Role of the BCA2 Ubiquitin E3 Ligase in Hormone Responsive Breast Cancer

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Abstract: The BCA2 protein contains a RING H2 finger and a Zn finger near the N-terminus and has E3 ligase activity. RING finger proteins play critical roles in mediating the transfer of ubiquitin and ubiquitin like modifiers to heterologous substrates as well as to the RING finger proteins themselves. Protein modification by ubiquitin and small ubiquitin-related modifier (SUMO) plays a pivotal role in protein homeostasis and is critical to regulating basic cellular processes such as proliferation, differentiation, apoptosis, intracellular signaling, and gene-transcriptional regulation. The addition of ubiquitin or SUMO can modulate the ability of proteins to interact with their partners, alter their patterns of sub-cellular localization and control their stability. It is clear that SUMO influences many different biological processes however recent data suggest that it is specifically important in the regulation of transcription. BCA2 is an E3 ligase that interacts with the SUMO conjugating enzyme Ubc9. It could therefore function as an E3 in the sumoylation of various transcription factors. We have found that the BCA2 is co-expressed with the estrogen receptor in 74% of ER-positive invasive ductal carcinomas from a 635 member breast cancer cohort ($p = 0.004$). At the cellular level, BCA2 co-localizes with ER and it appears that at the transcriptional level BCA2 mRNA expression is regulated by estrogen. Bioinformatic analysis of the BCA2 promoter region revealed ER and PR binding sites as well as that of other more general transcription factors. The data presented here provides an overview of the potential involvement of the BCA2 in hormone responsive breast cancer and opens up avenues that should be exploited to better understand the regulation of ER expression, growth of breast cancer cells, and the importance of BCA2.

Keywords: BCA2, SUMO, hormone-responsive, breast cancer, transcription.

INTRODUCTION

At the cellular level, transcription factors are tightly controlled by their rates of synthesis and degradation. Many transcription factors are maintained at an appropriate level by targeted addition of polyubiquitin chains and subsequent degradation in the proteasome [1]. While polyubiquitination targets proteins for degradation, monoubiquitination or their modification by small ubiquitin-like modifiers such as SUMO, alters subcellular localization and can change their activity [1]. Important transcription factors known to be regulated by ubiquitination or sumoylation are HIF1-α, c-Myb, c-Jun, Oct4, ETS1, and the ER among others [2-4]. Each of these transcription factors regulates the expression of a large number of target genes. Alterations of these transcription factors are frequently involved in tumorigenesis [1,5,6].

In response to circulating estrogen, the estrogen receptor (ER) regulates the genetic programs of cell cycle progression and growth in normal mammary gland and breast cancer epithelial cells. This critical transcription factor has two receptor forms, ERα and ERβ. ERβ demonstrates lower hormone-dependent transcriptional activity [7]; therefore ERα is considered the primary receptor for mammary gland development and function [8]. However, little is known of how the stability and expression of ER is regulated. Recent studies indicate that the ER is monoubiquitinated and sumoylated when interacting with BRCA1, which might lead to repression of ER transcriptional activation [9,10]. Furthermore, cancer-predisposing mutations in BRCA1 were observed to abrogate ER ubiquitination [9], implicating ubiquitin E3 ligases as playing a major role in ER regulation and hormone responsive breast cancer.

We had previously identified Breast Cancer Associated gene 2, BCA2 (synonymous with T3A12/ZNF364/Rabring7/ RNF115), a novel RING-finger ubiquitin E3 ligase, by subtractive hybridization cloning in breast carcinoma cell lines [11]. Subsequently we found that BCA2 is expressed in primary invasive breast cancers and is associated with a positive estrogen receptor status and outcome [12,13]. Here we describe the interaction of the BCA2 protein with the SUMO conjugating enzyme Ubc9, its regulation by ER, and its potential involvement in transcriptional regulation of hormone responsive breast cancers.

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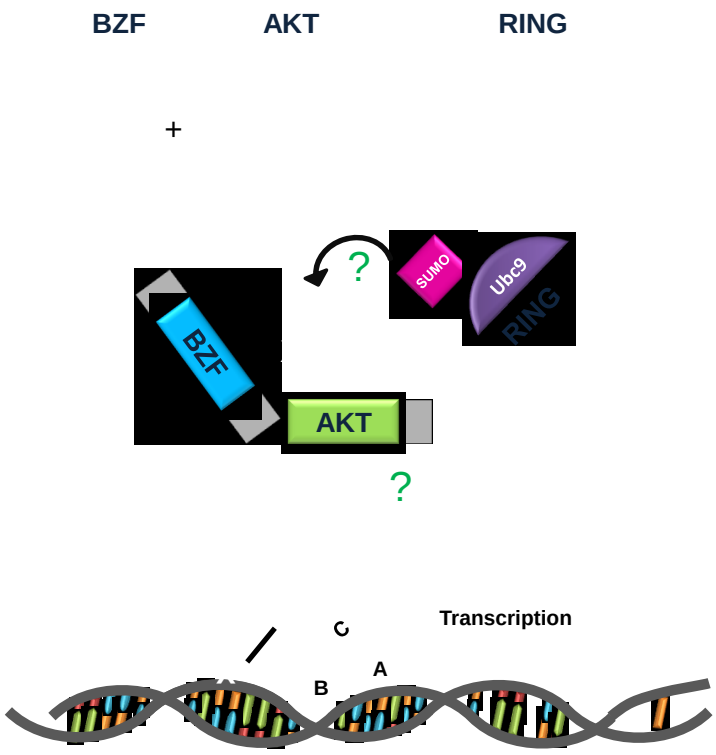

Fig. (5). Proposed model for BCA2 involvement in sumoylation. BCA2 with its known domains: BZF, BCA2 zinc finger domain; AKT, BCA2 AKT phosphorylation site; RING-finger domain, catalytic E3 ligase and probable E2 binding site. Ubc9, the SUMO-conjugating enzyme for the sumoylation pathway binds BCA2 likely in the RING domain, similar to the binding ubiquitin-conjugated E2s. This brings SUMO-conjugated Ubc9 into close proximity with the substrate (indicated by X) and allows the transfer of SUMO. Following sumolyation, the substrate may act as a factor in gene transcription though protein-protein interaction (with A, B, C) or DNA binding.

In the context of hormone-receptors, androgen receptor (AR), ER and PR, all have SUMO-conjugation sites; however the consequence of modification differs between the different receptors. ER is predominantly activated upon sumoylation. In contrast, when PR and AR are sumoylated, an inhibitory effect is observed [34].

ER sumoylation occurs strictly in the presence of estradiol and it appears that SUMO-1 regulates ER-dependent transcription (Figs. **4**, **5**) [10]. The SUMO E3 ligases PIAS1 and PIAS3, as well as Ubc9 were found to modulate ER-dependent transcription independently from their SUMO-1 conjugation activity and provide a link between the SUMO and estrogen pathways [10].

Several of the transcription factors that bind in the BCA2 promoter region (Fig. **3**, Table **1**) have been shown to be modified by sumoylation including YY1, SP1 and IκB [32,35-37].

YY1 protein is sumoylated at lysine 288 as the major sumoylation site [35]. Many post-translational modifications have been implicated in the regulation of Sp1 activity including SUMO-1. SUMO-1 is covalently conjugated to Sp1 within the N-terminal negative regulatory domain of Sp1. Compared with Sp1, sumoylation-deficient Sp1 mutants exhibit enhanced cleavage, increasing transcriptional activation. This is in contrast to the constitutively SUMO-1-modified Sp1, which is deficient in proteolytic processing and is associated with inhibition of Sp1 transcriptional activity [36].

The NF-κB inhibitory protein IκB is modified by SUMO-1 on lysine 21, which is also used for ubiquitin conjugation. Importantly, SUMO-1-modified IκB cannot be ubiquitinated and is resistant to proteasome mediated degradation, increasing the stability of the protein. As a result, signal-induced activation of NF-κB dependent transcription is inhibited [32]. While Ubc9 is the SUMO conjugating enzyme of IκB, the cooperating E3 ligase remains unknown.

BCA2 Interacts with the SUMO-Conjugating Enzyme UBC9

RING-E3 ligases are major players in post-translational modification and protein degradation. Known to be imbalanced in cancer, many E3s have emerged as mutated, lost or overexpressed in breast cancer. E3 ligases together with kinases make-up approximately 15% of all cancer genes, suggested to be major regulators in cell growth and death pathways [38]. More recently RING-E3 ligases have been known to play a role in the SUMO pathway, which is thought to be an antagonist of the ubiquitination pathway, by stabilizing proteins and sterically competing for ubiquitin modification sites [2].

To identify potential substrates for the BCA2 E3 ligase, we had previously performed bacterial and yeast-two hybrid screens [39, unpublished data]. Several binding partners

were isolated, including, Rab7, ubiquitin, 14-3-3, and the SUMO conjugating enzyme Ubc9 (Table **2**) [40]. Ubc9 was confirmed by pull down assays using GST-tagged Ubc9 expressed from the pGEX4T3 bacterial expression vector. BCA2 variations were constructed in pET100 vectors containing N-terminal 6xHis and Xpress tags. Purified bacterial recombinant wild-type BCA2, as well as RING (C228A, C231A) and AKT (S132A, S133A) BCA2 mutants, were incubated with GST-Ubc9 from bacterial lysates. Following incubation, mixtures were subjected to SDS-page and Western blotting. Membranes were probed with anti-Xpress antibody (Invitrogen). We found that Ubc9 binds to all BCA2 variants (Fig. **4A**). Moreover, when we expressed both BCA2 and Ubc9 in HEK293T cells, we saw co-localization with BCA2 in the cytoplasm and the nucleus suggesting that Ubc9 mediated sumoylation might be important to its nuclear as well as cytoplasmic function (Fig. **4B**).

CONCLUSION

Endocrine therapy for women with metastatic breast cancer is one of the options for systemic treatment in the battle against breast cancer. The "best" strategy depends in large part on the molecular biology of particular cancers, e.g., the expression of hormone receptors and HER2/neu.

Although, several agents that interfere with ER signaling are clinically available, the understanding of their mechanisms is more complex than expected. Tissue- and cell-specific estrogen mechanisms depend upon the formation of a wide variety of co-regulatory complexes as well as variable ER subtypes and extra-nuclear signaling events.

It is well known that post-translational modifications, such as sumoylation, affect breast cancer by altering important regulatory proteins, transcription factors, growth factors and oncoproteins [41]. Innumerate cellular pathways are impacted through sumoylation, affecting transcriptional activity, protein stability and protein sub-cellular localization. Examples of sumoylated oncogenes and tumor suppressor genes include Mdm2, c-Myb, Rb, ER, and p53, all of which undergo sumoylation [41,42]. Here we showed that BCA2 is upregulated by ER and that it in turn could mediate the sumoylation of ER and other proteins through its interaction with Ubc9.

Genomic and proteomic approaches are often used to determine targets differentially regulated by sumoylation. Such work has broadened our understanding of how sumoylation affects ER associated signaling molecules function and localization in breast cancer cells. An example of this is our work with BCA2, where we identified its interactions with the ER and SUMO conjugating enzyme Ubc9. Ubc9 is the only E2 currently known to conjugate SUMO to substrates. It is possible that BCA2 may act as an E3 and catalyze the addition of SUMO to substrates, or BCA2 may auto-sumoylate to regulate its own function, similar to the POU domain transcription factor OCT4 which has been shown to bind Ubc9 [2]. We believe the interplay of SUMO, BCA2, and ER in breast cancer warrants further examination for potential clinical interventions that may exploit their molecular characteristics.

ACKNOWLEDGEMENTS

This work was supported by Award Number R01CA127258 from the National Cancer Institute, Developmental Therapeutics Program and by the Canadian Breast Cancer Research Alliance special program grant on metastasis.

ABBREVIATIONS

BCA2	=	Breast cancer associated gene 2
SUMO	=	small ubiquitin-like modifier
AP1	=	Activator Protein 1
NF-kappa B	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
T3R	=	Triiodothyronine Receptor
ER	=	Estrogen Receptor
ERE	=	Estrogen responsive element
YY1	=	Ying Yang 1
PR	=	Progesteron Receptor
OCT1	=	Octamer-binding transcription factor 2
HBBCC	=	Henrietta Banting Breast Cancer Collection
FITC	=	Fluorescein isothiocyanate
TRITC	=	Tetramethylrhodamine isothiocyanate
DAPI	=	4',6-diamidino-2-phenylindole
His	=	Histidine
GST	=	Glutathione S transferase
SP1	=	Specificity Protein 1
OCT4	=	Octamer-binding transcription factor 4
AR	=	Androgen Receptor

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Received: October 29, 2009 Revised: November 10, 2009 Accepted: November 12, 2009