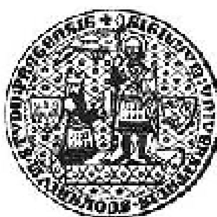
Univerzita Karlova v Praze

1. lékařská fakulta

Autoreferát dizertační práce



The functional *in vitro* analysis of the BRCA1 alternative splicing variants

Mgr. Jan Ševčík

Praha 2012

Doktorské studijní programy v biomedicíně

Univerzita Karlova v Praze a Akademie věd České republiky

Obor: Biochemie a patobiochemie

Předseda oborové rady: prof. MUDr. Jiří Kraml, DrSc

Školicí pracovište: Ústav biochemie a experimentální onkologie

Školitel: Doc. MUDr. Zdeněk Kleibl, Ph.D.

Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištené podobě na oddělení pro vědeckou činnost a zahraniční styky děkanátu 1. lékařské fakulty.

Obsah

1 INTRODUCTION - 6 -
2 THE WORKING HYPOTHESIS AND THE AIMS OF THE STUDY - 7 -
3 MATERIAL AND METHODS - 8 -
4 RESULTS - 9 -
4.1 A model system for functional analysis of BRCA1 alternative splicing variants .... - 9 -

4.2 The BRCA1Δ14-15 and BRCA1Δ17-19 splicing variants slow down the overall DDSB repair ... - 10 -

4.3 Kinetics of IRIF formation is influenced by BRCA1Δ14-15 and BRCA1Δ17-19 splicing variants after ionizing radiation-induced DNA damage .... - 10 -

4.4 The BRCA1Δ14-15 and BRCA1Δ17-19 splicing variants selectively change the sensitivity of cells to mitomycin C .... - 12 -

4.5 The activity of NHEJ is decreased in clones with modified expression of BRCA1.............. - 13 -

4.6 Overexpression of the BRCA1Δ14-15 or BRCA1Δ17-19 alternative splicing variants influences the viability and radiation sensitivity of MCF-7 cells. .... - 13 -

4.7 Overexpression of the BRCA1Δ14-15 or BRCA1Δ17-19 alternative splicing variants influences the proliferation of MCF-7 cells. .... - 15 -

5 DISCUSSION - 16 -
6 CONCLUSIONS - 18 -
7 REFERENCIES - 20 -
8 LIST OF PUBLICATIONS: - 21 -

ABSTRAKT:

Inaktivace tumor supresorového genu BRCA1 je důležitým predispozičním faktorem vzniku karcinomu prsu a ovaria. Možným mechanismem snižujícím aktivitu BRCA1 v procesu reparace dvouřetězcových zlomů DNA (DDSB) může být vedle patogenních mutací, rozsáhlých genomových přestaveb a epigenetických změn zahrnujících především hypermetylace promotorové oblasti i tvorba funkčně odlišných, nádorově specifických alternativních sestřihových variant. Alternativní (regulovaný) pre-mRNA sestřih je v mnoha případech zodpovědný za vznik proteinových izoforem s významným onkogenním potenciálem. V této práci jsme funkčně charakterizovali dvě alternativní sestřihové varianty genu BRCA1 Δ14-15 a Δ17-19, nalezené v průběhu genetického screeningu jedinců s vysokým rizikem vzniku karcinomu prsu. Pro funkční *in vitro* analýzu jsme vytvořili modelový systém klonů buněčné linie MCF-7 se stabilně modifikovanou expresí zkoumaných variant a s potlačenou expresí endogenní wtBRCA1. Pomocí comet assay a konfokální imunomikroskopie jsme v tomto systému sledovali vliv BRCA1Δ14-15 a Δ17-19 variant na kinetiku reparace DDSB a proliferaci. Aktivita DNA reparačních procesů byla stanovena přímo pomocí *in vitro* NHEJ assay a nepřímo testem senzitivity na mitomycin C. Proliferační aktivita byla stanovena klonogenním testem a růstovými křivkami. Výsledky funkční analýzy ukazují, že exprese alternativních sestřihových variant BRCA1Δ14-15 a Δ17-19 zvyšuje v MCF-7 buňkách úroveň endogenního poškození DNA, zpomaluje reparaci DDSB, negativně ovlivňuje tvorbu reparačních komplexů v počáteční fázi reparace a prodlužuje jejich trvání. Varianty BRCA1Δ14-15 a Δ17-19 rozdílně ovlivňují aktivitu HR, NHEJ a radiosensitivitu MCF-7 buněk. Vliv BRCA1Δ14-15 a Δ17-19 na DNA reparační pochody a proliferaci není v MCF-7 buňkách závislý na přítomnosti wtBRCA1 a vykazuje fenotypový projev srovnatelný se snížením exprese endogenní wtBRCA1 pomocí RNA interference. Zvýšení exprese BRCA1Δ14-15 nebo Δ17-19 má v MCF-7 buňkách dominantně negativní vliv na reparaci DDSB. Možným mechanismem působení vyšetřovaných alternativních sestřihových variant je narušení procesu vzájemné komunikace hlavních DDSB reparačních pochodů – HR a NHEJ. Důsledkem toho je ztráta flexibility procesu reparace DDSB a tím i snížení stability genomu. Výsledky naší práce ukazují, že vyšetřované sestřihové varianty nejsou v MCF-7 buňkách schopny funkčně nahradit wtBRCA1 v procesu reparace DDSB. Alternativní sestřihové BRCA1Δ14-15 a Δ17-19 negativně ovlivňují aktivitu BRCA1 v procesu udržování genomové homeostazi a mohou se tedy podílet na maligní transformaci.

ABSTRACT:

Inactivation of the tumor suppressor gene BRCA1 is a predisposing factor for a breast/ovarian cancer development. Beside the pathological mutations of BRCA1, large genomic rearrangements and epigenetic factors (mainly promoter hypermethylation), a formation of cancer-specific alternative splicing variants with aberrant biological properties can represent additional mechanism decreasing the overall BRCA1 activity in DNA double strand break (DDSB) repair. In this study, we analyzed BRCA1 alternative splicing variants BRCA1Δ14-15 and Δ17-19 ascertained previously during the screening of high-risk breast cancer individuals. We established a MCF-7 cell line-based model system of clones with stably modified expression of analyzed alternative splicing variants with coincidental RNA interference-mediated suppressed expression of endogenous wtBRCA1. We analyzed the impact of BRCA1Δ14-15 and Δ17-19 variants on DNA repair kinetics using comet assay and confocal immunomicroscopy. The capacity of DNA repair was assessed directly by an *in vitro* NHEJ assay and indirectly by a mitomycin C sensitivity test. The proliferation activities were determined by a clonogenic assay and growth curves. The result of functional analysis show, that overexpression of BRCA1Δ14-15 and Δ17-19 increases the endogenous level of DNA damage, slows down the DDSB repair, decelerates the initial phase of radiation-induced foci formation and prolongs their persistence. Moreover, BRCA1Δ14-15 and Δ17-19 differentially influence the activity of HR and NHEJ and sensitivity of MCF-7 cells to ionizing radiation. The overexpression of analyzed splicing variants exerts a phenotype comparable with RNAi mediated suppression of endogenous wtBRCA1 expression and does not depend on the presence of wtBRCA1. The overexpression of BRCA1Δ14-15 or Δ17-19 impairs the DNA repair capacity in a dominant-negative fashion in MCF-7 cells. We hypothesize, that BRCA1Δ14-15 and Δ17-19 impair the balance and communications between main DDSB repair pathways – HR and NHEJ. This leads to the loss of flexibility in DDSB repair and could contributes to the increased genomic instability. We conclude that alternative splicing variants BRCA1Δ14-15 and Δ17-19 are not able to functionally replace the wtBRCA1 in the DDSB repair process. Both analyzed variants negatively influence the BRCA1 functions in maintaining of genome homeostasis in MCF-7 cells and thus can potentially exert a malignant transformation potential

1 INTRODUCTION

BRCA1 is a large and predominantly nuclear phosphoprotein. The majority of the BRCA1 functional studies have shown that the BRCA1 protein serves as a protein-protein interaction modulator with a central position in the DNA damage signaling pathway [1]. The best described is its direct participation in the homology-directed DDSB repair [2]. Besides that, it has been proposed that BRCA1 also participates on non-homologous end joining (NHEJ) [3]. Moreover, BRCA1 participates in cell cycle and apoptosis regulation in response to DNA damage [4]. All together, functions of BRCA1 are strongly associated with the molecular response to genotoxic stress indicating the key position of BRCA1 in the maintenance of genomic integrity.

Except the constitutive splicing that results in formation of full-length mRNA, it is estimated that almost 60% of human genes undergo alternative splicing leading to the formation of different mRNA isoforms from a single primary transcript [5]. The process of alternative pre-mRNA splicing can affect the biological activity of relevant transcript by alteration of protein-coding sequences, introduction of premature stop codon, or changes in the 5' or 3' untranslated regions of mRNA.

Malignant transformation is governed by genomic DNA alterations accompanied by epigenetic insults. In cancer cells, splicing processes could be perturbated at various levels. Few examples indicated possible links between splicing control and oncogenic signaling pathways [6]. Moreover, changes in quantity of splicing factors and activity of their kinases have been documented in some cancer types [7]. These facts together with observations that many oncogenes and tumor suppressor genes are spliced into isoforms with distinct or completely different functions indicate that alterations in splicing during malignant transformation could be a part of its pathogenic mechanism.

Individuals carrying a germ-line mutation in the BRCA1 gene are predisposed to early-onset breast and/or ovarian cancer. Regarding to its tumor suppressor function, loss of BRCA1 expression causes the BRCA1-associated cancers. Beside to the inactivating mutations, large deletions, genomic rearrangements, and epigenetic changes, production of alternative splicing variants can be another mechanism altering BRCA1 mRNA and protein levels.

2 THE WORKING HYPOTHESIS AND THE AIMS OF THE STUDY

During the ongoing genetic screening program of high risk breast/ovarian cancer families, performed at the Institute of Biochemistry and Experimental Oncology, various splicing variants of the main hereditary BC predisposing gene BRCA1 have been repeatedly detected at mRNA level [8]. The absence of relevant gDNA rearrangement and a relatively broad spectrum of variants missing discrete exons indicate their origin in the mis-regulated pre-mRNA alternative splicing process.

Alternative splicing of pre-mRNA is capable to generate protein isoforms which can exert different properties and thus can markedly influence biological processes regulated by a full-length isoform [9,10]. There is a growing evidence of possible role of mis-regulated splicing in malignant transformation. However, the majority of experiments have been focused on functional analyzes of point mutations and variants resulting from aberrant mRNA splicing while products of alternative pre-mRNA splicing and their functional impact are almost unknown.

The aim of this project was to functionally analyze two BRCA1 alternative splicing variants Δ14-15 and Δ17-19 retaining the original BRCA1 open reading frame and lacking short exons in areas coding for the important BRCA1 structural motifs: a serine-containing domain (SCD) in BRCA1Δ14-15 and first of tandem BRCT domains in BRCA1Δ17-19. Both these variants have been ascertained during the screening of high risk BC individuals. Thus their detail analyses would be beneficial for understanding the role of alternative pre-mRNA splicing in malignant transformation.

The purpose of this study was:

- Establishing of a reliable system for the *in vitro* functional analysis of the BRCA1 sequence variants
- Determining of the effect of BRCA1Δ14-15 and BRCA1Δ17-19 alternative splicing variants on the DNA repair and growth properties of cells stably expressing analyzed variants.

The obtained results will be used for evaluation of the BRCA1 alternative splicing variants in the process of mammary malignant transformation.

3 MATERIAL AND METHODS

A human breast adenocarcinoma derived stable cell line MCF-7 was used as a model system for functional analysis of **selected BRCA1 sequence variants (BRCA1Δ14-15 and BRCA1Δ17-19**). Their coding sequences were constructed by **PCR-splicing approach** using a plasmid containing the entire human BRCA1 full-length coding sequence. Endogenous wtBRCA1 expression in MCF-7 cells was downregulated by **shRNA-mediated RNAi**. **Stable clones** expressing studied variants with/without coincidental downregulation of endogenous wtBRCA1 expression were selected after two steps **calcium-phosphate transfection**. Expressions of BRCA1Δ14-15, BRCA1Δ17-19, and wtBRCA1 in isolated stable clones were quantified on mRNA level by **qPCR** and on protein level by **western blotting**.

Functional analysis was initiated by estimation of overall repair capacity following ionizing radiation induced DDSB by **comet assays**. Kinetics of DNA repair complexes assemble was scored by counting the γH2AX and 53BP1 colocalizations in particular times after the γ-irradiation using a confocal **immunofluorescence microscopy**. Impairment of main DDSB repair pathways HR and NHEJ capacities were analyzed directly by an ***in vitro*** **NHEJ assay** and indirectly by **mitomycine C sensitivity test**, respectively. Influences of analyzed splicing variants on cellular growth were analyzed by **clonogenic assays** and **growth curves** under the standard cultivation conditions and following γ-irradiation.

Particular used methods:

PCR splicing approach

Transfection and selection of stable MCF-7 clones

Quantitative PCR (qPCR) and western blot

Comet assay

Confocal immunofluorescence microscopy

Mitomycin C sensitivity assay

In vitro NHEJ assay

Clonogenic assay

Proliferation – growth curves using a crystal violet staining

4 RESULTS

4.1 A model system for functional analysis of BRCA1 alternative splicing variants

The pcDNA3.1 BRCA1Δ14-15 and pcDNA3.1 BRCA1Δ17-19 expression constructs were prepared by a PCR splicing approach with a direct ligation into the expression pcDNA3.1 Hygro vector. A stable human breast adenocarcinoma-derived cell line MCF-7 expressing BRCA1 +/+ was used as an *in vitro* model system. With respect to the further experimental purposes, clones with expression constructs fully integrated into the MCF-7's genome ensuring stable expression of BRCA1 variants were prepared. The calcium-phosphate transfection with subsequent long term selection was chosen as reliable and reproducible method. Cells were first transfected by a pcDNA3.1 Hygro-based expression constructs enabling the overexpression of studied BRCA1 alternative splicing variants with endogenously expressed wtBRCA1. To assess the biological effect of studied BRCA1 alternative splicing variants (BRCA1Δ14-15 and BRCA1Δ17-19) solely, MCF-7 clones combining the expression of analyzed BRCA1 splicing constructs together with a shRNA-mediated downregulation of endogenous wtBRCA1 were prepared. A highly specific downregulation of expression of endogenous wtBRCA1 only was achieved by a human H1 promoter-driven expression of interfering shRNAs targeting the BRCA1 coding sequences lacked in the examined BRCA1 splicing variants. To rule out the nonspecific effect of transfection procedure and construct's integration into the host genome, three stable MCF-7 clones of each engineered expression construct were further examined. The stable MCF-7 clones expressing used shRNAs only, irrelevant shRNAs (targeted to mouse CEBPγ), empty pSUPER and pcDNA3.1 vectors, and non-transfected MCF-7 cells were used as controls for functional analyses.

The integration into the MCF-7 genome was proved by a PCR using gDNA as a template. Results of RT-PCR, qPCR and WB analysis in the stably transfected clones in the two consecutive passages proved functionality of the model system and temporal stability of the modification of BRCA1 expression on both mRNA, and protein levels respectively. By the interfering shRNA, the expression of endogenous wtBRCA1 mRNA was downregulated up to 10% of its normal level. The total BRCA1 expression in the combined clones was 95% (in the case of BRCA1Δ14-15; normalized to the GAPDH), and 80% (in the case of BRCA1Δ17-19; normalized to the GAPDH) of the control MCF-7 cells BRCA1 expression level, respectively. The modification of BRCA1 expression on the mRNA level correlated directly with the results of quantitative WB.

4.2 The BRCA1Δ14-15 and BRCA1Δ17-19 splicing variants slow down the overall DDSB repair

Both protein structural motifs, the SCD lacking in BRCA1Δ14-15, and first of tandem BRCT lacking in BRCA1Δ17-19, were shown to be important determinants of BRCA1 activity in the DNA repair process. With regard to that, we first examined whether the expression of these BRCA1 alternative splicing variants influences the DNA repair capacity in MCF-7 cells. A time course of DNA damage level was directly scored by a comet assay after a single 1.5 Gy dose of γ-radiation in 15, 30, 60, and 120 minutes post irradiation (PI) respectively.

In all analyzed cells, the peak DNA damage was detected at the time of 15 minutes PI with significant differences in the rate of DNA damage between controls and clones with modified BRCA1 expression. In control cells, the degree of the DNA damage decreased progressively over the further analyzed time period reaching the level comparable to the initial DNA damage rate in the time 120 min PI. In the clones with modified BRCA1 expression, the DNA repair velocity was slower though the differences in the degree of DNA damage between controls and clones with modified BRCA1 expression decreased during the examined time period. However, at the time of 120 min PI, the DNA damage rate was still significantly higher in all clones with modified BRCA1 expression compared with controls. There were not detected significant differences in the DNA repair time course between clones with downregulated expression of wtBRCA1 and clones with overexpressed BRCA1Δ14-15 and BRCA1Δ17-19 alternative splicing variants.

These results showed that both downregulation of wtBRCA1 or overexpression of BRCA1Δ14-15 or BRCA1Δ17-19 splicing variants slows down the DDSB repair particularly in the initial phase after the DNA damage. Moreover, the presence of either of examined BRCA1 splicing variants interferes with the activity of wtBRCA1 in a dominant-negative fashion.

4.3 Kinetics of IRIF formation is influenced by BRCA1Δ14-15 and BRCA1Δ17-19 splicing variants after ionizing radiation-induced DNA damage

Assuming the main role of BRCA1 as a central protein-interaction modulator in orchestration of the DNA damage response, we next examined whether the BRCA1Δ14-15 or BRCA1Δ17-19 splicing variants influence the kinetics of IRIF formation by scoring the *in vitro* co-localization of γH2AX with 53BP1 using the immunofluorescence confocal microscopy. The number of γH2AX/53BP1 foci was

determined in different times after the same single dose of 1.5 Gy of γ-radiation as in the previous experiment and in non-irradiated clones. As all analyzed clones originated from the identical MCF-7 line, it could be expected that the γ-irradiation induces an identical initial number of DDSBs.

Under the standard cultivation conditions, the rate of endogenous DNA damage was higher in clones with downregulated expression of wtBRCA1, in clones expressing the alternative splicing variants BRCA1Δ14-15 or BRCA1Δ17-19 variants in comparison with controls. This suggests that low expression of full-length BRCA1 as well as overexpression of BRCA1Δ14-15 or BRCA1Δ17-19 slightly increases the level of endogenous DNA damage and could thus contribute to the genome instability in model MCF-7 clones.

Following the γ-irradiation, the maximal number of γH2AX/53BP1 foci in controls was detected at the time of 5 minutes PI. A dissociation of γH2AX/53BP1 foci was apparent since that time as the number of foci progressively decreased over the entire analyzed time period. In MCF-7 clones expressing the BRCA1Δ14-15 or BRCA1Δ17-19 variant with coincidentally downregulated expression of wtBRCA1, the number of IRIFs further increased at the time beyond 5 minutes PI reaching the peak values at the time of 30 minutes PI. In all clones with modified expression of BRCA1, the number of IRIF successively decreased since the time of 30 minutes PI, however, the initial delay (compared with control cells) was sustained throughout the overall analysis period. The slowest decomposition of γH2AX/53BP1 foci was registered in the clones expressing BRCA1Δ17-19 variant. The difference between the number of persisted IRIF in clones expressing BRCA1Δ17-19 variant, other clones with modified BRCA1 expression, and control cells was most apparent at 24 hours (1440 min) PI.

The majority (more than 80%) of the DNA lesions is repaired within first 120 minutes PI in control cells. The rest of the persisted IRIF is repaired within subsequent 1220 minutes. From the IRIF kinetics point of view, the DDSB repair can be thus subdivided into two main phases. i) The rapid one, represented by the fast dismantle of IRIF within the first 120 minutes PI, and ii) the slow one, represented by a deliberate decomposition of the rest of persisted IRIF. The rapid phase of the DDSB was significantly harmed in all clones with BRCA1 modified expression as the number of IRIF increased after the 5 minutes PI, reaching its peak value at 30 minutes PI. In cells with downregulated expression of wtBRCA1 or overexpressed BRCA1 splicing variant BRCA1Δ14-15, the kinetic of IRIF dismantle correspond to the control cells after the 30 minutes PI. In cells with overexpressed BRCA1 splicing variant BRCA1Δ17-19 variant, the kinetics of IRIF decomposition was slowest without any different phases apparent in control cells.

These results show that either downregulation of wtBRCA1 or overexpression of any of studied BRCA1 splicing variants impairs the kinetics of formation of IRIF compared with controls. In either of BRCA1Δ14-15 or BRCA1Δ17-19 splicing variant, the effect on slower IRIF formation was independent on the presence of wtBRCA1 indicating that neither of analyzed splicing variants is able to functionally substitute for wtBRCA1 in the process of the γH2AX/53BP1-containing DNA repair protein complexes formation. In concord with the previous comet assays, the most obvious difference in the DDSB level between control and clones with modified expression of BRCA1 appears at the time 30 minutes PI. The kinetics of IRIF decomposition was significantly decreased during the first rapid phase (within the first 120 minutes PI) in the clones with modified expression of BRCA1, while the second phase was comparable to the controls. The most prominent impairment of IRIF decomposition was seen in BRCA1Δ17-19 variant suggesting the ultimate importance of BRCA1 BRCT domain-mediated protein/protein interaction for DDSB repair. Finally, our results support the evidence about the importance of BRCA1 on the process of IRIF formation during both initial as well as delayed phases of DDSB repair.

4.4 The BRCA1Δ14-15 and BRCA1Δ17-19 splicing variants selectively change the sensitivity of cells to mitomycin C

It has been documented, that BRCA1 protein directly participates on the process of HR. Mitomycin C causes DNA interstrand covalent cross-links repaired exclusively by HR. Thus, the sensitivity of cells to mitomycin C treatment indirectly reflects the HR capacity. With respect to the previous results, we further examined the response of MCF-7 clones with a modified expression of BRCA1 to the different concentrations of mitomycin C on the proliferation level.

Based on the results of growth curves and the EC-50 calculated from a dose response curve, we concluded that clones with a shRNA-downregulated expression of wtBRCA1 were the most sensitive to mitomycin C as the lowest used mitomycin C concentration (2 μg/ml) was closed to lethal. Significantly increased sensitivity to mitomycin C was also observed in cells expressing the BRCA1Δ17-19 splicing variant alone or together with wtBRCA1. On the contrary, the sensitivity of clones expressing the BRCA1Δ14-15 splicing variant alone or together with wtBRCA1 was comparable with controls.

These results are consistent with previously published findings that a downregulation of wtBRCA1 impairs the HR pathway and causes hypersensitivity to the DNA cross-linking agents. A similar

phenotype exerts the cells expressing variant BRCA1Δ17-19 lacking first BRCT domain. On the other hand, the BRCA1Δ14-15 splicing variant did not influence mitomycin C sensitivity. This suggests that unlike the depletion of wtBRCA1, or overexpression of the BRCA1Δ17-19, the BRCA1Δ14-15 alternative splicing variant does not substantially impair the HR.

4.5 The activity of NHEJ is decreased in clones with modified expression of BRCA1

Negative effect of BRCA1 inactivation on capacity and fidelity of DDSB repair has been described in both pathways - HR and NHEJ. Hence, after finding that the cells expressing BRCA1 alternative splicing variants have decreased DNA repair capacity, however, exert differential sensitivity to the DNA cross-linking, we further analyzed the influence of the analyze BRCA1 alternative splicing variants on NHEJ. We used the indirect *in vitro* assay based on measurement of the lucipherase activity recovery from pGL-control expression vector linearized prior to its transfection to model clones.

The activity of both overall and precise NHEJ was significantly lower in all examined clones with downregulated expression of wtBRCA1, and expression of BRCA1Δ14-15 or BRCA1Δ17-19 splicing variants compared with controls.

Though the activity of precise NHEJ was reduced in clones with modified expression of BRCA1, the decrease was proportional as the ratio of overall to precise NHEJ was not significantly distinct from controls. In all clones the precise NHEJ ranged between 20% to 25% of the overall NHEJ activity. The only exception constituted clones expressing the BRCA1Δ17-19 splicing variants with or without coincidentally downregulated endogenous wtBRCA1. The precise NHEJ accounted only 10% of overall NHEJ activity and was thus decreased in comparison with controls and other analyzed clones.

4.6 Overexpression of the BRCA1Δ14-15 or BRCA1Δ17-19 alternative splicing variants influences the viability and radiation sensitivity of MCF-7 cells.

After finding that BRCA1 alternative splicing variants decrease the efficiency of DDSB repair, we further examined whether modifications of the BRCA1 expression can influence vital and growth characteristics of MCF-7-based stable clones. A clonogenic assay was used to evaluate the possible

effect of the BRCA1Δ14-15 or BRCA1Δ17-19 alternative splicing variants on the viability and radio-sensitivity of MCF-7 clones.

Cells of clones with modified BRCA1 expression were treated by a single dose of ionizing γ-radiation (1 and 5 Gy) and seeded in a dilution ensuring the formation of single cell colonies. After 10 days of cultivation, the plating efficiency (PE) and surviving fraction (SF) of cells were determined by a crystal violet staining. In this assay, the plating efficiency (number of colonies containing more than 10 cells) reflects the viability of particular clones under the standard conditions and upon γ-radiation DNA damage, while the average number of cells per colony is an indirect marker of the cell proliferation and radiation sensitivity.

Under the standard cultivation conditions, the highest PE was scored in the control non-transfected MCF-7 cells and in the cells transfected by an empty expression vector. Compared with that, the PE of clones with stably modified expression was generally lower. Cells expressing the BRCA1Δ14-15 alternative splicing variant had the lowest PE in comparison with other analyzed clones.

The MCF-7 clones with stably downregulated expression of endogenous BRCA1 had the lowest PE in both used γ-radiation doses as well as the highest decrease of PE in relation to the standard cultivation conditions. The decrease of PE of the cells expressing the BRCA1Δ14-15 was comparable with controls. On the contrary, the PE of BRCA1Δ17-19 cells treated with γ-radiation was highest and the decrease in its rate lowest from all analyzed clones and controls.

Results of SF were in consistence with that of PE for all analyzed clones and controls which responded to ionizing radiation-induced DNA damage by the decrease in the SF of cells. However, the relative SF upon γ-radiation treatment was highest in clones expressing the BRCA1Δ17-19 alternative splicing variant and lowest in the clones with stably downregulated expression of endogenous wtBRCA1. The relative SF of cells with upregulated expression of BRCA1Δ14-15 alternative splicing was comparable with controls after the γ-radiation treatment.

These results showed that downregulation of endogenous wtBRCA1 significantly decrease the clonal viability while increasing the radiation sensitivity of MCF-7 cells. The overexpression of the BRCA1Δ17-19 alternative splicing variant increases the radioresistance of MCF-7 clones on both PE and SF levels, while the overexpression of the BRCA1Δ14-15 alternative splicing variants leads to slight increase in radio-sensitivity of MCF-7 cells. These results indicates, that expression of distinct BRCA1 alternative splicing variants can differentially influence the viability and radio-sensitivity of MCF-7 cells under the DNA damaging conditions.

4.7 Overexpression of the BRCA1Δ14-15 or BRCA1Δ17-19 alternative splicing variants influences the proliferation of MCF-7 cells.

After the finding that modification of BRCA1 expression can influence the viability of MCF-7 clones, we further directly examined the proliferation activity of clones under the standard cultivation conditions and upon the different rate of γ-radiation-induced DNA damage by an independent assay. To prove that the possible change in the proliferation activity is attributed to BRCA1 and its downstream but not upstream signaling, the cells were cultivated with caffeine, a potent inhibitor of the BRCA1 upstream activator ATM, prior to irradiation in a parallel experiment.

Growth curves of clones with modified expression of BRCA1 irradiated by a single dose of ionizing radiation showed different proliferation tendencies in comparison with controls. As supposed from previous experiment, the control non-transfected MCF-7 cells responded to the irradiation by the decrease in a proliferation rate proportionally to the γ-radiation dose. The slope of growth curves of control MCF-7 cells showed that after the initial proliferation deceleration (caused by the DNA damage), the cells grew with the proliferation rate comparable to the non-irradiated ones. Compared to that, clones with downregulated expression of endogenous wtBRCA1 and cells expressing the BRCA1Δ14-15 alternative splicing variant were more sensitive to the ionizing radiation showing markedly decreased proliferation rate during whole time period. On the contrary, cells expressing the BRCA1Δ17-19 alternative splicing variant exerted an increased radio-resistance.

The results of parallel experiment with the same set of clones from the identical passage, cultivated under the same conditions but treated with the caffeine showed, that inhibition of ATM causes hypersensitivity of cells to the DDSB with a total abrogation of proliferating activity. The proliferation tendencies were comparable within all analyzed clones and control cells irrespectively to the BRCA1 expression modification.

Overall results of proliferation assays, consistent with the result of clonogenic assay, showed that modulation of BRCA1 expression can differently influence the proliferation of MCF-7 cells upon the ionizing radiation-induced DNA damage. The altered proliferation capacity of particular clones was independent on the presence of the endogenous wtBRCA1 which indicates the dominant effect of BRCA1Δ14-15 and BRCA1Δ17-19 alternative splicing variants. Moreover, the observed shift in the proliferation rate in MCF-7 clones was completely abolished by a caffeine-mediated ATM inhibition and is thus not caused by the superordinate DDSB signaling pathway, but it is most likely the specific response to modified expression of BRCA1.

5 DISCUSSION

In this study, we focused on a detail characterization of the effect of BRCA1Δ14-15 and BRCA1Δ17-19 alternative splicing variants on the DDSB repair. The variants were selected for functional analyzes due to their repeated ascertainment during the screening program of high-risk breast cancer individuals. Both Δ14-15 and Δ17-19 alternative splicing variants retain the original BRCA1 reading frame while lacking several short N-terminal exons coding for portions of structural domains – the serine-containing domain (SCD) in BRCA1Δ14-15, and first BRCT domain in BRCA1Δ17-19. These protein motifs were previously described as important determinants of BRCA1 function within DDSB repair pathways. Translation of these sequence variants can lead to the production of protein with altered biological properties with a relevant impact on the BRCA1-mediated processes.

Our results showed that depletion of wtBRCA1 in MCF-7 cells slightly increased the level of endogenous DDSBs, decelerated the initial IRIF assembling after the γ-radiation-induced DNA damage, slowed down the IRIF disassembling, and prolonged their persistence. A retarded formation of γH2AX/53BP1-containing IRIF correlated directly with slower DDSB repair. Moreover, the cells with downregulated expression of wtBRCA1 were increasingly radiosensitive and less viable in comparison with controls. The similar, but not same, phenotypic pattern exerted the cells with overexpressed BRCA1Δ14-15 or BRCA1Δ17-19 alternative splicing variant.

We hypothesized, that the overexpression of the BRCA1Δ14-15 variant leads to preferential usage of homology-directed DDSB repair in MCF-7 cells while the expression of BRCA1Δ17-19 compromise both the NHEJ and HR with substantial impact on precise NHEJ and final steps of HR. In our study, we worked with non-synchronized population of cells where most cells were resting in G1 cell cycle phase. Thus, it can be assumed that the majority of DDSB repair after irradiation by the ionizing γ-radiation relayed on the NHEJ pathway. A block of NHEJ pathway caused by either downregulation of wtBRCA1, or overexpression of BRCA1Δ14-15 or BRCA1Δ17-19 leads to the functional switch to alternative DDSB repair pathway. This causes that DDSB initially determined to undergo NHEJ must be redirected to HR. Homology-directed repair of DDSB is relatively slow process comparing to the NHEJ . Assuming that,"confusion" in the initial decision-making process leads to an accumulation of stalled NHEJ IRIF until triggering of HR. This produces a delay observed during the initial formation of IRIF within 30 minutes PI in our model cells.

The expression of BRCA1Δ14-15 does not change the sensitivity to mitomycin C which indicates that HR is sufficiently active to deal with radiation-induced DNA damage. This is supported by the finding

that the number of IRIF at the time 24 hours PI is comparable to the initial endogenous DNA damage level in cells expressing the BRCA1Δ14-15 alternative splicing variant. On the other hand, the expression of BRCA1Δ17-19 increases sensitivity to mitomycin C which indicates that HR is impaired. Because the number of IRIF progressively decreased during the time PI we hypothesize that the problem relies to the later phases of HR, probably during the final dismantle of IRIF. This is in consistence with previously described finding that the inability to finalize HR properly leads to hyperrecombination and hypersensitivity to DNA cross linking agents [11].

Recently, it has been described that BRCA1 is an important factor ensuring the proper and well-timed IRIF dismantle preventing the undesirable crossing-over events [12]. Though the mechanism is not fully understood yet, it is known that both RING finger and BRCT protein-interaction motifs of BRCA1 are indispensable for this process [13]. In consistence with this hypothesis is a finding that cells expressing the BRCA1 with mutation in BRCT domain exert hyperrecombination and genomic instability [14]. Thus, the formation of specific BRCA1-containing complexes seems to be a clue to the BRCA1 function in a DNA repair. Structural changes in the BRCA1 protein-interaction modules can influence its binding capacity. Moreover, the majority of protein-protein interactions in the process of DNA repair take place in the phosphorylation- or ubiquitin-dependent manner. Upon DNA damage, specific BRCA1 serine residues are phosphorylated by upstream kinases, which could in turn regulate the BRCA1 binding specificity [15]. The phosphorylation status was shown to be an important factor influencing the BRCA1 intracellular localization and trafficking [16]. The availability of the BRCA1 protein at the site of its requirement can thus influence the formation of specific complexes. The exons 14 and 15 deleted in analyzed variant BRCA1Δ14-15 contain six serine residues known to be targets of ATM upon DNA damage stimulation. This indicates, that phosphorylation of these serine residues is a part of DNA damage-induced reaction network which governs the activity of BRCA1 to the exact place in the DNA repair pathway. Thus, the BRCA1Δ14-15 variant, lacking the substantial part of SCD, results in formation of protein isoform with impaired sensitivity to the DNA damage up-stream signaling resulting in impaired DDSB repair as indicated by our results. Similarly to that, BRCA1Δ17-19, lacking the substantial part of first BRCT domain, has probably altered protein-binding capacity. So, even properly localized BRCA1Δ17-19 cannot elicit the relevant protein-protein interactions and blocks the step which is dependent on this interaction in a dominant-negative manner. The DDSB were repaired even in cells with the downregulated BRCA1 to a low level. This indicates that BRCA1 is not absolutely indispensable for the process of DNA repair and rather enhances then enables this process in general. However, a delayed processing of potentially highly dangerous DNA lesions can contribute to a genomic instability. An increased number of endogenous IRIF testify for this assumption.

6 CONCLUSIONS

The BRCA1 alternative splicing variants have been repeatedly ascertained during the ongoing screening of high breast cancer risk individuals. Their relatively frequent occurrence and discrete changes in structurally and functionally important BRCA1 protein domains suggest a different biological activity to the full-length BRCA1 isoform. This work is primarily focused on the establishment of a model system that will allow to functionally characterize sequence variants of *BRCA1* gene, described during the population specific screening of high breast and/or ovarian risk individuals in Czech Republic, and to assess their possible relevance in the process of malignant transformation. Regarding to the working hypothesis we summarize the crucial results of the functional *in vitro* analysis of the BRCA1 alternative splicing variants:

- Assuming the limits of cancer cell line, stable transfection and expression modification, the MCF-7-based clones combining the PCR splicing approach and stable overexpression of studied BRCA1 sequence variants with coincidental RNAi-mediated downregulation of endogenous wtBRCA1, constitutes a **reliable model system for the functional *in vitro* analysis**.

- **Overexpression of BRCA1 Δ14-15 or Δ17-19 increases the degree of endogenous DNA damage and decreases the overall DDSB repair capacity**. This effect does not depend on the presence of endogenous wtBRCA1 and exerts phenotypic signs comparable to RNAi-mediated depletion of endogenous wtBRCA1in MCF-7 cells.

- **Overexpression of BRCA1 Δ14-15 or Δ17-19 decelerates the assembly of γ-radiation-induced γH2AX/53BP1 foci at the initial phases of DDSB repair and prolonged their persistence**.

- Stable clones expressing BRCA1 Δ17-19 and clones with downregulated expression of endogenous wtBRCA1, but not clones with overexpressed BRCA1 Δ14-15 exert hypersensitivity to mitomycin C. This result shows that **BRCA1 Δ17-19 and BRCA1 Δ14-15 differentially impair HR**. Both **alternative splicing variants** as well as downregulation of endogenous wtBRCA1 significantly **decrease overall NHEJ capacity**.

- **The overexpression of BRCA1 Δ14-15 or Δ17-19 increases the viability and clonogenic potential of MCF-7 cells**.

- The downregulation of endogenous wtBRCA1 and upregulation of BRCA1 Δ14-15 causes the radiation sensitivity while the upregulation of BRCA1 Δ17-19 cause a radiation resistance of MCF-7 cells.

The results of our study indicate, that the BRCA1 alternative splicing variants Δ14-15 and Δ17-19 are not able to functionally replace the full-length BRCA1 in a γ-radiation induced DDSB repair process in MCF-7 cells. Both variants exert a dominantly-negative effect on the DDSB repair, cell viability and clonogenic potential. The overall decrease of DDSB repair capacity caused by the overexpression of BRCA1 Δ14-15 or Δ17-19 could be a factor negatively influencing the genomic stability. This together with disregulated proliferation of MCF-7-based clones could contribute to the malignant transformation.

Hence, studied BRCA1 alternative splicing variants could potentially negatively influence the BRCA1 tumor suppressive activity indicating that alternative pre-mRNA splicing may represent an important regulatory pathway contributing to malignant transformation

7 REFERENCIES

Reference List

1. R.Roy, J.Chun, S.N.Powell, Nat. Rev. Cancer. 12 (2011) 68-78.
2. B.Wang, Cell Biosci. 2 (2012) 6-
3. D.T.Bau, Y.C.Mau, C.Y.Shen, Cancer Lett. 240 (2006) 1-8.
4. C.X.Deng, Nucleic Acids Res. 34 (2006) 1416-1426.
5. J.M.Johnson, J.Castle, P.Garrett-Engele, Z.Kan, P.M.Loerch, C.D.Armour, R.Santos, E.E.Schadt, R.Stoughton, D.D.Shoemaker, Science. %19;302 (2003) 2141-2144.
6. C.Cheng, M.B.Yaffe, P.A.Sharp, Genes Dev. 20 (2006) 1715-1720.
7. M.T.Pind, P.H.Watson, Breast Cancer Res. Treat. 79 (2003) 75-82.
8. P.Pohlreich, J.Stribrna, M.Zikan, J.Novotny, (2006) p260–
9. A.Garcia-Sacristan, M.J.Fernandez-Nestosa, P.Hernandez, J.B.Schvartzman, D.B.Krimer, Cell Res. 15 (2005) 495-503.
10. A.Ghosh, D.Stewart, G.Matlashewski, Mol. Cell Biol. 24 (2004) 7987-7997.
11. M.E.Moynahan, T.Y.Cui, M.Jasin, Cancer Res. 61 (2001) 4842-4850.
12. L.J.Barber, J.L.Youds, J.D.Ward, M.J.McIlwraith, N.J.O'Neil, M.I.Petalcorin, J.S.Martin, S.J.Collis, S.B.Cantor, M.Auclair, H.Tissenbaum, S.C.West, A.M.Rose, S.J.Boulton, Cell. 135 (2008) 261-271.
13. L.J.Barber, S.J.Boulton, DNA Repair (Amst). 5 (2006) 1499-1504.
14. S.M.Dever, S.E.Golding, E.Rosenberg, B.R.Adams, M.O.Idowu, J.M.Quillin, N.Valerie, B.Xu, L.F.Povirk, K.Valerie, Aging (Albany. NY). 3 (2011) 515-532.
15. J.N.Glover, R.S.Williams, M.S.Lee, Trends Biochem. Sci. 29 (2004) 579-585.
16. S.Okada, T.Ouchi, J. Biol. Chem. 278 (2003) 2015-2020.

8 LIST OF PUBLICATIONS:

The BRCA1 alternative splicing variant Δ14-15 with an in-frame deletion of part of the regulatory serine-containing domain (SCD) impairs the DNA repair capacity in MCF-7 cells. **Sevcik J**, Falk M, Kleiblova P, Lhota F, Stefancikova L, Janatova M, Weiterova L, Lukasova E, Kozubek S, Pohlreich P, Kleibl Z. Cell Signal. 2012 May;24(5):1023-30. Epub 2012 Jan 3. **IF = 4.058**

Influence of dihydropyrimidine dehydrogenase gene (DPYD) coding sequence variants on the development of fluoropyrimidine-related toxicity in patients with high-grade toxicity and patients with excellent tolerance of fluoropyrimidine-based chemotherapy. Kleibl Z, Fidlerova J, Kleiblova P, Kormunda S, Bilek M, Bouskova K, **Sevcik J**, Novotny J. Neoplasma. 2009;56(4):303-16. **IF = 1.192**

The CHEK2 gene I157T mutation and other alterations in its proximity increase the risk of sporadic colorectal cancer in the Czech population. Kleibl Z, Havranek O, Hlavata I, Novotny J, **Sevcik J**, Pohlreich P, Soucek P. Eur J Cancer. 2009 Mar;45(4):618-24. Epub 2008 Nov 6. IF = **4.121**

Long-term BRCA1 down-regulation by small hairpin RNAs targeting the 3' untranslated region. Vondruskova E, Malik R, **Sevcik J**, Kleiblova P, Kleibl Z. Neoplasma. 2008;55(2):130-7. **IF = 1.179**

Establishment, growth and in vivo differentiation of a new clonal human cell line, EM-G3, derived from breast cancer progenitors. Brozova M, Kleibl Z, Netikova I, **Sevcik J**, Scholzova E, Brezinova J, Chaloupkova A, Vesely P, Dundr P, Zadinova M, Krasna L, Matouskova E. Breast Cancer Res Treat. 2007 Jun;103(2):247-57. Epub 2006 Oct 25. **IF = 4.453**

RNA regulation and cancer development. Scholzová E, Malík R, **Sevcík J**, Kleibl Z. Cancer Lett. 2007 Feb 8;246(1-2):12-23. Epub 2006 May 3. Review. **IF = 3.398**

Dipeptidyl peptidase-IV activity and/or structure homologues (DASH) in transformed neuroectodermal cells. Malík R, Busek P, Mares V, **Sevcík J**, Kleibl Z, Sedo A. Adv Exp Med Biol. 2003;524:95-102.

Total impact factor: **18.401**

Presentations: