Association of recurrent mutations in \textit{BRCA1}, \textit{BRCA2}, \textit{RAD51C}, \textit{PALB2}, and \textit{CHEK2} with the risk of borderline ovarian tumor

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Abstract

\textbf{Background:} There are several genes associated with ovarian cancer risk. Molecular changes in borderline ovarian tumor (BOT) indicate linkage of this disease to type I ovarian tumors (low-grade ovarian carcinomas). This study determined the prevalence and association of mutations in \textit{BRCA1}, \textit{BRCA2}, \textit{PALB2}, \textit{RAD51C}, and \textit{CHEK2} with the risk of BOTs.

\textbf{Methods:} The study group consisted of 102 patients with histologically confirmed BOT and 1743 healthy controls. In addition, 167 cases with ovarian cancer G1 were analyzed. The analyses included genotyping of 21 founder and recurrent mutations localized in 5 genes (\textit{BRCA1}, \textit{BRCA2}, \textit{PALB2}, \textit{RAD51C}, and \textit{CHEK2}). The risk for developing BOT and low-grade ovarian cancer, as well as the association of tested mutations with survival, was estimated.

\textbf{Results:} The \textit{CHEK2} missense mutation (c.470T>C) was associated with 2-times increased risk of BOT (OR=2.05, \(p=0.03\)), at an earlier age at diagnosis and about 10% worse rate of a 10-year survival. Mutations in \textit{BRCA1} and \textit{PALB2} were associated with a high risk of ovarian cancer G1 (OR=8.53, \(p=0.005\) and OR=7.03, \(p=0.03\), respectively) and were related to worse all-cause survival for \textit{BRCA1} carriers (HR=4.73, 95%CI 1.45–15.43, \(p=0.01\)).

\textbf{Conclusions:} Results suggest that \textit{CHEK2} (c.470T>C) may possibly play a role in the pathogenesis of BOT, but due to the low number of BOT patients, obtained results should be considered as preliminary. Larger more in-depth studies are required.

\textbf{Keywords:} Borderline ovarian tumor, Low-grade ovarian cancer, Recurrent mutations, \textit{BRCA1}, \textit{BRCA2}, \textit{RAD51C}, \textit{PALB2}, \textit{CHEK2}

Background

Borderline ovarian tumors (BOTs) are neoplasms of epithelial origin, characterized by upregulated cellular proliferation and the presence of nuclear abnormalities, but in contrast to ovarian cancer, they do not show infiltrative growth patterns [1, 2]. Borderline tumors constitute approximately 10–20\% of all epithelial ovarian neoplasms [3] and are histologically classified based on the epithelial cell type, similarly to invasive carcinomas. The most common subtypes are serous (50\%) and mucinous (45\%), while the less common include endometrioid, clear cell, seromucinous, and borderline Brenner tumor [1–3]. BOTs are usually limited to the ovaries and are diagnosed at FIGO stage I [4]. Although the prognosis in BOT patients is generally excellent, serous borderline tumors can implant on peritoneal surfaces and progress
to low-grade serous ovarian carcinoma [2, 3]. There are several genes associated with the risk of ovarian cancer [5–9]; however, very little is known about germline mutations related to BOTs [10–17].

Most cancer predisposition genes are considered molecularly heterogeneous, displaying hundreds of different disease-causing sequence alterations. However, in certain populations, founder mutations can be identified, which are located within a genomic region that is in linkage disequilibrium and therefore segregates as a unit. The founder mutations are inherited and often remain restricted to one or a few populations or specific geographic regions. The Polish population has been recognized as relatively homogeneous in terms of the genetic make-up. Studies on genetic predisposition to cancer diseases revealed the existence of a number of founder alleles and recurrent mutations in several genes, including BRCA1, CHEK2, and PALB2 genes [18–22].

Recently, 21 founder and recurrent mutations in BRCA1/2, PALB2, RAD51C, and CHEK2 genes have been analyzed in a large Polish case-control study including 2270 ovarian cancer patients and 1743 controls. Results have shown a significant association of mutations in BRCA1/2 and RAD51C with cancer risk [5].

In the current study, we analyzed the prevalence of 21 recurrent germline mutations in BRCA1, BRCA2, RAD51C, PALB2, and CHEK2 genes among Polish patients with BOTs and decided to determine whether are associated with the risk of developing this tumor.

Methods

Study group

The study group consisted of 102 patients with histologically confirmed BOT diagnosed between 1975 and 2018 in Independent Public Clinical Hospital No. 2 of the Pomeranian Medical University in Szczecin and were recruited between 2001 and 2017 years. The mean age of BOT patients at the time of recruitment was 49 years (range 17–83 years). Based on reported pathological and molecular similarities of BOTs and low-grade invasive ovarian tumors [23, 24], 167 women with low-grade ovarian cancer (grade 1—G1) were additionally included in analyses. The patients with ovarian cancer G1 were selected from a group of 2270 consecutive ovarian cancer cases described earlier [5]. The obtained clinical information included age at diagnosis, tumor histology, and family history of ovarian cancers. Data on the vital status was collected from the Ministry of Digital Affairs in July 2019. The characteristics of patients are presented in Table 1.

The control group consisted of 1743 healthy women selected from the registry of the International Hereditary Cancer Center in Szczecin, as described earlier [5]. Briefly, they were women recruited in the West Pomerania region between 2002 and 2019 year. For all controls, demographic and clinical data, as well as information about family history of ovarian cancer, were collected. The mean age of controls at the time of recruitment was 55.8 years (range 18–90 years). Healthy

| Feature                          | BOT cases, n=102 (%) | Ovarian cancer G1 cases, n=167 (%) |
|----------------------------------|----------------------|------------------------------------|
| Mean age at diagnosis (range)    | 47.76 (17–83)        | 54.25 (19–84)                      |
| Mean follow up in months (range) | 126 (10–528)         | 82 (1–216)                         |
| The histological type of tumor   |                      |                                    |
| Serous                           | 61 (59.8%)           | 79 (47.3%)                         |
| Mucinous                         | 22 (21.6%)           | 38 (22.8%)                         |
| Endometrioid                     | 0 (0%)               | 36 (21.6%)                         |
| Clear-cell                       | 0 (0%)               | 2 (1.2%)                           |
| Other or undefined/mixed         | 1 (0.1%)             | 2 (1.2%)                           |
| Missing                          | 18 (17.6%)           | 10 (6.0%)                          |
| Family history of OC             |                      |                                    |
| Yes                              | 12 (11.8%)           | 8 (4.8%)                           |
| No                               | 88 (86.3%)           | 152 (91.0%)                        |
| Missing                          | 2 (2.0%)             | 7 (4.2%)                           |
| Death                            |                      |                                    |
| Yes                              | 10 (9.8%)            | 44 (26.3%)                         |
| No                               | 88 (86.3%)           | 113 (67.7%)                        |
| Missing                          | 4 (3.9%)             | 10 (6.0%)                          |

*BOT borderline ovarian tumor
controls were followed since the time of recruitment, and the mean follow-up was 26.1 months (0–213 months).

Sample preparation molecular analysis
A blood sample was obtained from all cases and controls, and the DNA was isolated using a previously described method [25]. DNA samples were stored at 4 °C prior to genotyping. The analysis encompassed genotyping of 21 recurrent/founder germline mutations localized in five genes: *BRCA1* (9 mutations), *BRCA2* (4 mutations), *RAD51C* (3 mutations), *PALB2* (2 mutations), and *CHEK2* (3 mutations), as listed in Table 2.

Genotyping was performed as described earlier [5]. All mutations, with the exception of c.5266dupC in *BRCA1*, were genotyped by a real-time PCR using customized TaqMan Assays on LightCycler Real-Time PCR 480 System (Real-Time PCR System, Roche Diagnostics, Indianapolis, IN, USA), following the standard protocol. The reaction mix for analysis of each sample included GoTaq® Probe qPCR Master Mix (Promega, Madison, WI, USA), TaqMan Genotyping Assays × 40 or TaqMan Genotyping Assays × 80 (Applied Biosystems, Foster City, CA, USA), and deionized water (Promega, Madison, WI, USA). Samples were analyzed on 384-well plates. On each analyzed plate 3 control samples were included: positive and negative for tested mutations and a water-blind control. The genotyping was performed using LightCycler 480 Instrument, and data were analyzed using LightCycler 480 Basic Software Version 1.5 (Roche Diagnostics, Indianapolis, IN, USA). The analysis for c.5266dupC mutation in *BRCA1* was performed using allele-specific amplification PCR (ASA-PCR) as described previously [20]. The sequences of primers and probes used for genotyping are available on request.

All study participants provided written informed consent. The study was approved by the Ethics Committee of the Pomeranian Medical University in Szczecin (protocol code KB-0012/153/16 (BN-001/33/04), 20.12.2016).

Table 2 Recurrent mutations tested in *BRCA1*, *BRCA2*, *RAD51C*, *PALB2*, and *CHEK2* genes

| Gene | cDNA | Protein change | Molecular consequence |
|------|------|----------------|----------------------|
| *BRCA1* | 5382insC (c.5266dupC) | p.Gln1756fs | Frameshift |
|       | 300T>G (c.181T>G) | p.Cys61Gly | Missense |
|       | 4153delA (c.4035delA) | p.Glu1346fs | Frameshift |
|       | 1806C>T (c.1687C>T) | p.Glu563Ter | Nonsense |
|       | 185delAG (c.68_69delAG) | p.Glu25fs | Frameshift |
|       | 3819delS (c.3700_3704delS) | p.Val1234fs | Frameshift |
|       | 3875del4 (c.3756_3759delGTCT) | p.Ser1253fs | Frameshift |
|       | 5370C>T (c.5251C>T) | p.Arg1715Ter | Nonsense |
|       | 794delT (c.794_795delCT) | p.Ser265Cysfs | Frameshift |
| *BRCA2* | 4075delGT (c.3847_3848delGT) | p.Val1283fs | Frameshift |
|       | 8138del5 (c.7013_7017delTTTCCT) | p.Ala2357_Phe2638insTer | Nonsense |
|       | 886delGT (c.658_659del) | p.Val220fs | Frameshift |
|       | 6174delT (c.5946delT) | p.Ser1982fs | Frameshift |
| *RAD51C* | c.905-2_905-1delAG | p.Glu303TrpfsX41 | Skipping of exon 7 |
|       | c.577C>T | p.Arg193Ter | Nonsense |
|       | c.502A>T | p.Arg168Ter | Nonsense |
| *PALB2* | c.172_175delTTGT | p.Gln60fs | Frameshift |
|       | c.509_510delG>A | p.Arg170fs | Frameshift |
| *CHEK2* | c.1100delC* | p.Thr367fs | Frameshift |
| PTT | c.444+1G>A* | p.E149fsX6 | Skipping of exon 2 |
| Missense | c.470T>C | p.Leu157Thr | Missense |

*PTT protein truncating mutations*
date of diagnosis until death from any cause. The hazard ratios (HRs) were estimated for mutation carriers in comparison with non-carriers using Cox proportional hazards. In addition, the 5- and 10-year survival among the \( \text{BRCA1} \), \( \text{BRCA2} \), \( \text{RAD51C} \), \( \text{PALB2} \), and \( \text{CHEK2} \) mutation carriers was calculated.

All analyses were done separately for each gene, \( \text{BRCA1} \), \( \text{BRCA2} \), \( \text{RAD51C} \), \( \text{PALB2} \), and \( \text{CHEK2} \) (separately for two protein-truncating mutations and one missense mutation), as shown in Table 2) using R Project for Statistical Computing (R version: 4.0.3). The \( p \)-values less than 0.05 were recognized as statistically significant.

Results
Association of \( \text{BRCA1} \), \( \text{BRCA2} \), \( \text{RAD51C} \), \( \text{PALB2} \), and \( \text{CHEK2} \) mutations with BOT risk

Genotyping of 21 mutations in \( \text{BRCA1} \), \( \text{BRCA2} \), \( \text{RAD51C} \), \( \text{PALB2} \), and \( \text{CHEK2} \) genes was performed for 102 BOT patients, 167 cases of ovarian cancer G1, and 1743 healthy controls. The prevalence of mutations was similar in both tested groups of ovarian tumor patients: 12.75% in BOT (13 out of 102 cases) and 12.57% in ovarian cancer G1 cases (21 out of 167). In the control group, mutations were detected in 6.77% (118 out of 1743) individuals.

Mutations in \( \text{BRCA1} \) and \( \text{PALB2} \) genes were significantly associated with the high risk of ovarian cancer G1, while \( \text{CHEK2} \) missense mutation (c.470T>C) was associated with 2-times elevated risk of BOT. The distributions of mutations in \( \text{BRCA1} \), \( \text{BRCA2} \), \( \text{RAD51C} \), \( \text{PALB2} \), \( \text{CHEK2} \) genes, and the ORs associated with the risk of ovarian tumors are shown in Table 3.

The mean age at diagnosis of BOT was lower among cases with any detected mutation than without mutation: 45 years vs. 49 years, respectively. Carriers of \( \text{CHEK2} \) missense mutation (c.470T>C) were diagnosed about 10 years younger than non-carriers (38 years vs. 49 years).

The mean age at diagnosis of ovarian cancer G1 was similar for carriers of any mutation and non-carriers: 55 years and 54 years, respectively.

Among \( \text{CHEK2} \) missense (c.470T>C) mutation carriers, the family history of ovarian cancer was present in 3 (27.3%) patients with BOT and none with ovarian cancer G1.

There was no association of tested mutations with the histological type of ovarian tumor, neither in BOT nor ovarian cancer G1 patients.

All-cause survival

Information on all-cause survival was available from 98 out of 102 BOT cases, and among them, 10 deaths (10.2%) were recorded. There was no association of all-cause survival with mutations in any of the 5 tested genes. The 5-year survival rate for all BOT patients was 90.48% and was similar for any mutation carriers and non-carriers (90% and 89.93%, respectively); the 10-year survival rate was 88.89% and was about 10% lower for mutation carriers (80%) than for non-carriers (89.80%).

Table 3 The prevalence and association of tested 21 founder/recurrent mutations in \( \text{BRCA1} \), \( \text{BRCA2} \), \( \text{RAD51C} \), \( \text{PALB2} \), and \( \text{CHEK2} \) genes with the risk of BOT and low-grade ovarian cancer

| Gene     | Controls, \( n=1743 \) (%) | Cases | Bot, \( n=102 \) (%) | Ovarian cancer G1, \( n=167 \) (%) |
|----------|-----------------------------|-------|-----------------------|-----------------------------------|
| BRCA1    | 5 (0.29)                    | 0 (0.00) | 4 (2.40)               |                                   |
| OR (95% CI) | ref.                      | -     | 8.53 (2.09–32.54)     | 0.005                             |
| p value  |                             |       |                       |                                   |
| BRCA2    | 0 (0.00)                    | 1 (0.98) | 1 (0.60)               |                                   |
| OR (95% CI) | ref.                      | -     |                       |                                   |
| p value  |                             |       |                       |                                   |
| RAD51C   | 3 (0.17)                    | 0 (0.00) | 0 (0.00)               |                                   |
| OR (95% CI) | ref.                      | -     |                       |                                   |
| p value  |                             |       |                       |                                   |
| PALB2    | 3 (0.17)                    | 0 (0.00) | 2 (1.20)               |                                   |
| OR (95% CI) | ref.                      | -     | 7.03 (0.92–42.72)     | 0.03                              |
| p value  |                             |       |                       |                                   |
| CHEK2    | PTT                         | 10 (0.57) | 1 (0.98)               |                                   |
| OR (95% CI) | ref.                      |       |                       |                                   |
| p value  |                             |       |                       |                                   |
|         | missense mutation (c.470T>C)| 97 (5.57)| 11 (10.78)             | 13 (7.78)                         |
| OR (95% CI) | ref.                      |       | 2.05 (1.01–3.81)      | 1.43 (0.75–2.53)                  |
| p value  |                             |       |                       | 0.03                              |
In a group of ovarian cancer G1 patients, the information about all-cause survival was available from 157 of 167 cases, and 44 deaths (28%) were recorded. The estimated HRs for all-cause survival among mutation carriers were not statistically significant for any of the tested genes except BRCA1 (HR = 4.73, 95%CI 1.45–15.43, p = 0.01). The 5-year survival rate for all patients was 71.32%, and the 10-year survival rate was 59.36%. There was no difference between mutation carriers and non-carriers (5 years: 69.23% and 69.72%; 10 years: 60% and 58.62%).

**Discussion**

Borderline ovarian tumors (BOTs) represent a heterogeneous group of noninvasive tumors of uncertain malignant potential. They have characteristic histology and usually occur in younger women, before 40–45 years of age. The majority of BOT is diagnosed at the early stage and has a favorable prognosis [1–3]. However, the symptomatic recurrence rate varies between 3% [26] and 34% [27] and is associated with decreased survival [28]. The molecular changes in BOTs indicate linkage of this disease to type I ovarian tumors (low-grade ovarian carcinomas) [2, 3, 29, 30]. Recently, we found that pathogenic founder/recurrent germline mutations in 4 genes (BRCA1, BRCA2, RAD51C, PALB2) are responsible for 12.5% of ovarian cancer cases among unselected patients in the Polish population [5]. In the present study, we aimed to estimate the prevalence of 21 founder/recurrent germline mutations in 5 genes (BRCA1, BRCA2, RAD51C, PALB2, and CHEK2) among Polish patients with BOTs and to assess an association of these mutations with the risk of development of BOTs. Because of morphological similarities of borderline tumors and low-grade ovarian cancer [2, 3], we also included in the analysis 167 patients with ovarian cancer G1. Pathogenic mutations were detected in 12.74% of BOT (13 out of 102 cases), in 12.57% of ovarian cancer cases (21 out of 167 cases), and in 6.71% controls (117 out of 1743 women).

In the group of BOT patients, the most common was the missense mutation (c.470T>C) in the CHEK2 gene. The frequency of this mutation was higher in BOTs than in controls (10.8% vs. 5.6%), which corresponded to a statistically significant 2-times elevated risk of BOT (OR = 2.05, p = 0.03). The prevalence of other tested recurrent mutations in BRCA1, BRCA2, RAD51C, and PALB2 and two protein-truncating mutations in CHEK2 among BOT patients was very low and was insufficient to perform a reliable association analysis (Table 3).

In a group of ovarian cancer G1 patients, statistically significant association with cancer risk was detected for mutations in BRCA1 (OR = 8.53, p = 0.005) and PALB2 (OR = 7.03, p = 0.03) genes; however, it should be noted that the number of mutation carriers was very limited (4 cases with BRCA1 and 2 cases with PALB2). The CHEK2 mutations, including missense and two protein-truncating, were not significantly associated with cancer risk (Table 3).

The CHEK2 missense mutation (c.470T>C) was previously analyzed in another study of 539 ovarian cystadenomas, 122 borderline ovarian tumors, and 447 ovarian cancer cases, including 88 low-grade (G1) tumors [15]. In that study, the significant association of CHEK2 missense mutation (c.470T>C) with the risk of non-invasive tumors (OR = 1.7, p = 0.005 for ovarian cystadenoma and OR = 2.6, p = 0.002 for BOTs) and borderline significant correlation with low-grade ovarian cancer (OR = 2.1, p = 0.04) were reported. In our study, the association of CHEK2 missense mutation (c.470T>C) was statistically significant for BOT risk, but not for the risk of ovarian cancer G1 (Table 3). The possible explanation of the discrepancy in the above results may be the difference in a number of tested low-grade ovarian cancer cases, which was almost 2-times larger in our study (167 cases) than in previous analysis (88 cases). The mutations in the CHEK2 gene were already reported to correlate with the risk of prostate and breast cancer [20, 21, 31], but not with ovarian cancer [5, 21, 32]. The results of this study seem to support this observation and may extend it, suggesting a possible association of CHEK2 missense (c.470T>C) mutation with the 2-times increased risk of BOT.

Mutations in BRCA1, BRCA2, RAD51C, and PALB2 genes were already shown to correlate with the risk of ovarian cancer [5–9], but not with BOTs [10–14, 33]. The two studies conducted on the Jewish population (including 117 BOT and 161 ovarian cancer cases, as well as 46 BOT and 59 ovarian cancer cases, respectively) showed a much lower incidence of thfoundersnder BRCA1/2 mutations in BOT patients than in invasive early-stage ovarian carcinoma patients—the prevalence varied between 2.2 and 4.3% in BOT cases and 24.2–32% in ovarian cancer cases [10, 11]. In other studies, from Norway (190 BOT and 478 ovarian cancer patients) and Canada (134 BOT and 515 ovarian cancer patients), the BRCA1/2 mutations were detected in 4% and 11.7% invasive cases, respectively, but none of the patients with BOT [12, 13]. There were performed several other smaller studies, in which only single cases of BRCA1/2 mutations among BOT patients were detected, and the cumulative prevalence in all tested patients was 1.3% for BRCA1 and 0.2% for BRCA2 genes [33]. In contrary to the reports mentioned above, in a study of 1333 Czech ovarian cancer patients and 152 borderline ovarian tumor cases recruited from seven centers, the prevalence of BRCA1/2 mutations was similar in high-grade ovarian cancers and BOT cases (30.9% and 28.9%) [14]. There is no obvious explanation for such a high frequency of BRCA1/2 mutations among patients with BOT. It may be the consequence of several factors, such as...
differences in sample size or population-related differences. It should be also considered that pathological differentiation of BOTs from invasive tumors is not easy and may differ, especially when evaluation is done by pathologists from different centers [34, 35]. In our study, the 102 patients with histologically confirmed BOTs were diagnosed in one hospital, and the histology was evaluated and reviewed by 3 independent pathologists, which indicates a high-quality assurance of pathology diagnosis.

In our analysis, the mean age at diagnosis was lower among BOT patients than among low-grade ovarian cancer patients (47.76 vs. 54.25; Table 1), which is consistent with reported data [3, 36, 37]. The occurrence of any detected mutation in BRCA1, BRCA2, RAD51C, PALB2, and CHEK2 genes was in general associated with earlier age of diagnosis of BOT, when compared to non-carriers (45 years vs. 49 years) and was 10 years younger in carriers of CHEK2 missense mutation (c.470T>C) than in BOT patients without mutation (38 years vs. 49 years). This observation is consistent with a previous study which also reported a correlation of CHEK2 missense (c.470T>C) mutation with earlier age of diagnosis among BOT patients [15].

The all-cause survival, as it was expected, was better among BOT patients than ovarian cancer cases (5-year survival rate: 90.48 vs. 71.32% and 10-year survival rate: 88.89 vs. 59.36%), which is consistent with other studies reporting favorable prognosis for patients with borderline ovarian tumors [4, 28, 38–40]. There was no significant association of mutation status among BOT patients and death. However, the 10-year survival rate was lower for mutation carriers (80%) than for non-carriers (89.8%). Among patients with ovarian cancer G1, the all-cause survival was significantly associated with a BRCA1 mutation (HR=4.73, 95%CI 1.45–15.43, \(p=0.01\)). This result is different than in our previously reported published analysis of 2270 ovarian cancer patients, in which we did not find an association of BRCA1 mutation with overall survival [5]. However, it should be noted that low-grade ovarian cancers are rare and in that study, 7.3% of all cases (167 out of 2270) were accounted and only 4 cases carried BRCA1 mutation.

Our study has several limitations. It is based on a relatively small number of cases, and obtained results should be considered as preliminary. In this study, we analyzed the 21 most common Polish founder mutations in 5 genes associated with breast/ovarian cancer risk. Therefore, we omitted other non-founder mutations which might be detected by full sequencing of these genes. In our study, the cases and controls were not matched. The time of recruitment and mean age of cases and control were similar. It is unlikely but it might be that other unknown genetic or lifestyle factors could cause a study bias.

Conclusions

The results of our study suggest that CHEK2 (c.470T>C) may possibly play a role in the pathogenesis of BOT, but not low-grade ovarian cancer. The presence of the CHEK2 missense (c.470T>C) mutation may be associated with earlier age at diagnosis of BOT and about 10% worse rate of a 10-year survival. On contrary, the low-grade ovarian cancer is associated with BRCA1 and PALB2 mutations and presents worse survival among BRCA1 carriers. These results may suggest that the genetic predisposition and the molecular mechanisms underlying tumor initiation differ between invasive and borderline tumors of the ovary.

Due to the low number of BOT patients, there remains the possibility that CHEK2 c.470T>C variant may not be directly related to tumor development, but rather may be a coincidental finding. Because of that, the obtained results should be considered as preliminary. Further studies conducted on larger groups are required to verify these observations.

Abbreviations
BOT: Borderline ovarian tumor; PTT: Protein truncating mutations

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Authors’ contributions
A.J. and A.O. conceptualized the study; P.B. and A.O performed the statistical analyses; A.O., KP, and KB performed the molecular analyses; A.J., J.L., J.M., J.G., J-T.S., M.S., C.C., T.D., T.H., A. T-G., and T.B. collected the data and material from cancer patients and controls; A.J. and A.O. drafted the manuscript; A.J. supervised the study; A.J., A.O., J.L., J.M., J.G., J-T.S., M.S., C.C., T.D., T.H., A. T-G., T.B., P.A., KP, and KB reviewed and approved the final version of the manuscript.

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Availability of data and materials
The data presented in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Pomeranian Medical University in Szczecin (protocol code KB-0012/153/16 (BN-001/33/04), 20.12.2016). Informed consent was obtained from all subjects involved in the study.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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