



Clinical value of molecular changes in ovarian carcinoma

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Summary

Studies carried out on cell lines and ovarian cancer xenografts have indicated that some disturbances of the expression of some groups of genes in cancer cells have a role in the efficacy of chemotherapy, including the response to given groups of drugs. Cisplatin is a DNA-damaging agent. On the basis of the data obtained from cell lines the level of the expression of TP53, BCL and BAX proteins affects the response of the ovarian cancer to cisplatin, however this effect has not been confirmed in clinical studies. In the last few years, in the framework of a multi-centre project, we have investigated a group of 233 patients with ovarian cancer treated with cisplatin regimen (cisplatin plus cyclophosphamide). We have shown that ovarian carcinomas with TP53 protein accumulation (TP53+) and those without accumulation (TP53-) constitute separate biological groups. In our studies the TP53 status had an impact on the clinical value of other proteins such as BAX and BCL-2, as well as of some clinical factors, eg. residual tumor size or clinical stage. BCL-2 expression had a negative influence on complete remission only in the TP53(-) group. On the other hand, the risk of a relapse was lower for the higher BAX expression in the TP53 (+) group. Now we have embarked on similar studies on a group of patients treated with taxanes. In the future, the evaluation of molecular markers may provide a basis for a more individualised chemotherapy of ovarian cancer patients.

Key words: ovarian cancer, TP53, BCL-2, BAX, cisplatin.

Kliniczne znaczenie zmian molekularnych w raku jajnika

Streszczenie

Badania na liniach komórkowych i ksenoprzeszczepach raka jajnika wykazują, że zaburzenia ekspresji niektórych grup genów w komórkach nowotworowych decydują o skuteczności chemioterapii, w tym o odpowiedzi na poszczególne grupy leków. Cisplatyna jest lekiem uszkadzającym DNA. Według danych z linii komórkowych poziom ekspresji białek TP53, BCL-2 i BAX wpływa na odpowiedź raka jajnika na cisplatynę, jednak nie potwierdzono tego w badaniach klinicznych. W ostatnich latach opracowaliśmy w ramach projektu wieloośrodkowego grupę 233 pacjentek z rakiem jajnika leczonych w oparciu o cisplatynę (cisplatyna plus cyklofosfamid). Wykazaliśmy, że raki jajnika z akumulacją (TP53+) i bez akumulacji (TP53-) białka TP53 stanowią odmienne biologiczne grupy. W tych badaniach status TP53 determinował kliniczne znaczenie innych białek, takich jak BAX i BCL-2, jak również czynników klinicznych (wielkość pozostawionych resztek nowotworu, stadium kliniczne). Ekspresja BCL-2 miała negatywny wpływ na całkowitą remisję jedynie w grupie TP53(-). Z kolei, ryzyko wznowy było niższe przy wysokiej ekspresji białka BAX jedynie w grupie TP53(+). Obecnie rozpoczynamy analogiczne badania dotyczące grupy pacjentek leczonych w oparciu o taksany. W perspektywie, ocena markerów molekularnych może być podstawą bardziej zindywidualizowanego doboru chemioterapeutyków.

Słowa kluczowe: rak jajnika, TP53, BCL-2, BAX, cisplatyna.

Introduction

Postoperative treatment of ovarian cancer patients has for many years involved the administration of cis- or carboplatin together with cyclophosphamide or in combination

with adriamycin and cyclophosphamide. In the last few years, in the treatment for ovarian cancer the optimal regimen in the first line chemotherapy has been that of administering taxanes (drugs impairing the mitotic spindle) and the platinum derivatives. There have been some reports,

however, that this combined form of treatment is not always efficacious. About 20-30% of patients do not respond to both platinum-based and taxane-based chemotherapy.

What is decisive in the outcome of treatment, apart from clinical and histopathological factors, is the sensitivity of the lesion to chemotherapy which may initially be low or may decrease in the course of treatment. The latter is a result of the defensive mechanism of cancer cells in a response to the toxicity and positive selection of clones resistant to the applied therapy.

The fundamental assumption underlying chemotherapy is the damage inflicted on the genetic material or structures essential in cancer cell division, which leads to death and elimination of cells. The efficacy of chemotherapy depends on many factors involved in molecular processes. Chemotherapeutic agents, since they act as cell toxins, lead to activation of cell defensive mechanisms that involve elimination of the drug from cells and its detoxification. If, however, the cell structures are damaged, mechanisms that detect the damage are set in motion. When the DNA is damaged, cell repair mechanisms are induced. If the damage cannot be repaired (which is an expected result of chemotherapy), the cell is put on a programmed cell death pathway, referred to as apoptosis. At each of the above stages there may appear molecular disturbances which will inevitably impair the cancer response to chemotherapy. The following factors may be responsible for the efficacy of chemotherapy: excessive elimination of the drug from the cell and its excessive detoxification, the impairment of DNA damage detection or excessive damage repair, and at the last stage of cell response to chemotherapy - apoptosis insufficiency. The two former mechanisms are referred to as pharmacological drug resistance, while others are called non-pharmacological drug resistance.

Mechanisms of pharmacological resistance

The genes responsible for multidrug resistance (*MDR1*, *MPR* and *LPR*) code for proteins which eliminate cytostatic agents from the cell; their activation contributes to a lower concentration of the drug in the cell. In particular, the *MDR1* gene encodes glycoprotein P (Pgp) bound to the cell membrane, which acts like an effusion pump [1]. Pgp eliminates doxorubicin, vincristin, VP16 and paclitaxel. Pgp expression seems not to affect the sensitivity to cisplatin [2-4]. In most reports on ovarian cancer patients treated with cisplatin no effect of Pgp on the cancer response to chemotherapy has been observed [3-7].

Another mechanism of pharmacological resistance involves the increase of the activity of glutathione and glutathione S-transferases. Glutathione is tripeptide thiol, which plays an important role in cell detoxification from various xenobiotics such as platinum derivatives [8,9]. On the other

hand, glutathione S-transferases (GST) take part in detoxification by catalysing the combination, among other things, of cisplatin and alkylating agents with glutathione [3,4,10]. In spite of the fact that these mechanisms are involved in cisplatin detoxification, no direct relationship has been noted between the glutathione or glutathione S-transferases expressions and ovarian cancer response to therapy based on cisplatin [5,6,11].

Mechanisms of non-pharmacological resistance

Experimental studies carried out in the last few years have indicated the potential importance of molecular mechanisms occurring after damage of DNA or other molecular structures on effects of chemotherapy. The efficacy of cell cycle checkpoints responsible for growth arrest after DNA damage, mechanisms of DNA-damage detection and repair as well as efficacy of the programmed cell death - all these factors may influence tumour response to chemotherapy. Disturbances in the expression of individual genes that control these processes (due to mutation or altered expression) make it possible to differentiate cancers as to their sensitivity to cytostatic agents or groups of cytostatic drugs.

Non-pharmacological resistance to chemical therapy, generally speaking, is a result of the failure in recognising the damage caused by an antineoplastic agent or the tolerance thereof, or the enhanced repair of the damage or ineffective processes of elimination of damaged cells.

Mechanism of action of some cytostatic agents

Cisplatin and cyclophosphamide exert a cytotoxic effect by damaging cell DNA [12,13]. Cisplatin forms DNA adducts, while cyclophosphamide leads to DNA damage by alkylation. This results in the loss of DNA properties as a replication matrix and, consequently, DNA strand-breaks are formed, which leads to initiation of apoptosis. The effect of therapy with platinum compounds and cyclophosphamide is reversely proportional to the efficiency of DNA repair mechanisms, and directly proportional to the efficacy of the mechanisms controlling apoptosis.

Taxanes (taxol, paclitaxel and taxotere) exert a cytotoxic effect by stabilising and inactivating microtubules responsible for the formation of mitotic spindle. This process takes place through tubuline (a component of microtubules) polymerisation. By continuous administration of taxol mitosis cannot be completed, which causes cells to become blocked in G2 and M phases and this activates apoptosis.

Thus, cisplatin and taxanes cause damage to other cell structures and activate other biochemical pathways. Any damage resulting from the administration of cisplatin leads to the activation of the TP53 pathway which, in its turn, is not activated by taxanes.

Enhanced DNA repair

In response to DNA injury the cell sets in motion, among other things, a direct repair or nucleotide-excision or base-excision mechanisms. The direct repair involves breaking the link between DNA and the damaging agents (alkyl groups) through the action of alkyltransferase [14]. The repair by base-excision consists in the elimination of the damaged base and replacing it by a normal one. Large injuries (27 to 29 nucleotides) are repaired by removing nucleotides. The latter process is quite complicated. It includes several stages such as recognition, incision and removal of the strand, gap stabilisation, initiation of the repair replication and linkage with the rest of the strand. A very large number of enzymes participate in this process. The repair of DNA damage caused by alkylating agents is a direct process or it is made by excising nucleotides or bases, while that caused by cisplatin is made by excising nucleotides [15].

Among the main factors that contribute to the efficacy of chemotherapy with cisplatin is the extent of DNA damage. This damage is usually considerably bigger for the cell repair mechanisms to eliminate. However, some ovarian cancer lines resistant to cisplatin treatment indicate enhanced DNA repair, which is revealed by increased elimination of adducts and greater synthesis of DNA repair enzymes [3,4,16-18]. It has been reported that the patients, whose malignancies respond to cisplatin treatment have more cisplatin adducts in normal tissues than the patients with carcinomas resistant to treatment [19]. Pilot studies have shown increased mRNA expression for some enzymes participating in DNA repair such as ERCC-1 and XPA in ovarian cancer cases resistant to chemotherapy [20,21]. On the other hand in various malignancies resistant to the therapy with alkyl drugs and in some ovarian tumours high alkyltransferase expression has been noted [22,23].

DNA mismatch repair system defects

A separate group of genes the damage of which predisposes to resistance to chemotherapy belongs to the system referred to as mismatch repair. Among the most important genes in this system are *MLH1*, *MSH2*, *PMS1* and *PMS2*. Under normal conditions in the cell there may appear errors in DNA replication. Proteins encoded by these genes are responsible for the repair of random errors in replication and for elimination of some changes in DNA sequence induced by carcinogens. During repair by this system long DNA units, sometimes up to 1000 bases are removed. Defects or disturbances in the expression of mismatch repair genes result in the formation and accumulation of genetic changes in the whole genome and thus in the genes responsible for the phenotype of resistance, and particularly in the so-called microsatellite sequences that is replications of the same DNA sequence [24]. The general opi-

nion is that in microsatellite sequences there exists an increased risk of errors in replication. These errors, however, are repaired when the mismatch repair system is sufficiently effective [25]. In tumours, where the mismatch repair system is disturbed, microsatellite sequences are considerably heterogeneous as compared with those in normal tissues. This effect is referred to as microsatellite instability or RER+ (replication error) phenotype [26].

The mismatch repair system also plays a leading role in the detection of DNA adducts that are formed as a result of cisplatin treatment and in inducing signals that eventually lead to cell death. The loss of the DNA mismatch repair system, eg. by mutations inactivating the system's genes or by their lower expression level, makes it more likely that neoplastic cells resistant to chemical treatment are formed and selected. If the DNA repair enzymes are unable to recognise injury, persistent adducts (tolerance for damage) may appear. Increased tolerance for the presence of adducts (together with DNA replication bypassing adducts) has been observed in ovarian cancer cases resistant to cisplatin.

In the cohort of 233 ovarian carcinomas studied in the Polish multicentre project (later in the text) we did not find any loss of expression of *MLH1* and *MSH2* genes. Also, the analysis of 66 ovarian cancers for microsatellite instability in 12 loci has not revealed such molecular changes, whereas other authors report instability in 6-30% of ovarian cancer cases [Plisiecka-Hałasa et al. unpublished results, 27]. It seems, therefore, that the loss of the mismatch repair system is not in fact a cause of resistance in ovarian cancer cases observed in Poland.

Impairment of the apoptosis pathway

Irrespective of the mechanism of the action of a chemotherapeutic drug the anticipated biological effect is to eliminate the cell by apoptosis. When the mechanisms regulating apoptosis are impaired the resistance to treatment may be increased. The programmed cell death is controlled by a large number of proteins such as TP53, BCL-2 family, IAP family, some proteins of the STAT family, phosphatidylinositol kinase pathway (PI3K, PKB), RAS-MEK-ERK pathway and others. Detailed data on the role of these proteins in the control of apoptosis and impairment of the response to chemotherapy together with a list of relevant literature has been published in previous reports [28,29].

An important role in the induction of apoptosis in DNA damage is played by TP53 protein, which causes the cell cycle arrest in the G1 phase. At the same time the DNA repair mechanisms are initiated [30]. TP53 protein induces transcriptional activation of the *WAF1/CIP1* gene, which codes for the protein of 21kD (p21) molecular weight [31-33]. P21^{WAF1} exists in the quaternary complex with a cyclin (proteins controlling each phase of the cell cycle), cyclin-dependent kinase and a proliferating-cell nuclear antigen (PCNA). P21^{WAF1} inhibits both the activity of kinase (arrest of

the cell cycle) and PCNA (arrest of DNA replication) [30,34]. If the DNA repair fails, then TP53 protein initiates programmed cell death (apoptosis) [35-37].

It has been shown in ovarian cancer cells that normal TP53 protein is required for the cell cycle to be arrested in the G1 phase after the administration of cisplatin. This effect has not been observed after the transfection of the mutant TP53 protein [12] nor in the cells lacking TP53 or its P21^{WAF1} effector protein [38,39]. Cell lines resistant to cisplatin treatment feature no arrest in the G1 phase after cisplatin administration [39]. Other reports have also shown that impairment of TP53 protein has an impact on the resistance to cisplatin in ovarian cancer cell lines [40-42].

Apoptosis caused by taxane (in therapeutic doses) is independent of TP53 protein and the majority of published reports seem to indicate that defects in *TP53* and *P21^{WAF1}* genes do not lead to diminished sensitivity to the treatment with taxanes and other drugs which impair the cell tubular system in the G2 and M phases [41,43-45]. Some data, however, suggest that the presence of the normal TP53 protein has an unfavourable effect on the action of taxol [46]. TP53 protein plays a role in the mitotic spindle check-point: it becomes associated with microtubules of the division spindle. After taxol treatment cancer cells lacking TP53 were arrested in the G2/M phase and underwent apoptosis, whereas cells with normal TP53 were able to overcome G2/M arrest and accumulated in the G1 phase, in which they are not susceptible to taxol. Thus, the presence of normal TP53 protein facilitates cycle progression through the mitotic phase after taxanes had become active. On the other hand, the loss of TP53 function, as well as TP53 mutations sensitise cancer cells to taxanes - dissociation of TP53 and tubulins has been noted, which probably facilitates polymerisation of tubulins by taxanes; the cycle is arrested in the G2/M phase, after which the cells yield to apoptosis.

The process of programmed cell death in its effector phase is controlled by genes of the BCL-2 group. The BCL-2 family includes both the promoters of apoptosis, such as BAX protein, and apoptosis inhibitors, of which the most important agent is BCL-2 protein [47,48]. BCL-2 family plays a key role in the effector phase of apoptosis [49,50].

The BCL-2 protein family is characterised by the presence of homology domains (BH), which make possible the interaction among BCL-2 family proteins and with other proteins structurally unrelated with BCL-2 [49,51]. The presence of domains allows for the formation of homodimers (e.g. BAX/BAX) and heterodimers (e.g. BAX/BCL-2), which have a decisive role in the fate of the cell. Enhanced BCL-2 expression inhibits apoptosis, which in turn, may be modulated by proteins of the BAX group that nullify the antiapoptotic effect of BCL-2 due to the formation of heterodimers with BCL-2. The predominance of BCL-2 homodimers supports proliferation and survival, whereas the predominance of BAX homodimers leads to cell death.

The balance between the products of *BCL-2* and *BAX* ge-

nes is a decisive factor in the efficacy of apoptosis induced by chemotherapeutic drugs. Increased levels of the BCL-2 protein or disturbances in the proportion of apoptosis promoters and inhibitors (e.g. lower BAX expression - the BCL-2 inhibitor) are likely to unfavourably affect the apoptotic index [52]. Studies on the ovarian cancer cell lines have revealed that there is a relationship between the high BCL-2 expression and the resistance to cisplatin [40,41,53], although contradictory results have also been observed [54]. In experimental investigations BAX expression increased cell sensitivity to cisplatin or was found to have no such effect [41,54].

One of BCL-2 functions is to maintain the integrity of microtubular cell structures in the G2 and M phases. Anticancer drugs which impair mitotic spindle structures (including taxol) induce BCL-2 phosphorylation and thus nullify its antiapoptotic effect, whereas cytotoxic drugs that impair DNA fail to exert such an effect [55].

Taxanes phosphorylate BCL-2 protein in serine 70 locus. Phosphorylation in this position makes it impossible for BCL-2 protein to bind with BAX, which in turn, leads to apoptosis [56]. The proteins which inhibit BCL-2 phosphorylation, eg. endotheline-1, protect ovarian cancer cells against paclitaxel-induced apoptosis [57]. It is not only impaired phosphorylation, but also excessive BCL-2 expression that cause higher resistance of cancer cells to taxanes, which has been shown in studies on cell lines [58]. In addition, it has been noted that ovarian cancer cells resistant to paclitaxel reveal low BAX expression in contrast to sensitive cell lines [59]. Stimulation of BAX expression leads to enhanced ovarian cancer cell sensitivity to taxol by a factor of 500-1000, which is shown by massive apoptosis [60].

TP53, BCL-2 and BAX in ovarian cancer clinical studies

Despite the essential role of TP53 protein in the response of neoplastic cells to cisplatin and the high frequency of *TP53* gene mutations in ovarian cancer impairing the protein function, clinical studies have not revealed unequivocally the existence of a link between TP53 status and the response of ovarian carcinoma to chemotherapy [61-68]. Similarly, most clinical investigations have not confirmed the prognostic value of BCL-2 and BAX expressions in ovarian cancer treated with cisplatin.

However, the analyses of molecular markers in large homogenous clinical groups of ovarian cancers cited in world medical literature are rare. Among a few such reports concerning TP53 only two teams have studied the predictive value of TP53 accumulation [61, 64-66]. It was only Ferrandina et al. [65] who found a better response to chemotherapy in lesions without accumulation of TP53. Hartmann et al. [61] and Eltabbakh et al. [64] did not observe any clinical significance of TP53 accumulation. No predictive value of BCL-2 and BAX expressions was noted in ovarian cancer cases, either [40,66,69-73]. Only

Mano et al. [70] found higher frequency of total remissions in patients with lesions without BCL-2 expression in single factor analysis.

In the last few years we have analysed (in the framework of a multicentre KBN 4 P05C 028 14 project) a group of 233 ovarian cancer patients treated with cisplatin (cisplatin plus cyclophosphamide). The main criteria for including ovarian cancer cases in the above project were the following factors: the advanced clinical stage (IIB-IV) as determined by FIGO standards, completion of the first line post-surgery chemotherapy with use of cisplatin or carboplatin and cyclophosphamide, availability of data on the residual tumour size, availability of clinical observations and the material obtained from the first surgery procedure performed. Both the histopathologic and clinical data have been verified by a unified system of evaluation by a multicentre team. Based on strict criteria only 42% of cases referred for the project have been accepted. In this way uniform and well prepared clinical material has been obtained [74-76].

We have evaluated the prognostic and predictive value of TP53 protein accumulation, as well as that of other proteins such as BCL-2 and BAX. The analysis was carried out on the whole group of patients and in two subgroups: those without TP53 protein accumulation (TP53-) and with accumulation (TP53+). In our study, TP53 accumulation, as an independent variable, was of no clinical value, like in some reports published by other authors [61,64]. However, we have pointed out that ovarian cancer cases with and without TP53 protein accumulation form two distinct biological groups. We have also found that TP53 status was essential in determining the clinical value of other proteins such as BAX and BCL-2, as well as that of clinical factors, e.g. the residual tumour size or clinical stage.

Due to the novel approach to the analysis of ovarian carcinoma, based on two subgroups depending on the presence or lack of TP53 protein accumulation, we have demonstrated the prognostic value of BCL-2 expression, which had a negative impact on the complete remission only in the TP53(-) subgroup. On the other hand, the risk of relapse was lower for a high BAX expression only in the TP53(+) subgroup. In the whole group of patients the high BAX expression affected adversely complete remission. Our results seem to prove that BCL-2 and BAX, substantiated by a multifactorial analysis, have a predictive clinical value.

TP53 status also had an effect on the clinical value of clinical factors, particularly of the residual tumour size. Total removal of the lesion, as compared with 0.5-2 cm residual mass left, significantly increased the probability of complete remission and diminished the risk of death in the TP53(-) subgroup. However, this observation was not confirmed in the TP53(+) subgroup. All this seems to indicate that pre- or intraoperational determination of the TP53 status would be most desirable in ovarian cancer patients

to determine a TP53 - negative group, in which major effort should be made to completely extirpate the tumour mass [76].

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