BRCA1 deficiency and synthetic lethality in leukemias; not only gene mutation matters

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Abbreviations: BRCA1 – breast cancer 1 susceptibility protein, CML – chronic myeloid leukemia, AML – acute myeloid leukemia, PARP1 – Poly [ADP-ribose] polymerase 1, HR – homologous recombination, cNHEJ – classical non-homologous end-joining, TKI – tyrosine kinase inhibitors, SAC – spindle assembly checkpoint, GEMA – gene expression and mutation analysis, LSCs – leukemia stem cells, LPCs – leukemia progenitor cells, DSBs – DNA double-strand breaks, SG – stress granules, AREsite – AU-reach sequences, UPR – unfolded protein response, PERK – *PKR*-like ER-localized eIF2α *kinase,* eIF2α – eukaryotic initiation factor 2 alpha subunit

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ABSTRACT

BRCA1 (breast cancer 1 susceptibility protein) is one of main regulators of cellular genomic stability. It is responsible for proper segregation of chromatides to daughter cells during mitosis as well as DNA double strand breaks repair by homologous recombination (HR). Genetic alterations of *BRCA1* gene are cancer predisposition markers. Mutations or epigenetic alterations have been noticed in breast, ovarian and prostate cancers, significantly increasing risk of cancer development. Such gene alterations are not connected with leukemias. Importantly, BRCA1 deficiency is a factor which makes patients susceptible for personalized therapy with PARP1 inhibitors, which is based on the phenomenon called synthetic lethality. In this review we present our discoveries of novel mechanism leading to BRCA1 deficiency in leukemia, which is not connected with *BRCA1* gene mutations or epigenetic alterations, but with attenuated translation of BRCA1 protein linked to the cellular stress response and controlled by RNA binding proteins. Moreover, we found that some treatments or genetic alterations in leukemias might also result in BRCA1 deficits. Our studies provide evidence that PARP1 inhibitors should be considered as efficient treatment in BRCA1-deficient leukemias, leading to elimination of cancer cells, including stem and progenitor cells. Finally we propose a strategy to select leukemia patients which might be sensitive to therapy with PARP1 inhibitors.

INTRODUCTION

BRCA1 is an acronym of **br**east **ca**ncer **1** susceptibility protein, which is encoded by *BRCA1* gene localized on 17th chromosome in humans. It has been discovered as a result of intensive investigation of inherited susceptibility to breast cancer. Those studies led to identification of chromosome 17q12-21 as the first human genomic region that harbored an autosomal dominant susceptibility gene for breast cancer (BRCA1) [1,2,3,4]. Genetic alterations of *BRCA1* are cancer predisposition markers. Woman who carry mutations in *BRCA1* gene have 60–80% increased risk of breast cancer, and 25–40% increased risk of ovarian cancer [5,6,7]. These observations led to the conclusion that *BRCA1*, a gene that confers susceptibility to ovarian and early-onset breast cancer, encodes a tumor suppressor. Further studies showed that BRCA1 mutations also significantly increase risk of prostate cancers up to 30% [8]. Later, it has been shown that approximately 50% of epithelial ovarian cancers exhibit defective DNA repair due to genetic and epigenetic alterations of homologous recombination (HR) pathway genes [9].

BRCA1 gene encodes a big protein containing 1863 aminoacids. BRCA1 protein contains Zinc finger (RING) domain, BRCA1 C Terminus (BRCT) domain, Nuclear Localization Signal (NLS) domain and Nuclear Export Signal motifs [10]. The basic function of this protein is to maintain the integrity of genome, due to involvement in the control of cell cycle [11] and DNA damage repair [12]. BRCA1 plays a role in DNA double strand breaks repair by homologous recombination (HR), when the missing fragment is copied from the sister chromatid and thus allows for faithful repair of DNA. In this process BRCA1 interacts with several proteins such as PALB2, CtIP, 53BP1, and BRCA2 to bring RAD51 to DNA double-strand breaks. Thus, BRCA1 deficiency leads to defective DNA repair processes and which increases cancer risk, promotes cancerogenesis and supports cancer progression.

GENOMIC INSTABILITY AND BRCA1 DEFICIENCY IN CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia (CML) is a blood cancer caused by chromosomal translocation between chromosomes 9th and 22nd, leading to generation of the fusion chromosome called Philadelphia (Ph) Chromosome. Philadelphia Chromosome has been discovered in 1960 by Peter Nowell at the University of Pennsylvania School of Medicine in Philadelphia [13] and further character-

ized by cytogenetic techniques by Jane Rowley [14]. The new abnormal chromosome, named after the city in which it has been discovered, was not found in normal blood cells but was present exclusively in malignant leukocytes of CML patients. Further studies by J. Rowley provided evidence that reciprocal translocation between the long arms of chromosomes 9 and 22 is responsible for generation of Philadelphia Chromosome [14]. The consequence of such translocation is expression of the fusion gene *BCR-ABL1* and consequently the protein – BCR-ABL1. BCR-ABL1 protein possess strong constitutive tyrosine kinase activity and is the main driver of development of CML. BCR-ABL1 is the first oncogenic molecule which is targeted by the specific drug designed *in silico*. Imatinib (also known as Gleevec/Glivec), is tyrosine kinase inhibitor, which revolutionized the treatment of chronic myeloid leukemia. Imatinib was invented in the late 1990's by Nicholas Lyndon from Ciba-Geigy (now Novartis) and its use to treat CML was introduced by Brian Druker, an oncologist at the Dana-Farber Institute [15]. Imatinib shows activity against ABL, BCR-ABL1, PDGFRA, and c-KIT. It binds close to the ATP binding site, what results in the closed or self-inhibited conformation, leading to inhibition of the enzyme activity. Inhibition of BCR-ABL1 tyrosine kinase activity in consequence blocks the downstream pathways that promote leukemogenesis. The first clinical trial of imatinib took place in 1998 and the drug received FDA approval in May 2001. Introduction of imatinib revolutionized the therapy and rapidly modified treatment of CML patients, leading to prolonged remission in patients in chronic phase (CML-CP) of the disease. The IRIS study on a 1106 cohort of CML-CP patients showed that imatinib induced complete hematological response in 95.3% patients and complete cytogenetic response in 73.8% patients, significantly increasing also their quality of life [16]. At 6-year follow-up of IRIS trial, imatinib induced complete hematological response in 98% of patients in chronic phase and complete cytogenetic response in 87% patients [15]. Nevertheless, despite the big success of imatinib in CML therapy, imatinib resistance occurs in a cohort of patients. First, patients with the primary resistance showed very weak and short initial responses, as well as development of secondary resistance occurred in many patients which progressed into the blast crisis phase of the disease. This can result from mutations in BCR-ABL kinase domain, amplification of BCR-ABL gene, overexpression and activation of additional signaling pathways promoting survival growth of leukemia cells [17]. New generation tyrosine kinase inhibitors (TKIs) have been developed, such as dasatinib and nilotinib, to overcome imatinib resistance [18]. Especially, leukemia stem and progenitor cells residing in the bone marrow characterize by resistance to TKIs (for review of current state of knowledge please see [19]). This is an extremely important, as complete eradication of those cells is necessary to cure CML patients. Thus, studies looking for novel therapeutic targets and novel therapeutic strategies are carried out to overcome the resistance and to target and eradicate leukemia stem cells.

Studies performed by our group concentrated on elucidation of the prosurvival mechanisms participating in the development of CML as well as development of the therapy resistance. Our general goal it to propose novel targets for therapeutic intervention and novel strategies, which can be used alone or in combination with the existing anti-leukemic treatments. One of the features crucial for cancer development and progression is genomic instability and defective DNA repair. CML cells also belong to the genetically unstable ones. The percentage of cells with improper number of chromosomes and additional mutations significantly increases during the disease progression [20]. In blast crisis phase more than 80% of leukemia cells show symptoms of genomic instability, in comparison with 20% of cells in chronic phase. Moreover, CML cells undergo improper divisions as a result of centrosomes multiplication. Multiple centrosomes cause formation of multipolar spindles and defective segregation of chromosomes. Multiplication of centrosomes is one of the features of genetically unstable tumors.

We discovered that CML cells suffer from disturbed mitosis and improper segregation of chromosomes [21], leading to formation of multinucleated cells or lagging chromosomes. Both are typical symptoms of genomic instability and aneuploidy. We found that those abnormalities are a result of dysfunctional spindle assembly checkpoint (SAC). SAC is a big multi-protein complex which regulates all phases of mitosis including proper formation of the mitotic spindle, segregation of chromosomes, controls midbody and proper separation of daughter cells. Cells expressing BCR-ABL1 characterized by lower expression of SAC members, such as Mad, Bub1, Bub3, BubR1. This suggested an upstream common regulation. Finally we have found that decreased level of BRCA1, which acts as a transcription co-factor of *bubr1, bub1, bub3* and *mad2* genes, was responsible for their affected transcription [21]. Defective mitosis and aneuploidy resulting from BCR-ABL1 expression and dependent on BRCA1 was also reported by others. Authors shown that BCR-ABL1 evoked by viral transduction of CML progenitor CD34+ cells led to aberrations in chromosome segregation and centrosome formation, whilst ectopic expression of BRCA1 in *BCR-ABL1*-transduced cells reverted these anomalies [22]. In addition to our *in vitro* observations, we confirmed BCR-ABL1-dependent BRCA1 deficiency also in blood cells from CML patients [23]. This was one of the two first observations that BRCA1 is downregulated in leukemia cells that express BCR-ABL1. Before our studies, decreased level of BRCA1 in BCR-ABL1-expressing cells has been described by Deutsch [24], however the mechanism was not clarified. Importantly, chronic myeloid leukemia as well as other leukemias have not been considered as BRCA1-deficient type of cancer before. Altogether, our data implicated a novel, mutation-independent mechanism of BRCA1 downregulation, which may exist in CML leukemia cells.

STRESS RESPONSE AND DEFECTIVE BRCA1 TRANSLATION IN LEUKEMIA CELLS

At the time of our discovery, the mechanism of BRCA1 down-regulation in leukemia cells was unknown. Important observation was that the level of protein was decreased whilst its mRNA remained at high level [24]. This

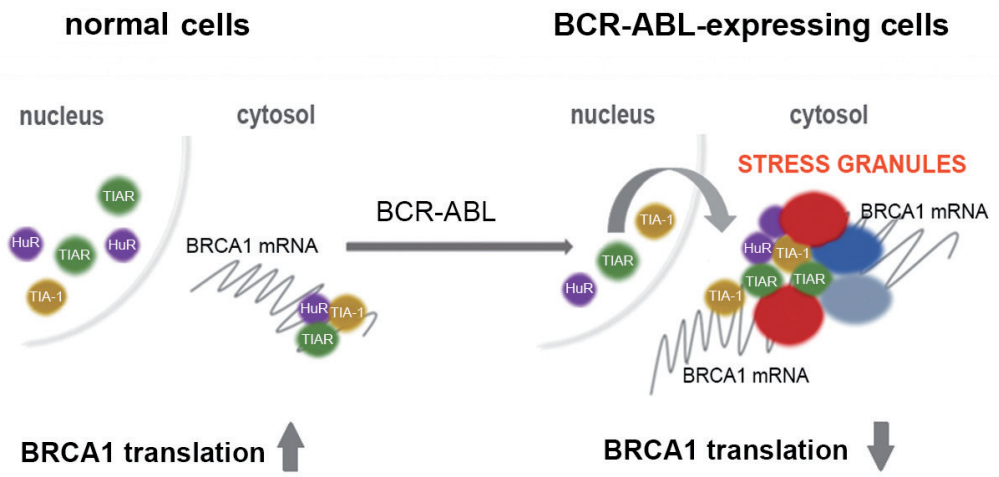

Figure 1. The scheme presenting the mechanism of BRCA1 deficiency in chronic myeloid leukemia cells.

has been confirmed in our model of progenitor CD34+ mouse cells expressing ectopic BCR-ABL1. Moreover, primary cells from CML patients also showed increased *BRCA1* mRNA level correlating with lower BRCA1 protein. This data suggested that such down-regulation visible as BRCA1 protein deficiency occurs post-transcriptionally. Using BRCA1 lucyferase reporter we have shown that BRCA1 translation is lower in cells expressing BCR-ABL1 and is upregulated by treatment with imatinib, what suggested dependence on BCR-ABL1 activity. In model cell lines we have found that RNA binding proteins play a central role in stabilization of the BRCA1 mRNA. Additionally, binding in RNA-protein complexes enables to keep the mRNA sequestered from translation machinery. We found that *BRCA1* mRNA is protected in stress granules (SG) formed by Tia proteins, and by this way of storage, mRNA is not available for translation [23,25]. We provided evidence that BRCA1 synthesis is regulated by stress granules formation. Increased formation of stress granules caused by chemical stimuli – thapsigargin, correlated with decreased BRCA1 protein level, whereas disassembly of SG caused by emetine resulted in increase of BRCA1 protein level [25]. Formation of stress granules and storage of mRNAs coding proteins involved in DNA damage response in complexes with Tia RNA binding proteins has recently been described also for non-malignant B cells [26]. This is a very efficient strategy to keep mRNAs protected from translation, however ready to release in a very fast mode, once they are needed as part of the remodeling of cancer cells function or quick adaptation.

The Tia proteins belong to RNA binding proteins, which interact with ARE site (AU-reach sequences) regions in 3′ UTR of mRNA. Apart from mRNA stabilization, these proteins also participate in mRNA splicing in the nucleus. In response to stress stimuli, Tia proteins shift to the cytoplasm and form aggregates called stress granules. Their main function is protection of mRNA and prohibition of mRNA translation. We found that activity of Tia proteins upon *BCR-ABL1* expression was evoked by previously reported stress response induced upon the oncogene activity [27]. We demonstrated that BCR-ABL1 leads to the endoplasmic reticulum stress and activation of unfolded protein response (UPR). As a result, PERK (*PKR*-like ER-localized eIF2α *kinase*) is activated and phosphorylates eIF2α (eukaryotic initiation factor 2 alpha subunit) – its only substrate known so far [28]. Phosphorylation of eIF2α generally attenuates global mRNA translation by inhibition of the ternary complex, however some mRNAs are preferentially translated under these conditions [29,30,31]. Simultaneously, phosphorylation of eIF2α stimulates formation of stress granules, leading to storage and sequestration from translation of selected mRNAs [32,26]. We have found that activation of stress response at moderate level (mild stress) and enhanced phosphorylation of eIF2α promotes survival of CML cells and resistance to imatinib [28]. Moreover, it stimulates invasiveness of CML cells and stromal cells by enhancing release of matrix modifying enzymes [31]. The scheme presenting the mechanism of BRCA1 deficiency in chronic myeloid leukemia cells discovered in our studies is shown in figure 1.

BRCA1-DEFICIENT LEUKEMIAS AND PERSONALIZED THERAPY BY SYNTHETIC LETHALITY CAUSED BY PARP1 INHIBITORS

Synthetic in ancient Greek stands for ‘putting together’. This phenomenon has been first described in fruit flies by Calvin Bridges [33] and coined by Theodore Dobzhansky [34]. In cancer the term ‘synthetic lethality’ refers to simultaneous occurrence of deficiency in two distinct proteins/pathways which is lethal, whereas lack or inhibition of only one of the proteins/pathways does not lead to cell death. Such strategy had a great advantage, as did not lead to the strong side effects towards healthy cells. The general idea of synthetic lethality is presented in figure 2A.

High throughput screen of various cancer cell types revealed frequent aberrations in DNA damage repair (DDR) pathways, which favor proliferation of cancer cells despite acquired DNA mutations. Defects in DNA repair system amplify the mutation occurrence in cancer cells.

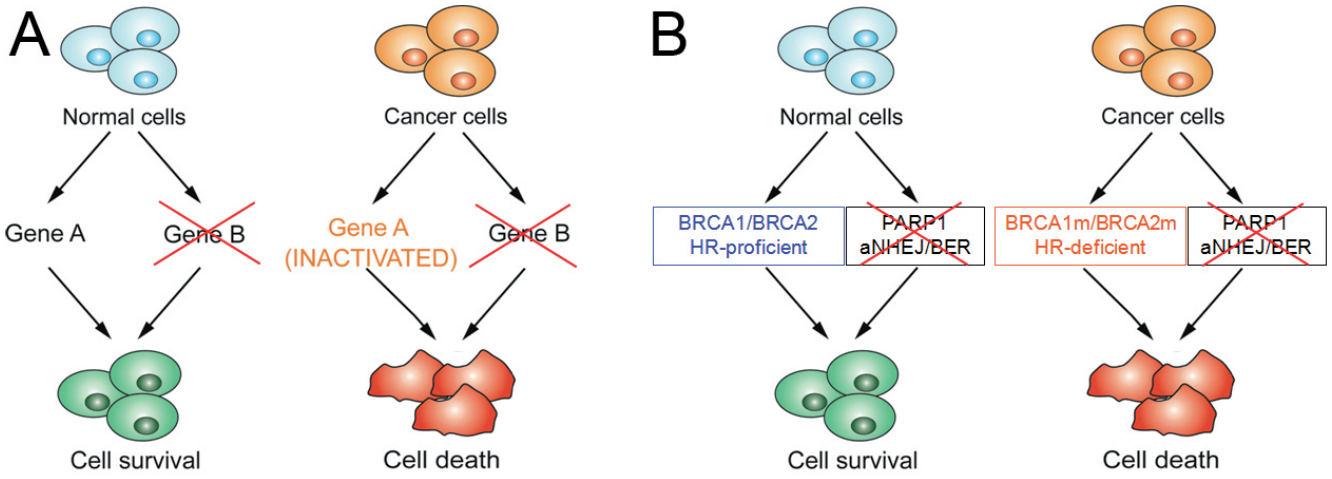

Figure 2. Targeting PARP1 triggers synthetic lethality in BRCA-deficient tumors. (A) The concept of synthetic lethality. (B) Inactivation of PARP1 triggers synthetic lethality in tumor cells displaying HR deficiency caused by inactivating mutations in BRCA1/BRCA2 (BRCA1m/BRCA2m).

Since there are several parallel DDR pathways, which can substitute one another to some extent, then cancer cells can survive but become dependent on the one, unmutated active pathway. One of the most promising strategies is based on the synthetic lethality caused by PARP1 inhibitors in cancers with dysfunctions in BRCA1/BRCA2-dependent homologous recombination (HR) DNA damage repair pathway [35]. PARP1 inhibitors have been developed to target poly-ADP ribose polymerase 1 (PARP1), which is involved in alternative non-homologous end-joining (aNHEJ) and base excision repair [36]. These mechanisms prevent/repair DNA damage in HR-deficient cells, thus PARP1 has been selected as a target for synthetic lethality in BRCA1 and BRCA2-deficient ovarian and breast cancers [37,38]. The principle of induction of synthetic lethality to target DDR pathways by PARP1 inhibitors in presented in figure 2B.

The first pre-clinical data showing therapeutic potential of PARP1 inhibitors were published in 2005, and the first clinical trials were performed in 2009/2010. Finally, Olaparib (Lynparza, Astra Zeneca) – first generation PARP1 inhibitor, has been approved by FDA in 2014, to treat BRCA1 mutated ovarian cancer [39], followed by FDA approval of Rucaparib (Rubraca, Clovis Oncology) in 2016 and Niraparib (Zejula, Tesaro) in 2017. Advanced trials are already ongoing to use PARP1 inhibitors in ovarian, breast and prostate cancers, as well as metastatic melanomas. Talazoparib (a next generation version of Olaparib), is in final phases of clinical trials to be used in treatment of breast and ovarian cancer [https://www.pfizer.com/news/press-release/-news from Dec 8, 2017]. Importantly, the therapeutic strategy based on the synthetic lethality assumes that healthy cells should remain intact, as inhibition of one pathway by the drug can be compensated by activity of the parallel pathway. Thus, such therapeutic approach might reduce side effects.

Even if PARP1 inhibitors are already approved or are in clinical trials to treat some types of prostate, ovarian and breast cancers, leukemias have not been included into these studies, due to lack of *BRCA1* mutations. However, we reported before that CML-CP leukemia stem cells (LSCs) and leukemia progenitor cells (LPCs) accumulate high numbers of potentially lethal DNA double-strand breaks (DSBs) [20,40]. Moreover, BCR-ABL1 tyrosine kinase causes downregulation of BRCA1 and DNA-PKcs proteins, which play key roles in two major DSB repair pathways: homologous recombination (HR) and classical non-homologous end-joining (cNHEJ), respectively [23,24,25,41]. Additionally we found that BRCA1 or DNA-PKc deficiency makes CML cells susceptible to synthetic lethality triggered by PARP1 inhibitors [42]. As a result of these studies, we proposed the strategy called GEMA (Gene Expression and Mutation Analysis) [42], to select leukemia patients which will be susceptible to PARP1 inhibitors. On the basis of gene and protein expression and mutation profiles analysis we have found that leukemia cells displaying BRCA1 deficiency are sensitive to treatment with PARP1 inhibitors and that combination of first line therapeutics (TKIs) with PARP1 inhibitors is much more efficient in eradication of leukemia [42].

Moreover, we found that some treatments of leukemia cells, even those which do not possess BRCA1 deficiency, may result in defective HR pathway, thus making these cells accessible for combined therapy with PARP1 inhibitors. Such combination of treatments can lead to more effective eradication of cancer cells. Imatinib-mediated inhibition of BCR-ABL1 kinase caused downregulation of RAD51 (key role in HR) and LIG4 (key role in cNHEJ) [41], what made the cells more prone to PARP1 inhibitor-mediated synthetic lethality. Altogether, we showed that imatinib-naive and imatinib-treated CML cells display specific defects in DSB repair and are sensitive to synthetic lethality induced by PARP1 inhibitors. We showed this in standard *in vitro* liquid culture conditions under normoxia as well as *in vivo* using mice xenografts [42]. Our data indicate that BRCA1 deficiency in leukemia can be even broader and BRCA1 can be downregulated also in other cases, thus making cells sensitive to PARP1 inhibitors. Stability of BRCA1 protein might be decreased [43] and novel *BRCA1* mutations can occur [44]. Interestingly, we have shown that inhibition of FLT3 (FMS-like tyros-

ine kinase 3), one of the mutations connected with occurrence of acute myeloid leukemia, leads to BRCA1 deficiency in an unknown way [45]. Even if the mechanism is not identified, we provided evidence that combination of FLT3 inhibitors and PARP1 inhibitors efficiently eliminates FLT3-positive quiescent and proliferating leukemia cells. Moreover, the combination of those drugs significantly delayed disease onset and effectively reduced leukemia-initiating cells in mouse xenograft model. Using the proposed strategy (GEMA) to analyze gene mutation and protein profiles followed by *in vitro* cell cultures and mouse models we found that inhibition of PARP1 activity evokes synthetic lethality in various types of leukemia [42,45-47]. Altogether our data clearly confirm the potential of PARP1 inhibitors in selected cohorts of leukemia patients.

LEUKEMIA MICROENVIRONMENT AND SENSITIVITY TO PARP1 INHIBITORS

Elimination of so called leukemia stem/progenitor cells, which remain in quiescent state protected by the bone marrow niche, is one of the main therapeutic challenges. The unique microenvironment created by bone marrow cells plays pivotal role in the cells resistance to therapy and eventually cancer relapse. The mechanism and signaling pathways involved in regulation of leukemia cells stemness and resistance vary dependently on the leukemia type (for review see [48,49]). In case of CML, treatment with imatinib leads to upregulation of CXCR4 receptor [50]. Stimulation of CXCR4 by a chemokine CXCL12/SDF-1 secreted by stromal cells directs CML cells migration to the bone marrow, promotes their quiescence and plays crucial role in CML resistance to therapy [50]. Later studies demonstrated, that inhibition of CXCR4 and SDF-1 interaction allows to overcome protection of leukemia cells by the stroma [51,52]. Nowadays, there are clinical trials verifying potency of inhibitors targeting CXCR4 in leukemia treatment. Similarly in AML expressing Fms-like tyrosine kinase 3 with internal tandem duplication (FLT3-ITD) stimulation of this receptor tyrosine kinase by FlT3 ligand cytokine supports drug resistance of these cells. Targeting of FLT3-ITD with a specific inhibitor AC220 overcomes this protective effect [53]. Another factor, which supports leukemia cells resistance to therapy is low oxygen level (hypoxia) in the bone marrow microenvironment. Hypoxia was demonstrated to support quiescence of leukemia cells [54]. First line therapeutics used for leukemia treatment efficiently eliminate cancer cells from the blood stream but fail to eradicate the leukemia stem cells residing in the bone marrow. It became apparent recently, that verification of novel drugs should be performed in the context of human stroma environment. In order to verify efficacy of PARP1 inhibitors in CML cells we co-cultured the cancer cells with human stromal fibroblasts under hypoxia (1% oxygen). Under such conditions the treatment sufficiently eliminated imatinib refractory cells [55].

FINAL CONCLUSIONS

BRCA1 mutations are strong cancer predispositions markers. Altogether our data showed that some types of leukemia belong to the BRCA1-deficient cancers. However the mechanisms leading to the BRCA1 deficiency and defective HR DNA repair pathway can be different, and not necessarily connected with *BRCA1* mutations. We have also shown that attenuated BRCA1 protein synthesis can be responsible for BRCA1 deficiency. We found that RNA binding proteins can be responsible for decreased translation of BRCA1 in leukemia. Additionally, some treatments can also result in BRCA1 downregulation, further increasing a cohort of BRCA1-deficient leukemia patients. We showed in *in vitro* and *in vivo* studies that BRCA1-deficient leukemias should be considered to be treated by PARP1 inhibitors, which can be used alone or with the existing therapies. Finally, we proposed a strategy to select a cohort of leukemia patients characterized with defective HR pathway and by this sensitive to PARP1 inhibitors. Thus, our studies provided strong evidence that the number of potential cancers which can be treated with PARP1 inhibitors might be higher than it is proposed today, and some types of leukemias represent BRCA1-defficient cancers.

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Deficyty BRCA1 i syntetyczna letalność w białaczkach; nie tylko mutacje mają znaczenie

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Słowa kluczowe: BRCA1, białaczka, inhibitory PARP1, syntetyczna letalność

STRESZCZENIE

BRCA1 jest jednym z głównych regulatorów stabilności genomowej w komórce. Odpowiada ze kontrolę segregacji chromosomów oraz komórek potomnych po podziale oraz reguluje naprawę dwuniciowych pęknęć DNA poprzez rekombinację homologiczną (HR). Zmiany genetyczne na poziomie genu *BRCA1*, takie jak mutacje i zmiany epigenetyczne są markerem predyspozycji w kierunku zachorowania na nowotwór piersi, jajnika i prostaty, istotnie zwiększając ryzyko wystąpienia nowotworu. Zmiany takie nie są obserwowane w białaczkach. Co ważne, deficyty BRCA1 są czynnikiem wskazującym na wrażliwość pacjentów na personalizowaną terapię inhibitorami PARP1, opartą o zjawisko tzw. syntetycznej letalności. W niniejszej pracy przedstawiamy nasze badania prowadzące do odkrycia nowego mechanizmu prowadzącego do deficytów BRCA1 w białaczkach, który nie jest związany z mutacjami lub innymi zmianami na poziomie genu, lecz z zahamowaną translacją i deficytem białka BRCA1. Zaburzenia te są efektem aktywacji komórkowej odpowiedzi na stres i kontrolowane przez białka wiążące RNA. Co więcej, wykazaliśmy, że pewne terapie jak i zmiany genetyczne w białaczkach także mogą wywołać deficyty BRCA1. Nasze badania dowiodły, że inhibitory PARP1 powinny być rozważane jako skuteczne terapeutyki w przypadku białaczek z niedoborem BRCA1, prowadząc do skutecznej eliminacji komórek nowotworu, w tym także komórek macierzystych i progenitorowych. W końcu, w wyniku naszych badań, zaproponowaliśmy strategię selekcji pacjentów z białaczkami, którzy będą wrażliwi na terapię inhibitorami PARP1.