11 cases with no recurrence and 9 with local recurrence/metastases. In this group, homozygous deletion at 9p21 involving CDKN2A gene was reported in two cases with progression, while in two other cases with progression single copy loss at 9p21 was noted. None of these aberrations were observed in cases that did not develop recurrences [13]. In our study, we detected pathogenic variants in the CDKN2A gene in as many as three out of eight pairs of primary and recurrent tumours. Tan et al. and our findings may suggest that CDKN2A gene is involved in the development of primary PTB and local recurrences/metastases.

It is worth to mention that all mutations detected in the paired samples affected the suppressor genes:

- **CDKN2A** – encodes a protein that is a CDK4-dependent cyclin kinase inhibitor and a P53 stabilizing protein,
- **PTEN** – encodes a protein that negatively regulates the PI3K/AKT signaling pathway,
- **TP53** – encodes a protein involved in, among others, a mechanism of controlling cell arrest in the cell cycle, apoptosis and DNA repair.

The allelic frequency of variants detected in the sequenced material from the primary tumour and recurrence (40-60%) suggests that they might be hereditary changes. In order to confirm this hypothesis in the future research, an attempt will be made to assess the presence of the detected variants in archival material derived from normal tissue (from the paired samples) by Sanger sequencing.

Only in one pair, in the recurrence but not in the primary tumour, pathogenic variant in the **EGFR** gene was found. This might be a possible molecular hallmark of progression. In order to determine if this variant is associated with progression, the frequency of this mutation should be examined in a larger group of malignant and borderline tumors.

**Conclusions**

The **CDKN2A** gene might be involved in the development of phyllodes tumor of the breast and its local recurrences/metastases.

Mutations in the **EGFR** gene might be a possible molecular hallmark of progression.

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**References**

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