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The Impact of M1775K Variant in *BRCA1* Regulation

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Abstract

The missense mutations of the BRCT domain of human *BRCA1* have been argued to be associated with predisposition to hereditary breast/ovarian cancer. Mutations at the C-terminus of the protein are critical for function and structural excellence. This mutation has been associated with changes in the structure of the protein and loss of interaction with other peptides involved in cell cycle control. In previous studies we have shown that the modification of various mutants in BRCT domain, such as Met1775K is strongly affecting the functional and structural integrity of the domain and the binding affinity with phospopeptides.

In the present study we have attempted to determine the effect of the M1775K mutation on the intracellular localization of the protein. For this purpose, GFPBRCA1-BRCT M1775K mutant was created and expressed in MCF-7 cell lines. Due to fluorescence microscopy determined that the mutation affects the protein's mislocalization in relation to wt*BRCA1*-BRCT protein. Mutant EGFP-*BRCA1*-BRCT M1775K form is mainly determined in the cytoplasm in contrast to the EGFPwt*BRCA1*-BRCT protein that localizes into nucleus and cytoplasm.

Cytoplasmic mislocalization of M1775K mutation, presented here, may be related to disruption of *BRCA1*-BRCT folding. The M1775K mutation influences the nuclear transport of *BRCA1*-BRCT domain to the nucleus, suggesting that the impact of the integrity of the *BRCA1*-BRCT domain is crucial in functional and structural level.

Keywords: Cancer; Phospopeptides; Mutation

Introduction

Breast cancer gene, *BRCA1*, is one of the well-studied genes. Many mutations of *BRCA1* have been identified, several of which are located in the *BRCA1*-BRCT domain. The particular functional significance of the *BRCA1*-BRCT region may be determined by involvement of cell cycle control and interaction with other proteins such as BRIP1 and DNA helicase BACH1 [1].

M1775K is a rare variant of the *BRCA1*-BRCT domain appears to be critical for *BRCA1* function and structural integrity. The M1775K mutation disrupts the pocket phosphopeptide binding of the *BRCA1* BRCT domain, thereby inhibiting the *BRCA1* interaction with the BRIP1 and CtIP proteins. These results indicate that the integrity of BRCT is critical to *BRCA1* function. *BRCA1* has two domains involved in protein-protein interactions which are often mutated in human cancers: the N terminal RING domain and the two BRCT sequential domains at the C-terminal end of the protein. The structure of the carboxy terminal domain of *BRCA1* consists of two identical BRCT repeats each containing 90 amino acids [2,3].

Each repeat of BRCT folds forming a parallel quadrilateral beta sheet located in the central part of the field, surrounded by three α-helices. The C-terminal BRCT regions are believed to mediate *BRCA1* transcriptional activity [2,3]. The most frequently detected mutations occur within the BRCT domains and have been reported to affect the *BRCA1* nuclear functions, including DNA repair [1] and transcriptional activity [4]. It has previously been shown that BRCT mutations: (i) have affected field folding *in vitro* to varying degrees depending on the induced destabilization; and (ii) have abrogated binding affinity of the BRCT region with synthetic phosphopeptides such as pBACH1/BRIP1 and pCtIP, affecting the structural integrity of the active BRCT sites involved in interaction with the partner molecules [5,6].

While the structural implications of many *BRCA1*-BRCT protein mutants have already been studied, the effect of these mutations on the localization of the protein at the cellular level has been determined only for a few of them [7]. The *BRCA1* mutations may alter *BRCA1* localization and restrict the protein out of the core. Two of the C-terminal mutations (M1775R and Y1853X) restrict nuclear localization disrupt the folding of the C-terminal of *BRCA1* [7-9], suggesting that the conformation changes they cause may be detrimental to *BRCA1* nuclear transport [6,10].

Methods

Mutagenesis and plasmid construction

The EGFP-*BRCA1* fusion construct containing the full length *BRCA1* cDNA [11,12] in-frame with an EGFP-containing vector (pEGFP-C3, Clontech Laboratories, Inc.), was kindly provided by Dr. Ody Sibon [11]. To assess the impact of *BRCA1* cancer mutation on subcellular localization used PCR-based mutagenesis to create *BRCA1* mutant cDNA corresponding to the detected M1775K *BRCA1*-BRCT mutation. Mutagenesis was performed by the system QuickChange (Stratagene), according to the manufacturer's instructions. The variant

in pEGFP-C3 -*BRCA1* demonstrated by site-directed mutagenesis. Then inserted by substituting restriction fragments into wild-type *BRCA1* by using appropriate restriction enzymes (pEGFP-C3-*BRCA1*wt). The mutant's integrity was determined by DNA sequencing.

Cell culture and transfection conditions

MCF-7 human breast cancer cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal calf serum (FCS) and grown at 37°C in a humidified 5% CO_2 atmosphere. Cells were transfected with the eukaryotic expression vector pEGFP-C3 carrying the *BRCA1*-BRCT cDNA (mutant and wt) fused to EGFP. Cells were transfected at about 70% confluency (optical) with 1-2 μg of plasmid DNA per well of 6 well plate, using Lipofectamine Reagent (Life Technologies), according to the manufacturer's instructions. Transfected cells were cultured for 16 h post-transfection and subsequently analyzed by immunofluorescence.

Immunofluorescence microscopy

Cells expressing EGFP-tagged protein were fixed in 4% Paraformaheldyde in PBS for 20 min at room temperature, washed three times in PBS, stained with the DNA binding dye Hoechst 33285 (Sigma) (cell nuclei staining), washed in PBS, followed by a final wash in ddH_2O and then mounted in fluorescence preservative solution for direct detection of the EGFP fluorescence.

Results

Subcellular localization of fusion EGFP-*BRCA1*-BRCT mutant and wt*BRCA1*

In order to assess the proteins mislocalization BRCT fused to GFP (GFP*BRCA1*- BRCT-M1775K mutant) which inserted in MCF-7 human breast cancer cells. The expressed fusion proteins were detected by fluorescence microscopy and the transfection efficacy was about 30%. The EGFP-*BRCA1*-wild type seems to have nuclear-cytoplasmic distribution. Based on these observations, examined the effect of *BRCA1*-BRCT M1775K cancerrelated mutation in the protein's subcellular localization and its nuclear entry capacity. Based on its location, the selected BRCT mutation is located at crucial region of the BRCT domain function.

M1775K is located, at the interface region directly affecting the BACH1/BRIP1 and CtIP phosphopeptide binding pocket as we have already mentioned. In accordance with previously reported findings, the calorimetric results support the hypothesis that thermal unfolding mechanisms mainly involve the hydrophobic inter-BRCT-repeat interface [5,6]. The M1775K missense variant is referred not to bind the synthetic phosphopeptides pBACH1/BRIP1 or pCtIP.

Structural analysis of the interatomic interactions of Lys1775 shows a direct clash of its side chain with Phe13 of either phosphopeptide, a result arising from the disruption of the BRCT-phosphopeptide binding pocket [5]. Based on these results the potential effect of mutant in the sub-cellular localization of the protein determined by using a construct expression of *BRCA1* fused to EGFP in MCF-7 breast cancer epithilioma cells. As expected from structural and biochemical results, M1775K localized mostly in the cytoplasm in all cells examined, regardless cell cycle phase (dividing cells) whereas *wtBRCA1* detected in cytoplasm and the nucleus (Figure 1).

Discussion

Mutant M1775K is crucial for BRCT domain integrity and protein's localization. This residue is located near to the binding site with CtIP and BACH1, at the interface region of BRCT and affects through hydrophobic interactions the structural and functional integrity of the domain [6,13]. Fusion EGFP-*BRCA1* protein M1775K is localized from the nucleus to the cytoplasm, demonstrating a similar impact on nuclear localization with the mutation M1775R [8].

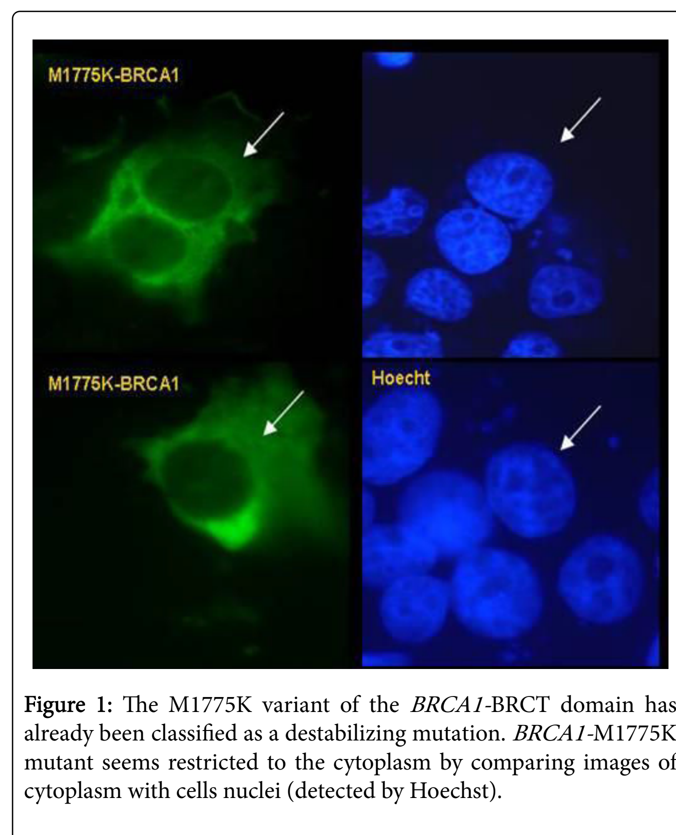

Figure 1: The M1775K variant of the *BRCA1*-BRCT domain has already been classified as a destabilizing mutation. *BRCA1*-M1775K mutant seems restricted to the cytoplasm by comparing images of cytoplasm with cells nuclei (detected by Hoechst).

The structure of the *BRCA1*-BRCT mutants M1775K implies modification of the BRCT domain which may interrupt the interaction with phosphopeptides such BACH1 and CtIP, possibly affecting *BRCA1* re-localization in the cell nucleus by disrupting the molecular protein complex during DNA repair processes. The CtIP protein binds to *BRCA1* in a CDK-dependent manner and formation of the functional complex MRN-CtIP-*BRCA1* is important for end resection to initiate HR-mediated DSB repair [14,15].

BRCA1 binds to CtIP through its BRCT domains when CtIP is phosphorylated by CDKs at Ser-327, and this interaction is believed to be important for HR [16,17]. Furthermore mutants of *BRCA1* Ring disrupt protein to co-localize in nuclear foci by affecting the interaction with proteins such as BARD1 and BACH1, which are important for DNA repair. This failure occurs despite the continued ability of the RING mutant protein to interact with BARD1 and the ability of the BRCT mutant to interact with BACH1 suggesting that both RING and BRCT domains of *BRCA1* are required for the functional and structural integrity of *BRCA1* to the sites of DNA damage and repair [18,19] .

Our results are in accordance with the literature. For example immunohistochemical microscopy studies of human breast tumors

have frequently detected a shift in *BRCA1* from nucleus to cytoplasm in cancers of increasing grade and from patients carrying germ-line *BRCA1* mutations [20], and similar observations have been made for breast cancer cell lines expressing mutant forms of *BRCA1* [21].

According to Solyom et al. abraxas directly interacts with the *BRCA1*-BRCT (*BRCA1* carboxyl-terminal) repeats and contributes to *BRCA1*-dependent DNA damage responses. A novel heterozygous alteration, c.1082G>A (Arg361Gln), results in abrogation of nuclear localization contributing to a BRCA-centered tumor suppression [22].

Moreover, two of the BRCT mutations (M1775R and Y1853X) are known to adversely affect *BRCA1* protein folding and nuclear function. The BRCT mutations reduced *BRCA1* nuclear import by a mechanism consistent with altered protein folding, as indicated by the restoration of nuclear staining by more extensive C terminal deletions suggesting that BRCT mutations alter nuclear targeting of *BRCA1*, and that this may contribute to the inhibition of nuclear DNA repair and transcription function [23].

Conclusion

Cytoplasmic mislocalization of M1775K *BRCA1*-BRCT mutant, presented here, which disrupt *BRCA1* C-terminal folding according to previous studies, influence the nuclear transport of *BRCA1*-BRCT domain to the nucleus, suggesting that the impact of the integrity of the *BRCA1*-BRCT domain is crucial at the functional and structural level. Such mutations may control subcellular compartmentalization and in turn regulation of *BRCA1*-related pathways and processes.

Ethical Approval and Consent to Participate

The study was justified by the ethics committee.

Consent for Publication

Written informed consent was obtained from author. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Availability of Data and Materials

Data are available in any case requested.

Declaration of Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

Drikos Ioannis, Effrosyni Boutou demonstrated the design of the study and draft the manuscript. Author approved the final manuscript.

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