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Clinical importance of *FANCD2, BRIP1, BRCA1, BRCA2* and *FANCF* expression in ovarian carcinomas

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ABSTRACT

Objective: DNA repair pathways are potential targets of molecular therapy in cancer patients. The *FANCD2, BRIP1, BRCA1/2*, and *FANCF* genes are involved in homologous recombination DNA repair, which implicates their possible role in cell response to DNA-damaging agents. We evaluated a clinical significance of pre-treatment expression of these genes at mRNA level in 99 primary, advanced-stage ovarian carcinomas from patients, who later received taxane-platinum (TP) or platinum-cyclophosphamide (PC) treatment.
Methods: Gene expression was determined with the use of Real-Time PCR. The *BRCA2* and *BRIP1* gene sequence was investigated with the use of SSCP, dHPLC, and PCR-sequencing.
Results: Increased *FANCD2* expression occurred to be a negative prognostic factor for all patients (PC+TP: HR 3.85, p = 0.0003 for the risk of recurrence; HR 1.96, p = 0.02 for the risk of death), and this association was even stronger in the TP-treated group (HR 6.7, p = 0.0002 and HR 2.33, p = 0.01, respectively). Elevated *BRIP1* expression was the only unfavorable molecular factor in the PC-treated patients (HR 8.37, p = 0.02 for the risk of recurrence). Additionally, an increased *FANCD2* and *BRCA1/2* expression levels were associated with poor ovarian cancer outcome in either TP53-positive or -negative subgroups of the TP-treated patients, however these groups were small. Sequence analysis identified one protein truncating variant (1/99) in *BRCA2* and no mutations (0/56) in *BRIP1*.
Conclusions: Our study shows for the first time that *FANCD2* overexpression is a strong negative prognostic factor in ovarian cancer, particularly in patients treated with TP regimen. Moreover, increased mRNA level of the *BRIP1* is a negative prognostic factor in the PC-treated patients. Next, changes in the *BRCA2* and *BRIP1* genes are rare and together with other analyzed FA genes considered as homologous recombination deficiency may not affect the expression level of analyzed genes.

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Introduction

Ovarian cancer ranks at the top of the list of the most lethal gynecological malignancies.[1] Therefore, it is of utmost importance to identify molecular biomarkers predicting prognosis and response to chemotherapy, and potential new targets for molecular inhibition.

Currently, taxanes combined with cisplatin or its analogs (the TP regimen) are the standard first-line treatment of ovarian cancer patients.[2,3] It replaced platinum-cyclophosphamide (the PC regimen) and other protocols based on DNA damaging agents. Nevertheless, in patients with advanced disease, the overall survival rates are still poor.

Platinum compounds induce DNA damage by the formation of DNA adducts and interstrand crosslinks (ICL). These lesions inhibit DNA replication, transcription and induce cell cycle arrest and apoptosis.[4] Homologous recombination (HR) during the S phase of the cell cycle is one of the mechanisms removing DNA adducts.[5–8] HR-mediated DNA repair requires activation of Fanconi anemia (FA) pathway. Abnormalities of genes involved in the FA pathway, resulting in the homologous recombination deficiency (HRD) have been described to be essential for cell sensitivity to DNA damaging agents (for reviews, see refs.[9–11]) and PARP inhibitors.[12-14] On the other hand, taxanes impair the cell tubular system through polymerization and stabilization of β-tubulin in G2 and M phases of the cell cycle. This leads to activation of the spindle assembly checkpoint (SAC). A prolonged cell-cycle arrest may lead to apoptosis or to mitotic exit, by slippage into G1 state, in which cells develop resistance to antimitotic agents.[15,16] Recently, studies on cell lines have shown that FA genes are involved in regulation of the SAC.[17]

Fanconi anemia is a genetic disease characterized by chromosomal instability and a high risk of cancer development. The FA pathway involves proteins encoded by 19 genes, including *FANCD2, BRIP1* (*FANCJ, BACH1*), *BRCA1* (*FANCS*), *BRCA2* (*FANCD1*) and *FANCF* (for a review, see ref.[18]). The FANCD2 is a central component of the FA DNA repair pathway, which

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protects the stalled replication fork and localizes to centrosomes during mitosis. BRIP1 is RECQ-like helicase that participates in FANCD2 loading onto chromatin and in ATR-mediated DNA damage checkpoint activation. BRCA1 and BRCA2 participate in the RAD51 loading to DNA, stalled replication fork protection, and interact with FANCD2. FANCF is a component of FA core complex that is responsible for the mono-ubiquitination of FANCD2. The *FANCD2* gene mutations have been found in breast cancer,[16] acute leukemia[19,20], and ovarian cancer.[21] Germline mutations in *BRIP1* have been associated with an increased risk of epithelial ovarian and breast cancers,[22–25] just as germline mutations in the *BRCA1/2* genes [for review see ref.[26–28]]. The *FANCF* mutation studies in breast,[16,29] cervical[30] and ovarian cancer[21] have not revealed any mutations leading to the loss of protein function. Still, epigenetic silencing of *FANCF* by promoter hypermethylation has been reported in several tumors.[30–33] To date, the prognostic and/or predictive significance of FA genes was analyzed in many cancers. However, there are few data available on the clinical importance of the expression of these genes at the mRNA level, especially in ovarian cancers.

TP53 is one of the most frequently mutated genes in ovarian carcinomas. TP53 dysfunction, as determined by TP53 protein accumulation in the nuclei of tumor cells, may influence the clinical importance of other molecular factors, particularly of those regulated by, or interfering with TP53.[34–37]

In the present study, the prognostic and predictive value of tumor *FANCD2, BRIP1* (*FANCJ, BACH1*), *BRCA1* (*FANCS), BRCA2* (*FANCD1*) and *FANCF* expression at the mRNA level, was investigated in ovarian cancer patients treated with PC- or TP-regimen. Moreover, the significance of the expression level was analyzed in the context of the TP53 protein accumulation status and HR-deficiency status. We also evaluated mutation frequency in the *BRCA2* and *BRIP1* genes.

Results

FANCD2 expression

Increased *FANCD2* mRNA level significantly enhanced the risk of recurrence (Figure1(a), Table 1) and death (Figure 2(a), Table 2) in all patients (TP+PC, n = 99), in both univariate and multivariate analyses. A particularly unfavorable prognosis, considering both the risk of recurrence (Figure 1(c), Table 1) and the risk of death (Figure 2(c), Table 2), was observed in TP-treated patients (TP, n = 66) with an increased expression of *FANCD2*. A mean disease-free survival time of patients with high and low *FANCD2* expression in this group was 507 days and 636 days, respectively, while the same values of overall survival time of patients with high and low *FANCD2* expression were 991 days and 1263 days, respectively. Kaplan-Meier survival curves also showed a trend toward poorer prognosis for patients with high *FANCD2* expression compared to those with low expression, in terms of both the risk of recurrence (Figure 1(b,d)) and death (Figure 2(d)).

Furthermore, we investigated whether mutations in FA genes: *FANCD2, BRIP1, BRCA1, BRCA2, FANCF*, and *PALB2* which contribute to the homologous recombination deficiency status, may affect the relevance of *FANCD2* expressions as an independent prognostic factor. Multivariate analysis revealed that HRD status was not significantly associated with the prediction of OS and DFS, and confirmed that patients with increased *FANCD2* expression had a significantly greater risk of death and recurrence (Tables 1,2).

FANCD2 expression did not associate with complete remission and platinum sensitivity in any of the analyzed groups.

BRIP1, BRCA1, BRCA2, and FANCF expression

Elevated *BRIP1* expression was the only molecular factor which enhanced the risk of recurrence in the PC-treated patients (n = 22, Table 1), in both univariate and multivariate analyses. Additional multivariate analysis with HRD status also confirmed obtained association. Although this association was not significant in Kaplan-Meier analysis (Log-rank p = 0.27).

We did not find any significant association between the *BRCA1, BRCA2* or *FANCF* gene expression at mRNA level and the analyzed clinical endpoints in the whole series of ovarian cancer patients, and separately, in the PC- and TP-treated groups.

Analysis of gene expression considering the TP53 protein accumulation status

In the TP-treated patients, the clinical importance of the *FANCD2, BRCA1*, and *BRCA2* genes expression was observed in small subgroups related to TP53 accumulation status. Increased *FANCD2* expression enhanced the risk of recurrence (Figure 1(e), Table 1) in patients with TP53-positive carcinomas (n = 29), and the risk of death (Figure 2(e), Table 2) in those with TP53-negative carcinomas (n = 25).

Increased expression of the *BRCA1* gene was associated with a higher risk of recurrence in patients with TP53-positive carcinomas (Table 1, n = 29). On the other hand, increased expression of the *BRCA2* gene negatively influenced the probability of complete remission (OR 0.063, p = 0.05) and increased the risk of death in patients with TP53-negative carcinomas (Table 2, n = 25). The latter association also proved significant in the Kaplan-Meier analysis (Log-rank p = 0.011).

BRCA2 and BRIP1 sequence analysis

Sequence analyses of the *BRCA2* gene revealed a previously known[38], four base-pair duplication c.3975_3978dupTGCT (exon 11, Figure S1) in one tumor sample (1%) of 99 investigated ovarian carcinomas. It resulted in p.(Ala1327Cysfs*4) and generated a premature stop codon at position 1330. Since no matched normal tissue was available, it was not possible to classify this alteration as somatic vs. germline in origin. In addition, eight previously known germline substitutions were revealed (Table S1). One of the identified substitutions was missense variant of unknown significance (VUS) and conflicting interpretations of pathogenicity (c.9371A>T, p.(Asn3124Ile)),

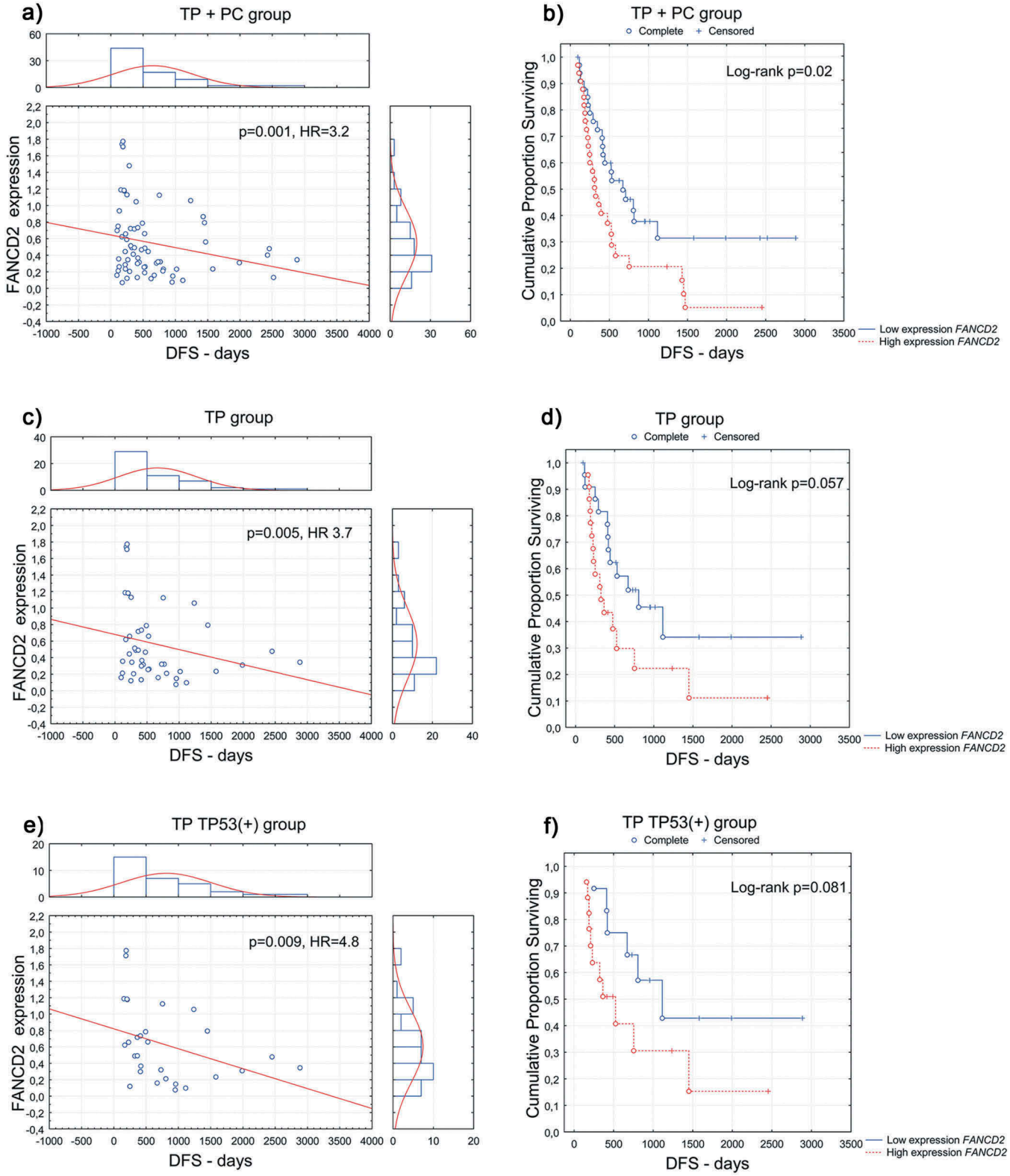

Figure 1. Disease-free survival (DFS) according to the *FANCD2* gene expression at the mRNA level in the (a, b) combined TP- and PC-treated groups of patients; (c, d) TP-treated group of patients (e, f) group of TP-treated patients with TP53-positive carcinomas; (a, c, e) univariate analysis of a continuous variable; (b, d, f) analysis of Kaplan–Meier curves, cut-off point at the median value of 0.4.

revealed in three (3%) of 99 investigated tumor samples (Table S1).

The *BRIP1* gene sequence analyzes, performed for 56 out of 99 tumors indicated the presence of nine previously known single nucleotide changes (Table S1).

Analysis of FANCD2, BRIP1, BRCA1, BRCA2, and FANCF expression considering the HR-deficiency status

A relationship between the expression of the studied genes at the mRNA level and the HR-deficiency (HRD) status based on mutations in the FA genes was assessed. To this end we found

Table 1. Statistically significant associations of the *BRCA1, BRIP1* and *FANCD2* mRNA expression with disease-free survival (DFS) in ovarian cancer patients, assessed in multivariate Cox proportional hazards models. Univariate analyses showed similar but weaker associations.

	DFS									
	TP group n=45		TP TP53(-) subgroup n=16		TP TP53(+) subgroup n=29		PC group n=22		TP+PC group n=67	
Variable name	HR	p	HR	p	HR	p	HR	p	HR	p
BRCA1	-		-		3.4	0.031	-		-	
BRIP1	-		-		-		8.37*	0.02*	-	
FANCD2	6.7*	0.0002*	-		7.7	0.0005	-		3.85*	0.0003*
Histological type										
Serous (0) vs Other types (1)	0.23	0.02	-		0.24	0.014	-		-	
TP53 protein accumulation status										
TP53-negative carcinomas (0) vs TP53-positive carcinomas (1)	4.05	0.003					-		1.9	0.05

* The supplementary, multivariate Cox proportional hazards regression analysis conducted with the recombination deficiency (HRD) status used as extra categorical variable, showed that the HRD status was not significantly contributed to the prediction of disease-free survival (p>0.05 in analyzed groups), and did not affect the clinical significance of analyzed genes expression.

no differences in the expression levels of the studied genes between tumors without mutation in *FANCD2, BRIP1, BRCA1, BRCA2, FANCF*, and *PALB2* (n = 71) and tumors harboring mutations in *BRCA1, BRCA2*, and *PALB2* (n = 28), either in the whole series of ovarian cancer patients (Figure 3(a)) or in the PC- and TP-treated groups (Table S2). In the TP-treated patients with TP53-negative carcinomas, the median *FANCD2* mRNA level was significantly higher in tumors with no FA mutations (0.39) than in mutation-positive tumors (0.24; p = 0.048; n = 25; Table S2; Figure 3(b)). We also found that HRD status did not affect the disease-free survival DFS and OS overall survival (Table S3, Figure S2). This relationship was observed for the DFS even if we extended the group of tumors harboring mutations for the p.(Asn3124Ile) variant, which deleteriousness is not well determined and the studies on the clinical importance of this variant are limited (data not shown). However, the revised HRD status had the slight impact on OS in the TP TP53(-) subgroup, where the risk of death was significantly lower for the mutation carries (log-rank p = 0.025).

Discussion

This study demonstrated that increased *FANCD2* mRNA expression is an independent negative prognostic marker for the risk of recurrence and death in ovarian cancer patients. To date, the *FANCD2* mRNA level in relation to prognosis of ovarian cancer patients was analyzed only in one study, and no association with patient outcomes has been found.[39] This discrepancy might partly be influenced by the statistical approach. While Ganzinelli et al. (2011)[39] analyzed *FANCD2* expression as a categorical variable with three values, our analysis comprised a continuous or dichotomous variable. Our results are in line with those obtained in other types of cancer, including breast, cervical and colorectal carcinomas, multiple myeloma, alveolar rhabdomyosarcoma, and hepatocellular carcinoma, where high *FANCD2* mRNA level has been shown to relate to poor prognosis.[40–45] With regard to response to treatment, RNAi silencing of *FANCD2* in NSCLC cell lines enhanced cisplatin and oxaliplatin sensitivity[46] and in taxol-treated HeLa cells led to inactivation of spindle assembly checkpoint (SAC), accumulation of extra centrosomes and multinucleation.[17] Recently, it was also shown that *BRCA1/2* mutated breast cell lines are hypersensitive to the loss of FANCD2, and that *FANCD2* gene overexpression was critical for the resistance of *BRCA1/2* deficient cells to PARP inhibitors, by stabilizing the replication fork.[47] Other literature data also indicate that mutations of *BRCA1/2* or other FA genes in ovarian cancers result in homologous recombination deficiency that promotes DNA repair through the FANCD2-dependent, mutagenic alternative end-joining (*alt-EJ*) pathway.[48] Taken together, these data suggest that inhibition of *FANCD2* expression may have a potential of sensitizing cancer cells to chemotherapeutic agents and PARP inhibitors, and may provide a tool to improve survival of cancer patients.

Further investigations of the *FANCD2* expression significance in relation to HR-deficiency showed that the prognostic significance of *FANCD2* expression was not affected by the HRD status. Moreover, the *FANCD2* gene mRNA levels did not relate to the HRD status. Contrary to this, Kais et al. (2016)[47], in a study based on the TCGA data set for which gene expression and the whole-exome DNA sequencing were available, presented increased *FANCD2* expression in tumors with *BRCA1/2* mutation-related HR-deficiency. This discrepancy may result from several reasons. Firstly, our analysis did not comprise the whole sequence of the analyzed genes; thus, some mutations might have been missed. Second, apart from germline and somatic mutations in FA genes, HRD may result from *BRCA1/2* silencing by promoter methylation and due to interactions with other proteins involved in DNA repair[13], the aspect that was not addressed in our study. Third, it has been hypothesized that not all *BRCA1* mutations are equal, and some may not induce HRD.[49] Moreover, a recent study based on the Next Generation Sequencing (NGS) allowed for a more specific description of HR-deficiency as an HRD score based on an analysis of Genome-wide LOH combined with HR gene mutation profiling, telomeric allelic imbalance, and large-scale state transitions.[50] Future studies with the use of NGS approach would be very helpful to assess the homologous recombination deficiency status more precisely.

Herein, we have shown that increased mRNA level of the *BRIP1* gene was associated with shorter disease-free survival (DFS) of the PC-treated ovarian cancer patients. Although this

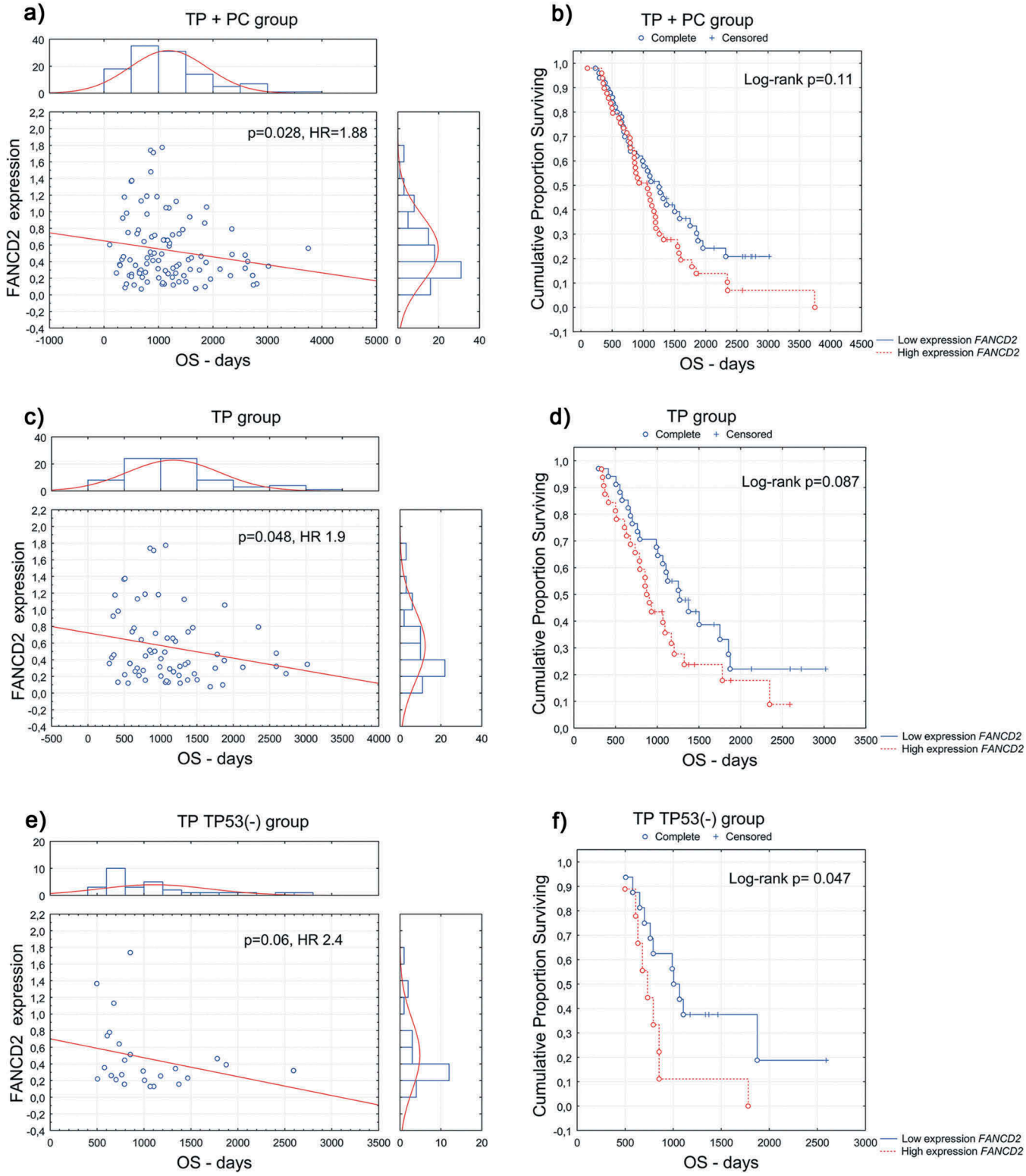

Figure 2. Overall survival (OS) according to the *FANCD2* gene expression at the mRNA level in the (a, b) combined TP- and PC-treated groups of patients; (c, d) TP-treated group of patients; (e, f) group of TP-treated patients with TP53-negative carcinomas. (a, c, e) univariate analysis of a continuous variable; (b, d, f) analysis of Kaplan–Meier curves, cut-off point at the median value of 0.4.

observation needs to be confirmed in a larger series of patients, this is consistent with the results in other cancers. Elevated level of *BRIP1* mRNA has been associated with a significantly shorter DFS in five-FU-treated patients with metastatic colorectal cancer[51] and with poor prognosis in breast cancer patients.[52] BRIP1 plays an important role in DNA repair mechanism. Available data indicate that BRIP1 and BRCA1 proteins co-localize before and after exposure to ionizing radiation in ovarian and breast cancer cell lines.[53] *BRIP1* silencing led to dissociation of BRCA1 protein from chromatin, which resulted in an

Table 2. Statistically significant associations of the *BRCA2* and *FANCD2* mRNA expression with overall survival (OS) in ovarian cancer patients, assessed in multivariate Cox proportional hazard models. Univariate analyses showed similar but weaker associations.

	OS									
	TP group n=66		TP TP53(-) subgroup n=25		TP TP53(+) subgroup n=41		PC group n=33		TP+PC group n=99	
Variable name	HR	p	HR	p	HR	p	HR	p	HR	p
BRCA2	-		4.28	0.019	-		-		-	
FANCD2	2.33*	0.01*	43.8	0.002	-		-*		1.96*	0.02*
Histological grade										
G1, G2 (0) vs G3 (1)	0.28	0.052	0.2	0.03	-		-		0.39	0.04
Residual tumor size										
0 cm (0) vs >2 cm (2)	0.3	0.02	43.6	0.04	-		-		0.4	0.006
≤ 2 cm (1) vs >2 cm (2)	-		118.4	0.01	-		-		-	
TP53 protein accumulation status										
TP53-negative carcinomas (0) vs TP53-positive carcinomas (1)	2.17	0.01					-		-	

* The supplementary, multivariate Cox proportional hazards regression analysis conducted with the recombination deficiency (HRD) status used as extra categorical variable, showed that the HRD status was not significantly contributed to the prediction of overall survival (p>0.05 in analyzed groups), and did not affect the clinical significance of analyzed genes expression.

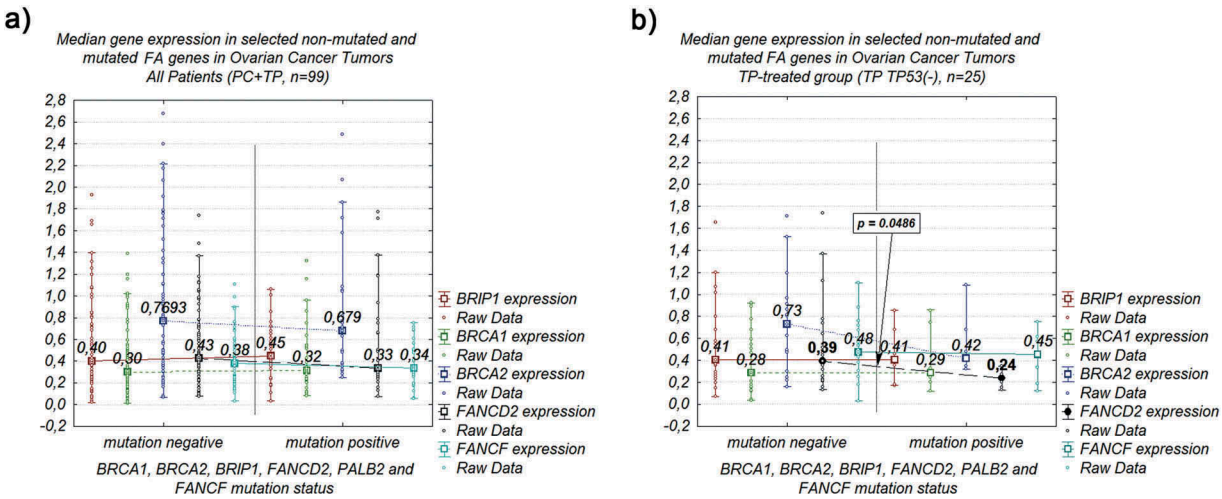

Figure 3. Association of analyzed FA genes median expression level and a mutation status of selected FA genes in ovarian cancer tumors: (a) combined TP- and PC-treated groups of patients – no significant relationship; (b) group of TP-treated patients with TP53-negative carcinomas – significant difference in median *FANCD2* expression level.

inhibition of RB1- and TP53-dependent DNA repair-activation of pro-apoptotic pathways, while restoration of *BRIP1* expression reversed this effect.[53,54] It has also been shown that overexpression of BRIP1 protein correlated with an increased cell proliferation rate,[54] which may contribute to earlier tumor recurrence.[55,56] Thus, both our analysis and published data indicate that high level of *BRIP1* in tumor cells is an unfavorable factor, especially in patients treated with DNA damaging compounds.

Our study did not reveal the association between *BRCA1/2* mRNA levels and clinical endpoints in PC- and TP-treated patients. To date, there is no consensus on the impact of *BRCA1* expression on ovarian cancer patients' outcome, and there are few studies on the clinical importance of *BRCA2* expression. Several studies have indicated the lack of relationship between the *BRCA1/2* expression and the response to TP-treatment,[56,57] while others have reported that low level of the *BRCA1* mRNA positively influenced prognosis (OS) of the PC-treated patients[58], and the TP-treated patients with the residual tumor less than 2 cm.[59] In some other cancers, high *BRCA1* gene expression has been considered to be a negative prognostic and/or predictive factor,[60–62] while high expression of *BRCA2* at mRNA level in breast cancer patients had a negative impact on docetaxel response.[63]

In the current study, there was no significant association between *FANCF* gene expression at the mRNA level and clinical endpoints, which is consistent with previous reports.[39,64]

Finally, analyses considering the TP53 protein accumulation status were performed. Due to the relatively small size of both TP53 subgroups, the results obtained should be interpreted with caution. Nevertheless, the clinical importance of increased *FANCD2* expression was observed in the TP-treated patients with TP53-positive (shorter DFS) and TP53-negative carcinomas (shorter OS). Moreover, gene expression analysis considering the HR-deficiency status revealed that the *FANCD2* mRNA level was higher in tumors with no TP53 protein accumulation and no mutations in any of the analyzed genes, as compared to tumors carrying those mutations. Although the obtained results appear ambiguous, there is an evidence in the literature, that TP53 status may influence the

biological and clinical importance of FANCD2. Studies on wild-type and TP53-mutant mice embryonic fibroblasts revealed that decreased expression of *FANCD2* mRNA and reduced capacity to repair the DNA interstrand crosslinks may depend on TP53 role in promoting the recruitment of the E2F4 repressor of the *FANCD2* promoter.[65] Interestingly, the authors reported that the analysis of the transcriptome data from the Australian Ovarian Cancer Study confirmed that the loss of TP53 function leads to an increased expression of the *FANCD2* gene in high-grade ovarian tumors. Moreover, Wysham et al. (2012)[66] observed that ovarian cancer patients with the co-expression of FANCD2, PARP, and TP53 proteins had unfavorable prognosis.

We also found a significant relationship between an increased *BRCA1* expression and shorter DFS in the TP-treated patients with TP53-positive carcinomas. The clinical significance of *BRCA1* gene expression in relation to the TP53 status was examined in ovarian cancer cell lines with wild-type TP53, mutant TP53 and without the TP53 protein.[67] The authors have shown that the reduced *BRCA1* mRNA expression resulted in approximately five-fold increase of platinum, but not taxane sensitivity of TP53 wild-type cells, but not of those with mutated *TP53*. Although our patient group was small, the obtained result is in line with our other study, where we have shown that TP53 accumulation status may determine the prognosis of patients who carry *BRCA1* mutations.[37]

The present study demonstrates the clinical importance of *BRCA2* mRNA level in ovarian cancer patients. A negative impact of increased *BRCA2* expression on complete remission (CR) and overall survival (OS) was observed in the TP-treated patients with TP53-negative carcinomas. The relationship between *BRCA2* and TP53 investigated in breast cancer cell lines pointed to the importance of TP53 status for the regulation of *BRCA2* gene promoter, as normal TP53 has been considered as a repressor of the *BRCA2* gene.[68] These observations together with our findings suggest that the prognostic and predictive value of *BRCA1/2* expression in the context of TP53 accumulation deserves further investigations.

To better characterize the molecular background of the analyzed tumor samples, sequence analyses of the studied genes were performed. One, protein-truncating mutation in the *BRCA2* gene (n = 1/99), and no mutations in the *BRIP1* gene (n = 0/56) have been identified. The *BRCA2* mutation was located in one of the high ovarian cancer risk-associated cluster regions (OCCR) of the *BRCA2* gene.[69,70] We also detected missense, germline substitution (c.9371A>T, p.(Asn3124Ile), Table 2), classified as a variant of uncertain significance and conflicting interpretations of pathogenicity (ClinVar). Previous studies have indicated that this *BRCA2* gene variant was frequently identified in patients with breast and ovarian cancers, especially from the Polish population.[71–73] Interestingly, in order to investigate significance of the HRD status in the context of DFS and OS prediction with c.9371A>T variant classified as mutation the present study demonstrated that revised HRD status had no impact on the risk of recurrence, however, it might have the slight impact on risk of death in the TP53(-) subgroup (data not shown). Because of the low number of patients with the c.9371A>T variant in our analysis and the lack of the literature data about the impact of this variant on patients outcome, the further studies are necessary in order to determine its clinical significance.

Our previous studies have revealed that the analyzed tumor samples harbored *BRCA1* (n = 26/99; 26.3%) and *PALB2* (n = 1/99; 1%) mutations[74,75] and no *FANCD2* and *FANCF* (n = 0/99) mutations.[21] Taken together, it may be concluded that besides the most common mutations of the *BRCA1* gene that frequently result in a loss of protein function (which are mostly point changes or small deletions and insertions, located across the entire coding gene sequence and at splice sites),[25,76] other deleterious variants in FA genes are relatively rare. This is in line with literature data which show germline and somatic mutations of *BRCA1/2* genes in about 20% of cases, and much less frequent mutations in other FA pathway genes, *BRIP1* (0,9–1,72%), *PALB2* (0,2–0,5%), *RAD51C* (0,41–2,9%).[77]

In summary, in the present study, we provided the evidence that the increased tumor *FANCD2* mRNA expression level is an unfavorable prognostic factor in ovarian cancer patients treated with a taxane-platinum regimen. For platinum-cyclophosphamide treated patients, only *BRIP1* expression turned out to be clinically significant. Our results also demonstrate that overexpression of the *FANCD2, BRCA1*, and *BRCA2* genes, depending on TP53 accumulation status, has a value of an adverse prognostic factor in TP-treated ovarian cancer patients. Additionally, we found no significant association between significance of analyzed genes expression and HR-deficiency based on *BRCA1/2* and other FA genes mutation status. Taken together, we showed that increased expression of HR DNA repair pathway genes may negatively influence prognosis in ovarian cancer patients.

Materials and methods

Patients and tumors

The study was performed on 99 fresh frozen samples of ovarian carcinomas from patients, who were subsequently treated with a taxane-platinum chemotherapy (TP, n = 66) or a platinum-cyclophosphamide chemotherapy (PC, n = 33). Tumors obtained during the surgical procedure as well as the relevant blood samples anticoagulated with EDTA were snap-frozen in liquid nitrogen and stored at −70°C.

The material was carefully selected, as previously described.[78] The study included only tumors containing less than 15% stromal cell contamination (scc) and meeting the following criteria: no chemotherapy before staging laparotomy; adequate staging procedure; International Federation of Gynecologists and Obstetricians (FIGO) stage IIB to IV disease[79]; tumor tissue from the first laparotomy available; moderate (G2) or poor tumor differentiation (G3); availability of clinical data, including residual tumor size and follow-up. All tumors were uniformly reviewed histopathologically, classified according to the criteria of the World Health Organization (WHO) and graded in a three-grade scale.[80] Clinicopathological characteristics are presented in Table S4. Previous sequence analysis of these tumor samples revealed mutations in the *BRCA1* gene (26/99; 26%),[74] the *PALB2* gene (1/70; 1%)[75] and no mutations in the *FANCD2* and *FANCF* genes (0/99)[21].

Response to chemotherapy was evaluated retrospectively, according to the WHO response evaluation criteria.[81] The evaluation was based on data retrieved from medical records referring to the patients' clinical condition and CA125 levels assessed in 3 to 4-week intervals. Complete remission (CR) was defined as the disappearance of all clinical and biochemical symptoms of ovarian cancer, evaluated after completion of first-line chemotherapy and confirmed 4 weeks later. Within the CR group, a platinum-sensitive group (PS), with disease-free survival (DFS) longer than six months was identified. Other tumors were described as platinum-resistant.[82]

The study was approved by the bioethics committee of Maria Sklodowska-Curie Institute – Oncology Center (ref. no. 39/2007).

DNA, mRNA extraction, cDNA synthesis

Genomic DNA was extracted from frozen tissues and relevant blood samples with the use of the QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Total mRNA was extracted from frozen tissues with the NucleoSpin RNA Kit (Macherey-Nagel), according to the manufacturer's protocol. mRNA quantity was measured with the use of UV spectrophotometer, and mRNA quality was assessed by the 260/280 ratio and in a 1% agarose gel. One microgram of total mRNA was transcribed to cDNA using the Super Script III First Strand kit (Invitrogen).

Gene expression

Expression of FANCD2, BRIP1, and BRCA1/2 at the mRNA level

Quantitative RT-PCR (Q-PCR) was run on the 7500 Fast Real-Time PCR System (Applied Biosystems), with the use of the FAM-labeled, TaqMan Gene Expression Assays (Applied Biosystems): *FANCD2* (assay ID: Hs00276992_m1), *BRIP1* (assay ID: Hs00230743_m1), *BRCA1* (assay ID: Hs00173233_m1), *BRCA2* (assay ID: Hs00609060_m1). All Real-Time PCR reactions were carried out in triplicates, in the final volume of 10 µl, with TaqMan Universal PCR Master Mix, no AmpErase™ UNG (Applied Biosystems) and about of 10 ng of cDNA, for 40 cycles, according to the following protocol: each cycle at 95°C for the initial 10 min, then at 95°C for 15 s and 60°C for 1 min. The obtained results were averaged, and gene expression levels were normalized to the *HGPRT*gene expression (VIC-labeled, Applied Biosystems, assay ID: 4326321E). A standard curve, used in all experiments, was prepared from serial dilutions of cDNA from one of the analyzed tumors.

Expression of FANCF at the mRNA level

FANCF gene consists of one exon. *FANCF* TaqMan probe (FAM-labeled, Applied Biosystems, assay ID: Hs00256030_s1) detects both the cDNA and genomic DNA (gDNA), which remains after the isolation of RNA. Purification of RNA from gDNA with the use of enzymatic method, i.e. digestion with DNase using RNase inhibitor has failed. Therefore, two separate Q-PCR experiments were performed. In the first experiment, gene expression was obtained based on both cDNA and gDNA, as described in the previous section. The second experiment was carried out for measurement of the gene expression based on gDNA, with the use of total mRNA instated of cDNA. Final values of *FANCF* expression were obtained by subtracting the value of gene expression evaluated in the second experiment from the value of gene expression obtained in the first experiment and were normalized to the reference gene expression (*HGPRT*). A standard curve was prepared as described in the previous section.

Sequence analysis of the BRCA2 and BRIP1 genes

DNA sequence analysis was carried out for the *BRCA2* and *BRIP1* genes in 99 and 56 ovarian carcinomas, respectively. Germline origin of the detected changes was confirmed in the corresponding DNA from blood samples (if available). The selected regions of *BRCA2* – exon 2,3, part of exon 11 – including the ovarian cancer cluster region (OCCR[70], nucleotides 3035 to 6629) and exon 25 [GenBank: NG_012772.3; NM_000059.3] were investigated with the use of the dHPLC method. The full coding sequence of the *BRIP1* gene (22 exons with the intron bounders [GenBank: NG_007409.2; NM_032043.2]) was analyzed with the use of the PCR-SSCP method.

Polymerase chain reaction (PCR)

DNA fragments were amplified with the use of primers designed by Wagner et al. (1999)[83] for *BRCA2* and by Lewis et al. (2005)[84] for *BRIP1*, or with the use of Primer3 software (Table S5). PCR mixtures were prepared according to the standard procedure (Applied Biosystems PCR Kit). PCR reactions were carried out for 36 cycles in a programmable thermal cyclers (Biometra, Eppendorff) with denaturation at 95°C, annealing at 54–64°C (depending on the exon) and extension at 72°C for 30 s each.

Denaturing high-performance liquid chromatography

Amplified DNA fragments of the *BRCA2* gene were screened by the dHPLC method with the use of automated dHPLC instrumentation (Transgenomic Inc). PCR products were eluted with linear acetonitryle gradient. The gradient and the temperature required for a successful resolution of heteroduplex molecules was determined with the use of the dHPLC melting algorithm (Transgenomic Inc).

Single strand conformational polymorphism analysis (SSCP)

All amplified DNA fragments of the *BRIP1* gene were analyzed with the use of the SSCP method. PCR products were denatured with 0.1 M NaOH and 2 mM EDTA at 55°C for 15 min. Subsequently, after 95% formamide, 0.05% xylene cyanol and 0.05% bromphenol blue were added, the samples were loaded to polyacrylamide gels (1:39 N,N'-methylenebisacrylamide to acrylamide in 0.5 x TBE with 10% glycerol). Electrophoresis was performed at 100 V, for 16–24 hours at room temperature. DNA bands were visualized with the silver-staining method compiled from several protocols. In our experience, this method detects 90% of all alterations, and 100% of deletions and insertions.[85]

Sequencing

All variants detected using SSCP and dHPLC were further sequenced with the Sanger method and BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) on automated ABI PRISM 3100 Sequencer (Life Technologies) according to the manufacturer's recommendations. Prior to sequencing, the PCR products were purified enzymatically with exonuclease I and alkaline phosphatase (Illustra ExoProStar, GE Healthcare Life Sciences).

TP53 protein accumulation status

Analysis of TP53 accumulation in the nuclei of tumor cells population was described previously by our team.[78,85] Briefly, TP53 accumulation was visualized by an immunohistochemical method, with the use of PAb1801 monoclonal antibody (1/3000, Sigma-Genosys) on paraffin-embedded material, after heat-induced epitope retrieval (HIER). It was described as present (>10% of positive cells; TP53(+)) or absent (TP53(-)).

Statistical analysis

Associations between *FANCD2, BRIP1, BRCA1, BRCA2, FANCF* expression, and clinical endpoints were analyzed with the use of the Kaplan–Meier method, log-rank test, univariate and multivariate Cox's proportional hazards models (OS, DFS) and logistic regression models (probability of CR, PS). Multivariate statistical analyses included the following independent variables: age of the patients (median: 53 years), the FIGO stage, histopathological type, grade, residual tumor size, and the TP53 accumulation status. The genes expression was analyzed as a continuous variable, and for Kaplan–Meier analysis – as a categorical variable (the median value of expression for the entire group was used as a cut-off point). Important factors were selected using a backward selection technique, where factors not significant at 0.1 (for OS, DFS), and 0.2 (for CR, PS) were removed stepwise from the model. To estimate the association between the analyzed genes' expression and the homologous recombination deficiency status (HRD, based on mutation analysis of six FA genes: *FANCD2, BRIP1, BRCA1, BRCA2, FANCF, PALB2*), the Kruskal–Wallis test was used. The analyses were performed in 1) the entire group of patients, 2) in the PC- and 3) TP-treated groups, and 4) in the TP-treated patients, subgrouped with respect to TP53 accumulation status. Additionally, associations between HRD status and clinical endpoints (OS, DFS) were analyzed with the use of the Kaplan–Meier method, log-rank test, and Cox's proportional hazard model. Furthermore, the clinical significance of the *FANCD2* and *BRIP1* gene expression (OS, DFS) was analyzed also with the HRD variable included in the multivariate Cox's proportional hazard model.

A p-value <0.05 was considered significant. All calculations were performed using SAS or Statistica softwares.

Abbreviations

CR complete remission
DFS disease-free survival
HR hazard ratio
OR odds ratio
OS overall survival
PC platinum-cyclophosphamide chemotherapy
PS platinum sensitivity
TP taxane-platinum chemotherapy
HRD homologous recombination deficiency

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors' contributions

JM-S designed the study, performed laboratory research, analyzed the data and wrote the manuscript. IKR analyzed the data and drafted the manuscript. JC carried out a statistical analysis. AD-M performed laboratory research and critically reviewed the manuscript. AB, LMS, RL performed laboratory research. PS, JK collected and described the clinical material. JK made a histopathological evaluation of the tumors, analyzed the data and drafted the manuscript.

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