

Nowadays, diagnostics enables detection of cancer-related mutations. It concerns germline mutations as well as de novo mutations. The number of genetics tests available for specific tumors increase incessantly. *BRCA1* and *BRCA2* are high-penetrant genes, but still their functions and mechanisms leading to cancer caused by their mutations remain unknown. Germline alterations in *BRCA1* and *BRCA2* sequences are related to hereditary breast cancer syndrome (HBC), hereditary breast and ovarian cancer syndrome (HBOC), hereditary ovarian cancer syndrome (HOC). Primary and secondary tumors prevention and choice of the best treatment are the benefits of the detection of *BRCA2* mutations. Research on correlation between the tumor phenotype and the type of mutation is continuously performed. This research may have an impact on the development of more effective drugs.

Key words: *BRCA2*, hereditary breast cancer, hereditary ovarian cancer, germline mutations.

Biological and clinical significance of *BRCA2*

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Homologous recombination

Homologous recombination is one of the mechanisms of DNA double strand breaks (DSBs) repair. It occurs in phases S and G2 of the cell cycle. Initiation of this process requires nucleolytic processing of the DSB in order to create single strand overhangs crucial for homologous recombination. The strand overhangs are substrates for RAD51 recombinase. In the presence of ATP, RAD51 builds a nucleoprotein filament on the single-stranded DNA (ssDNA). RAD51 finds a complete homologous template, which is identical to the sequence within the nucleoprotein complex and in the next step Rad51 catalyses attack of the ssDNA on the homologous double-stranded DNA (dsDNA) fragment – the sister chromatid or second chromosome – which results in a heteroduplex (Fig. 1). The single strand overhangs act as the primers and homologous DNA acts as the template for the DNA polymerase that extends the primers and fills the double strand breaks. That mechanism leads to the repair and to the restoration of the genetic material [1, 2]. The pathway of the DSB repair by homologous recombination is presented in Figure 1.

BRCA2 location, gene structure and features of the *BRCA2* protein

The *BRCA2* gene is located on the long arm of chromosome 13 (13q12.3) [3]. *BRCA2* consists of 27 exons and analogically to the gene *BRCA1* contains a very long exon 11 and the translation start point is located within exon 2 [4]. The gene encodes the protein *BRCA2* that has 3418 amino acid residues and is involved in DNA repair [3]. The protein is strongly charged because of the presence of negatively and positively charged amino acid residues [4].

There is no protein homological to *BRCA2* in the human proteome. Its structure can be divided into three main parts: the N-terminal region, central region and the C-terminal region (Fig. 2).

The N-terminal fragment of the *BRCA2* protein shows homology to the activation domain of the Jun transcription factor; this indicates the important role of *BRCA2* in transcription activation [5]. This region is encoded by exon 3; its function is negatively controlled by two inhibitory regions (IR1, IR2) located on both ends of exon 3 [5]. The first 906 amino acid residues of the N-terminus contain at least two phosphorylation sites but their exact position and influence on the functions of *BRCA2* remain unknown [6] (Fig. 2).

Within 2/3 of total *BRCA2* protein length a region including 1000 amino acid residues is located, which is composed of eight BRC motifs (BRC1-BRC8). A single BRC motif is about 70 amino acid residues long and its core includes 26 amino acids. BRC domains directly bind RAD51 recombinase. *In vitro* research has proved that BRC motifs differ in RAD51 binding affinity: BRC3-BRC4 have high affinity, BRC5 and BRC6 have very low binding affinity [7, 8]. It is believed that six of eight BRC repeats (BRC1-BRC4, BRC7, BRC8) bind RAD51 *in vivo* in mammalian

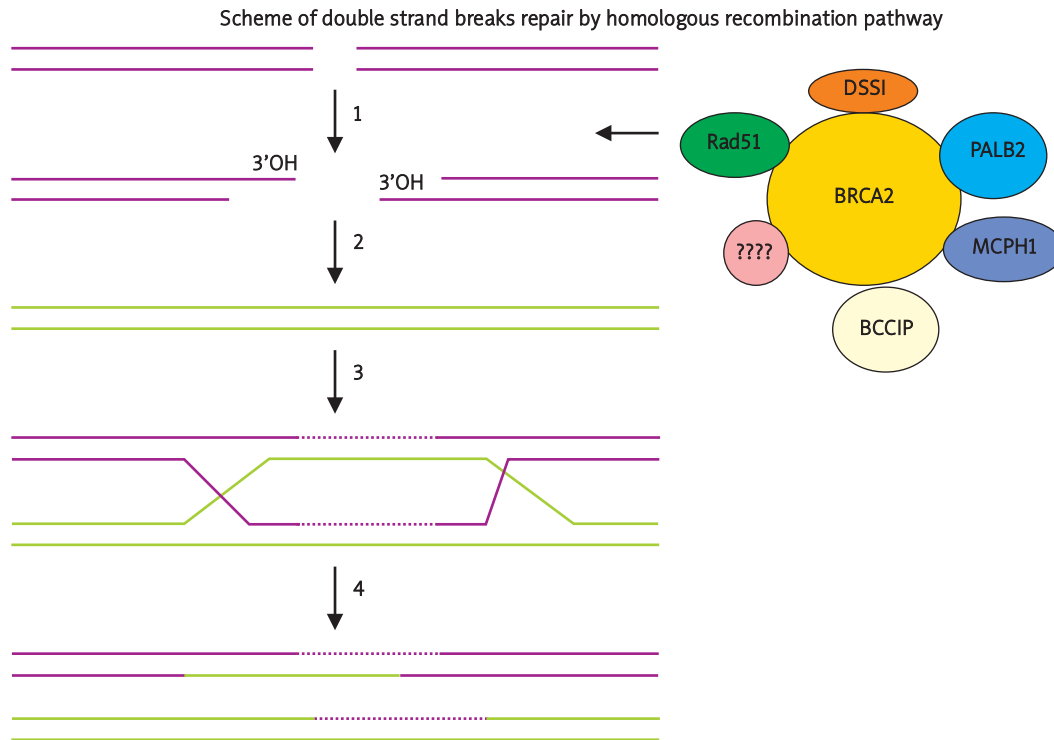


Fig. 1. Homologous recombination scheme. Exonucleolytic cleavage of the DSB ends results in the formation of ssDNA ends. The overhangs are recognized by the protein complex – including Rad51 [1, 2]. ssDNA invasion for the homologous dsDNA. dsDNA is the template for DNA polymerase. The gap is filled and the DSB is repaired [3, 4]

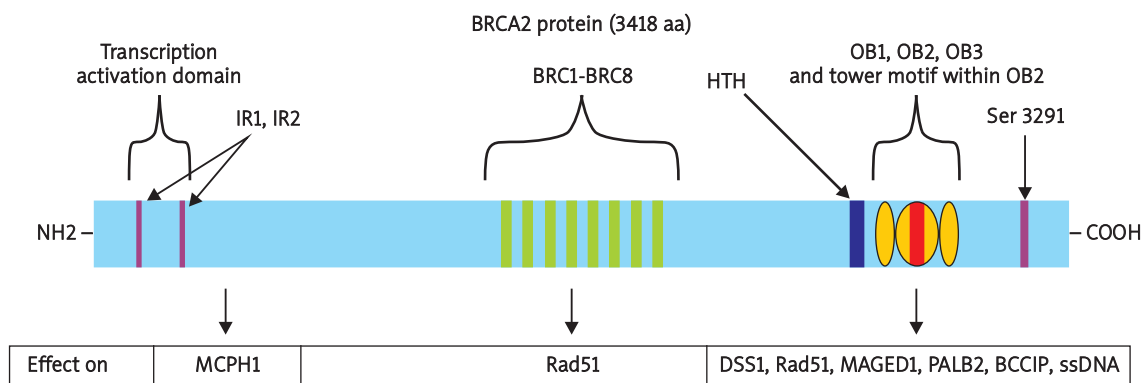


Fig. 2. BRCA2 structure

cells. The structural analysis of binding RAD51 by BRC motifs was performed using crystallography [9]. The core of the BRC domain consists of hydrophobic amino acids that ensure close contact with RAD51. Gly1523 and F1524HTASGK1530 sequence compose the region responsible for hydrophobic and polar interactions with RAD51 protein [9] (Fig. 2).

Like BRCA1, BRCA2 at its C-terminal end contains a motif that is characteristic of the granin family [10]. The C-terminal part of the BRCA2 protein contains an 800 aa residue long region, rich in many secondary structures [11, 12]. Within this region, a single helix-turn-helix motif and three OB (oligonucleotide/oligosaccharide binding) motifs are located. The second OB motif contains a tower motif. Through strong electrostatic interactions with OB1 and OB2 and by its helical domain, BRCA2 associates with DSS1. DSS1 is a neg-

atively charged polypeptide about 70 aa long, which is involved in the process of homologous recombination. The OB motifs also bind ssDNA with high affinity, whereas the tower motif shows affinity to dsDNA [11, 12] (Fig. 2).

The second RAD51 binding region was identified within the C-terminus of BRCA2; it is encoded by exon 27 and controlled by phosphorylation [6, 13, 14]. The most crucial role is played by the serine residue – Ser 3291. The level of 3291 serine phosphorylation is low during the S phase of the cell cycle, but increases when the cell enters the M phase. During exposure to DNA damaging factors, Ser 3291 is quickly dephosphorylated and BRCA2 associates with RAD51. The phosphorylation constitutes a “molecular switcher” that regulates homologous recombination by modulating the interaction between BRCA2 and RAD51 (Fig. 2).

BRCA2 protein functions

Various functions are attributed to BRCA2 protein. There are reports of its role in centrosome duplication, gametogenesis in mammals, and stabilization of replication forks [15-17].

It was shown that BRCA2 is a factor that regulates transcription and this activity is controlled by phosphorylation [5, 18].

Studies conducted on epithelial cells derived from mouse mammary glands showed that the expression of the *BRCA2* gene is co-regulated with *BRCA1* gene expression. Its highest level was detected in highly proliferating and differentiating cells; a relationship between cell cycle phase and expression of the two genes was noted – a peak of expression was observed in late stages of G1 / S. These data indicate the involvement of both gene products in cell cycle regulation, differentiation and proliferation [19].

Deficit of the BRCA2 protein disrupts normal cell division. Some researchers state that BRCA2 is located in the cytokinetic midbody and regulates cytokinesis, and its deficiency affects chromosomal stability [20]. However, recent studies refute this theory. Using time-lapse microscopy, the latest methods of cytokinesis analysis and BAC recombinants, it was shown that the BRCA2 protein has no effect on the regulation of cytokinesis in human cells. It is therefore unlikely that the chromosomal instability is caused by the cytokinesis defects in *BRCA2* mutation carriers [21].

RAD51, BRCA1 and BRCA2 colocalization in the nucleus indicates the cooperation of these three proteins in the DNA repair process. Although it is evident that both BRCA1 and BRCA2 associate with RAD51 (the interaction of BRCA1 with RAD51 is indirect), the biological significance of this impact has not been precisely established. BRCA2 interacts directly with RAD51 as an integral part of the process of homologous recombination in human cells [14, 22-24]. It is proven that BRCA2, as a component of the mechanism of homologous recombination, directly regulates RAD51 recombinase activity [25]. BRCA2 enables the binding of RAD51 to ssDNA coated with replication protein A (RPA) [26]. Both proteins not only share a common location during the DNA repair, but also interact with each other *in vivo* [7, 24, 27, 28]. In cells with a defective *BRCA2* gene, such as CAPAN-1, RAD51 is found mainly in the cytoplasm and is unable to repair double-stranded DNA breaks. This fact shows that BRCA2 is responsible for nuclear localization and efficiency of the RAD51 recombinase [29-31].

BRCA2 associates with other proteins relevant to the process of homologous recombination: PALB2, DSS1, microcephalin [11, 32, 33]. PALB2 recruits RAD51-BRCA2 complex to points where the DNA was damaged [32]. DSS1 stimulates homologous recombination, interacting with the BRCA2 C-terminus [11]. Microcephalin (MCPH1) is involved in the cellular response to DNA damage; at its C end it contains a BRCT domain, which interacts with the N end of BRCA2. BRCA2-MCPH1 interaction causes enrichment of DSB DNA foci in RAD51-BRCA2 complexes, but does not affect the formation of the complex itself. Research indicates that MCPH1 is responsible for the proper location of RAD51-BRCA2 [33].

Another protein involved in DNA damage repair through homologous recombination is BCCIP. This protein binds to

the C terminal domain of BRCA2. It stimulates the formation of BRCA2-RAD51 complex and is involved in homologous recombination [34].

Participation in meiotic recombination is attributed to the BRCA2 protein; it was found to form complexes with meiotic recombinase DMC1 [35].

It was shown that *BRCA2* expression inhibits tumour cell growth *in vivo* [36]. BRCA2 is a proliferation suppressor acting via stabilization of MAGE-D1 protein [37]. MAGE-D1 regulates apoptosis, transcription and cell cycle progression [38]. Simultaneous expression of these two proteins inhibits the proliferation of mammalian epithelial cells in a TP53-independent manner. The MAGE-D1 binding region of BRCA2 encompasses the DSS1 binding domain [12, 37].

BRCA2 gene mutations

Mutations within the *BRCA2* gene are linked to an increased risk of breast cancer (31-56%), ovarian cancer (10-27%), male breast cancer, prostate cancer, colon, pancreas, gallbladder and bile duct cancer, gastric cancer, and malignant melanoma [39].

Although the current diagnostic methods allow one to identify many different mutations of the gene, it still remains unknown how many *BRCA2*-dependent breast cancer cases are undiagnosed [40]. An additional difficulty is the fact that sequence analysis can detect many unclassified variants (UV) of unknown clinical value [41]. Researchers are trying to develop a test that would enable simultaneous detection of multiple clinically relevant *BRCA2* changes and *BRCA2*-dependent cancers [40].

Depending on the location and type of changes within the *BRCA2* gene, they may give rise to different phenotypes. Research within the Ashkenazi Jewish population and the population of Iceland discovered founder mutations (6174delT and 999del5, respectively). In Ashkenazi Jews founding 6174delT mutation increases the risk of breast cancer, ovarian cancer and prostate cancer. 999del5 mutation detected in the Icelandic population increases the risk of breast cancer in men, female breast cancer, ovarian cancer and prostate cancer [42, 43]. In the Polish population *BRCA2* mutations also occur with high penetration, but they are rare, diverse and characterized by low reproducibility; among others they predispose to breast cancer in men, and breast and ovarian cancer in women [44-48].

There are described clustered *BRCA2* mutations located in the section longer than 3.3 kb. This region, which is called OCCR (Ovarian Cancer Cluster Region), is located between 3035 and 6629 nucleotide [49]. Mutations in this area cause shortening of the protein product; they significantly increase the risk of ovarian cancer and are associated with a relatively low risk of breast and prostate cancer [49-52]. Due to the large diversity, as well as low frequency of repetitive mutations of the *BRCA2* gene, sequencing as a testing method is recommended in the majority of populations, including the Polish population [53].

BRCA2 mutations can be classified into different groups depending on their functional significance. Changes that are identified within the coding sequence of the BRC motifs prevent binding of RAD51 by BRCA2 and impair the process of

homologous recombination [9, 27]. Missense mutation of a single amino acid (T1526A) within the sequence encoding BRC4 motif blocks the ability of RAD51 binding by BRC4. There are also mutations within other motifs that bind RAD51 with high affinity, such as T1011R (BRC1), S1221P (BRC2) and T1980I (BRC7) – the molecular effect of these changes is the same as in the case of missense T1526A [9]. These mutations contribute to carcinogenesis [9].

6174delT mutation causes a loss of the signal part of the protein crucial for BRCA2 nuclear localization. It results in translation termination at codon 27 leading to significant shortening of the protein and loss of its function, which is partially retained in heterozygotes [43].

Clinical significance of BRCA2 gene mutations

Mutations within BRCA2 increase the risk of cancer: breast, ovarian, prostate, pancreas, gallbladder, bile duct, stomach cancer and malignant melanoma [39]. Published results show different values of the relative risk of disease.

In women with inherited mutations of BRCA2 the risk of developing breast cancer before 70 years of age is up to 80% [54].

According to the studies, the estimated risk of developing ovarian cancer before 70 years of age in 6174delT mutation carriers among Ashkenazi Jews is 18-21% [55, 56].

Struewing *et al.* state that the risk of developing prostate cancer before age 70 among Ashkenazi Jews is 16% for 6174delT BRCA2 mutation carriers and 3.8% for those without the BRCA2 mutation [55]. However, the results obtained by other centres were divergent and the risk ratio significantly differed [57-59]. The risk of prostate cancer varies depending on the population studied and the type of mutation [60].

In Poland, founder mutations of BRCA2 occur with high penetration but they are rare and unique. The repetitive change C5972T of low penetration, which increases the risk of early breast cancer (diagnosis below 40 years) and ductal carcinoma *in situ* with micrometastases were described [61].

Among people with mutations in BRCA2 the risk of breast and ovarian cancer varies and depends on environmental factors, hormonal factors and genetic factors [62-66]. It was shown, for example, that a mutation 135G>C in the gene RAD51, which affects the splicing of mRNA, significantly modifies the risk of breast cancer in BRCA2 mutation carriers [66]. Another example is the presence of BRCA2 change T1915M (C5972T), which does not interfere with the proper conduct of homologous recombination. T1915M dramatically increases the risk of breast cancer, when it is present with mutation of CHEK2 I157T. The CHEK2 gene encodes a kinase involved in the process of homologous recombination. In Poland, 1 per 500 women is a carrier of both mutations [67].

The BRCA1 and BRCA2 genes were classified into the group of caretaking genes, which care for the integrity of the genome. According to this classification, mutations in these genes are rarely found in sporadic tumours. Patients with diagnosed gene mutation may be subjected to genetically targeted therapies. The treatment applies a factor causing DNA damage that is repaired by the normal products of caretakers. Caretaker gene mutation prevents DNA damage repair that leads to the accumulation of errors and inhibition of cell proliferation, consequently causing its death [68].

It was found that most cells with a defective BRCA2 gene are sensitive to gamma radiation. Patients with breast cancer who are also carriers of mutations within this gene are more susceptible to radiation therapy than patients without the mutation [14].

It was observed that carriers of BRCA1 or mutation have a significantly better prognosis for ovarian cancer and have better response to cisplatin treatment than patients without the mutation within one of these genes. Cisplatin is a DNA crosslinking agent – it produces cross-linkages between DNA strands and thereby prevents replication and cell proliferation [69]. Preliminary studies indicate that the majority of BRCA1 mutation carriers undergoing cisplatin therapy achieve overall pathological remission [70, 71].

BRCA1 and BRCA2 genes are involved in DNA repair mechanisms; loss of their functions leads to genomic instability, but the exact mechanism involved in BRCA2-dependent breast and ovarian cancer remains unknown. It seems likely that in both cases (BRCA1 and BRCA2) the development of cancer begins in different types of mammary gland cells and/or by specific mechanisms, which is indicated by the different phenotype of both cancers. Cancers among BRCA1 mutations carriers in most cases are classified as negative for oestrogen receptor (ER) expression and human epidermal growth factor receptor (HER) expression, while tumours among BRCA2 mutation carriers often exhibit ER-positive phenotype [72].

Nowadays, research is aimed at developing therapeutic panels against cancer caused by deficient function of BRCA1 or BRCA2. Although mutations in both genes are typically found in the heterozygous state, in tumour cells a complete loss of the wild allele is observed. *In vivo* studies in Brca2 deficient mice demonstrated the strong cytotoxicity of three alkylating agents – chlorambucil, melphalan and nimustine. It was shown that melphalan and nimustine exhibit stronger cytostatic effects on cancer cells with BRCA2 deficiency than cisplatin [73].

Another type of chemotherapy includes PARP (polymerase poly-ADP-ribose) inhibitors. These inhibitors very effectively destroy cells with defective BRCA2 [74, 75]. In addition, their efficacy was proved when they were administered orally [76]. Polymerase poly-ADP-ribose is involved in the repair of single-stranded DNA breaks and affects the process of homologous recombination – inhibition of its activity induces a process of homologous recombination [77, 78]. Inhibition of PARP leads to the accumulation of single-stranded DNA breaks that block replication fork shifts, generating double-stranded breaks, which is necessary for homologous recombination repair. The normal process of homologous recombination is disrupted in cells with BRCA2 (–) phenotype; accumulation of DNA damage occurs, which leads to cell death [74]. The mechanism of action of PARP inhibitors is shown in Fig. 3. Patients generally inherit a mutant BRCA2 allele; the development of cancer occurs when the second (wild type) allele is inactivated by *de novo* mutation. Cancer cells do not possess the functional BRCA2 protein and are unable to perform homologous recombination, while the other body cells have the functional protein encoded by the wild-type allele and are capable of repairing double-stranded DNA breaks, thus being resistant

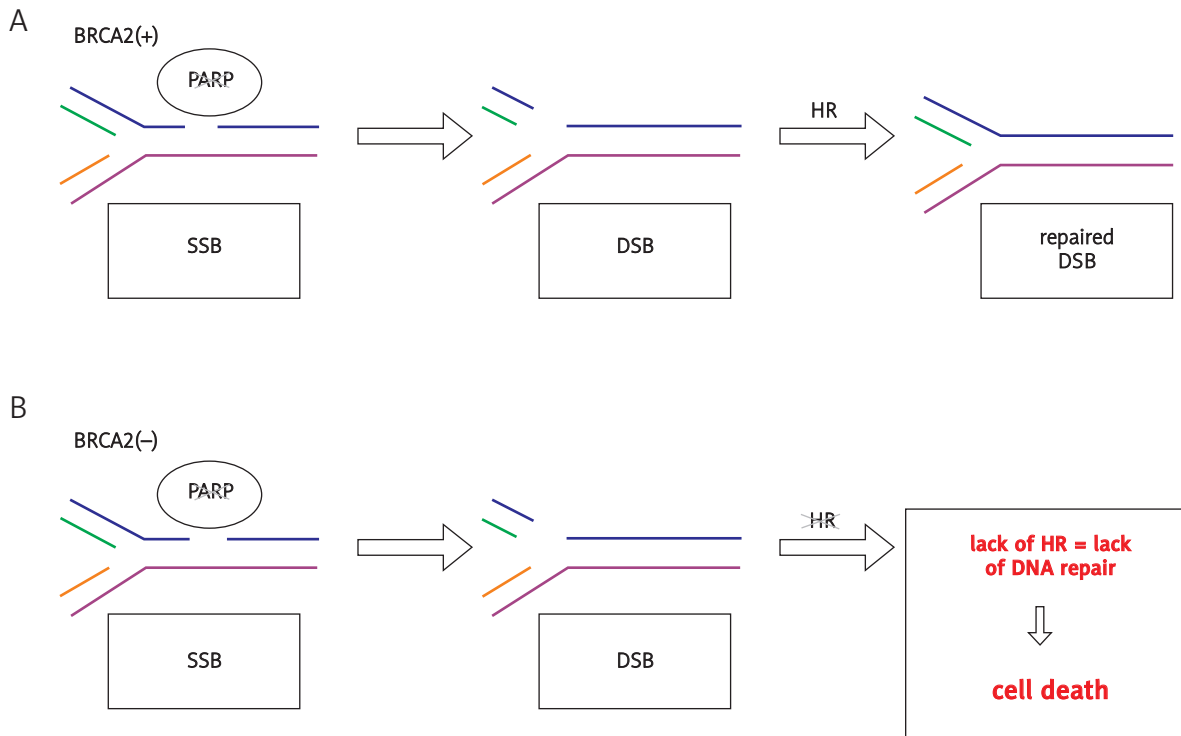


Fig. 3. Mechanism of action of PARP inhibitors: A – BRCA2 functional cells; PARP inhibition causes replication fork collapse at the single strand break site and the DNA double strand break is formed; DSB is repaired by HR pathway; B – BRCA2-deficient cells; PARP inhibition causes replication fork collapse at the single strand break site and the DNA double strand break is formed; DSB is unable to be repaired by HR pathway; accumulation of double strand breaks leads to cell death

Table 1. Examples of clinical trials of PARP inhibitors in treating patients with BRCA1/2 mutations [Based on data available at www.clinicaltrials.com]

PARP inhibitor	Clinical trial number	Status of clinical trial	Phase of clinical trial	Combined with	Localization of cancer
AG014699 (PF-01367338)	NCT00664781	patient recruitment	II	–	breast cancer, ovarian cancer
AZD2281 (KU-0059436; laparib)	NCT00647062	patient recruitment	I	carboplatin	breast cancer, ovarian cancer
PF-01367338	NCT01074970	patient recruitment	II	cisplatin	breast cancer
AZD2281 (KU-0059436; olaparib)	NCT00679783	activ	II	–	ovarian cancer, breast cancer
AZD2281 (KU-0059436; olaparib)	NCT00494234	finished	II	–	breast cancer
ABT-888 (veliparib)	NCT01104259	patient recruitment	I	cisplatin, vinorelbine	breast cancer, ovarian cancer, breast cancer in men
AZD2281 (KU-0059436; olaparib)	NCT01078662	patient recruitment	II	–	advanced stage of cancer
ABT-888 (veliparib)	NCT01009788	activ	II	temozolomide	breast cancer
ABT-888 (veliparib)	NCT00535119	patient recruitment	I	carboplatin, paclitaxel	breast cancer, ovarian cancer

to the cytotoxic effect of PARP inhibitors [79]. PARP-1 is the main protein of the PARP group; its activity covers 80% of the activity of poly-ADP-ribose in human cells. Clinically tested inhibitors are directed against PARP-1 functions. Various chemical compounds that are inhibitors of a very similar mode of action have different effects in clinical trials; however, blocking the function of PARP has a significant influence on the sensitivity of cells to genotoxic agents [79]. At present, the effectiveness of PARP inhibitors both in monotherapy and in combination with other cytostatics is being examined. Studies on PARP inhibitors in patients with *BRCA1/2* mutations are collected and presented in Table 1 [adapted from www.clinicaltrials.gov]. The problem with this type of chemotherapy is the resistance to treatment, which can develop from 18-77 weeks of treatment. This mechanism is not fully understood. It is believed that resistance to PARP inhibitors may result from genetic reversion of the *BRCA2* mutations caused by the secondary mutations, which restore a functional protein [80]. The phenomenon of resistance to chemotherapy by genetic reversion is also specific for cisplatin treatment [81]. PARP inhibitors enhance the antitumor efficacy of DNA-damaging factors: platinum, topoisomerase I inhibitors, temozolamide and radiation.

A phase II trial with oral olaparib administration (AZD2281) in monotherapy of advanced breast cancer with hereditary *BRCA1* mutation showed a greater clinical benefit (41% vs. 22%), partial response (37% vs. 22%) and longer time to progression (5.7 vs. 3.8 months) using a higher dose (400 mg 2× a day, compared to 100 mg 2× a day). There was also a good response in patients with *BRCA1/2* mutations [82].

A multicenter phase II study assessed the effectiveness of intravenous PARP1 inhibitor, BSI-201, in combination with gemcitabine and carboplatin. Addition of the PARP inhibitor to chemotherapy resulted in prolonging time to relapse (6.9 vs. 3.3. months), and longer overall survival (9.2 vs. 5.7 months) [83].

Treatment with PARP inhibitors was well tolerated and the most common side effects included fatigue, nausea and vomiting. 6-thioguanine (6-TG) is a compound that also selectively destroys *BRCA2* (–) tumour cells.

6-TG induces double-stranded DNA breaks, and cells lacking *BRCA2* function are not able to repair this damage by homologous recombination. This leads to accumulation of genomic changes and ultimately to cancer cell death. Tumours with *BRCA2* (–) phenotype are sensitive to 6-TG and do not develop drug resistance during therapy, as occurs in the case of PARP inhibitors [84].

Constitutional *BRCA1* or *BRCA2* mutation carriers often opt for mastectomy and removal of the ovaries and fallopian tubes (salpingo-oophorectomy) to reduce the risk of breast cancer and ovarian cancer. The effectiveness of such drastic prevention has been confirmed in 22 multicentre clinical trials in Europe and North America in the years 1974-2008. The study included patients diagnosed with mutations within the *BRCA1* or *BRCA2* gene [85]. Suggested age to remove the ovaries and fallopian tubes is 35-40 years; after surgery short-term supplementary hormonal therapy is recommended to reduce symptoms of menopause [40]. 72% decrease of breast cancer cases was observed among *BRCA2* mutation carriers who underwent prophylactic salpingo-oophorectomy; short-term

post-operative hormonal supplementation did not seem to affect the reducing effect [40, 86].

The average life expectancy of UK residents with *BRCA1* and *BRCA2* mutations was compared. It was observed that carriers of *BRCA1* mutations lived shorter than *BRCA2* mutation carriers; this was due to the increased incidence of ovarian cancer in the first group. In addition, longer survival in *BRCA2* mutation carriers for ovarian cancer diagnosed at an early stage of the disease was noted [87].

According to recent studies, oral hormonal contraception reduces the risk of ovarian cancer in *BRCA2* mutation carriers. However, these results remain at present quite controversial and require further analysis [88].

For people affected by cancer, the greatest benefit of genetic testing is the opportunity to participate in programmes of prevention and early diagnosis of secondary tumours. Laboratories that offer genetic testing for *BRCA1/2* should determine whether prevention programmes are available for families of patients affected. Carriers of mutations in these genes who are over 25 years of age should undergo preventive screening for early detection of breast cancer and ovarian cancer [53].

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