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REVIEW ARTICLE

Regulation and action of interferon-stimulated gene 15 in breast cancer cells

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Abstract

Interferon-stimulated gene 15 (ISG15) is a critical ubiquitin-like protein that can be conjugated to proteins via the ISGylation system to modify them posttranslationally. Furthermore, ISG15 can be detected as non-conjugated or free, intracellularly and/or extracellularly. Both conjugated and free ISG15 participate in different cancer types, including breast cancer. Here, we highlighted the findings on ISG15 and protein ISGylation, and their implications in the field of breast cancer research. ISG15 emerges as a central element in mammary tumors and may become a crucial protein in the strategies for detection, prognosis, and therapy of breast cancer.

Keywords Free ISG15 · ISGylation · IFN-gamma · Breast cancer cells

Introduction

Breast cancer is a severe health problem. Worldwide, according to the International Agency for Research on Cancer (IARC), breast cancer is the first cause of women's death by cancer and has the highest incidence among female cancer types. Breast cancer is defined as a collection of mammary tissue-associated neoplastic alterations [1–3]. More than 85% of breast cancers in women are not related to gene mutations, having multifactorial influences. At the histological level, almost 80% of breast cancers develop in the mammary ducts and are invasive (*invasive ductal carcinoma*). At the molecular level, mammary tumors are highly heterogeneous, leading to a deficiency of biomarkers for early detection, or prognostic biomarkers, as well as pharmacological targets of breast cancer [4, 5].

Interestingly, estrogen receptor alpha (ERα), progesterone receptor (PR), androgen receptor (AR), and human epidermal growth factor receptor 2 (HER2 or ERBB2) have been used as classical biomarkers for breast cancer. Among these, ERα is expressed in more than 70% of breast cancer cases, and the remaining 30% lack expression of this receptor; consequently, these cancer types are known as ERα+ and ERα−, respectively. ERα is a nuclear receptor, activated mainly by 17β–estradiol (E2), which acts as a pro-tumor transcriptional regulator in breast cancer cells. Thus, ERα is the therapeutic target for most cases of breast cancer. These treatments include the use of selective estrogen receptor degraders (SERDs), such as fulvestrant, and selective estrogen receptor modulators (SERMs), such as tamoxifen. In addition, aromatase inhibitors, such as letrozole, anastrozole, and exemestane, are used to inhibit the production of E2 hormone from androgens by blocking aromatase enzyme.

Breast cancer has been classified into four different subtypes based on the detection of ERα, PR, HER2, and Ki67 using immunohistochemical analysis: (1) luminal A-like (ERα+, PR ≥ 20%, HER2−, Ki67 < 20%); (2) luminal B-like (ERα+, PR < 20% and/or HER2+ and/or Ki67 ≥ 20%); (3) HER2-overexpressing (ERα−, PR−, HER2+); and (4) basal-like [ERα−, PR−, HER2− (triple-negative)]. The most common molecular subtype of invasive breast cancer is luminal A (~ 50% of breast cancers), which responds to endocrine therapy. The luminal B subtype (~ 20% of breast cancers) generally requires additional chemotherapy and has a worse prognosis than luminal A. The HER2-overexpressing subtype (~ 15% of all invasive breast cancers) is treated

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with anti-HER2-targeted therapy [6, 7]. Triple-negative breast cancer is aggressive and requires chemotherapy [8, 9].

Many research groups are trying to identify new biomarkers and pharmacological targets for the early identification and control of breast cancer. Here, we discuss ISG15/ISGylation as a potential factor in the context of breast cancer.

The basis of the ISG15 and ISGylation system

ISG15 protein (interferon-stimulated gene 15) is a protein of 156 aa and 15 kDa formed by two ubiquitin (UB)-like domains that are connected by a hinge sequence [10–12]. The ISG15 C-terminal domain has the sequence LRLRGG, which is also contained in UB, allowing its covalent interaction with target proteins' lysine residues through the E1-activating enzyme, the E2-conjugating enzyme, and the E3-ligase enzyme. These three enzymes are known as the ISGylation system, in which the E1-activating enzyme catalyzes the formation of a thioester bond with C-terminal glycine from ISG15 in an ATP-dependent manner. Next, via trans-esterification, ISG15 is transferred to the E2-conjugating enzyme. Finally, ISG15 is transferred from the E2-conjugating enzyme by E3 ligase enzyme to specific proteins in lysine residues [13–15]. Some enzymes have been identified in the ISGylation system; for example, in humans, UBE1L E1-activating enzyme, UBCH8 E2-conjugating enzyme, and HERC5, HHARI, and EFP as E3-ligases enzymes [16–24]. Interestingly, HERC5 is the E3 ligase for ISG15 in humans, whereas mice express the E3 ligase HERC6 [25, 26]. Contrarily, USP18 (UBP43) is an enzyme able to remove ISG15 from ISGylated proteins, generating free ISG15 by a process known as de-ISGylation [27] (Fig. 1).

ISGylation represents a posttranslational modification that has not been thoroughly studied in comparison with other modifications, and consequently, few ISGylated target proteins have been reported. However, some of the protein targets of ISGylation are central to several cellular processes such as Filamin B, Parkin, BECN1, PCNA, β-catenin, and TP53. Interestingly, ISGylation has been detected in one, two, or multiple sites in lysine residues as monoISGylation. However, the functions of ISGylation are not entirely determined, despite being related to an increase or decrease in protein stability and the regulation of protein–protein interactions [20, 28–32].

It has been demonstrated that an integrin-like receptor, in natural killer (NK) cells, can recognize extracellular free ISG15 to promote the secretion of IFN-γ, suggesting that ISG15 may act as a cytokine and trigger a signaling pathway in some cellular contexts [33]. Indeed, there is still a need to further investigate the roles of ISG15 and ISGylation, but its potential involvement in several pathophysiological processes, including malignance in mammary tissues, can already be glimpsed.

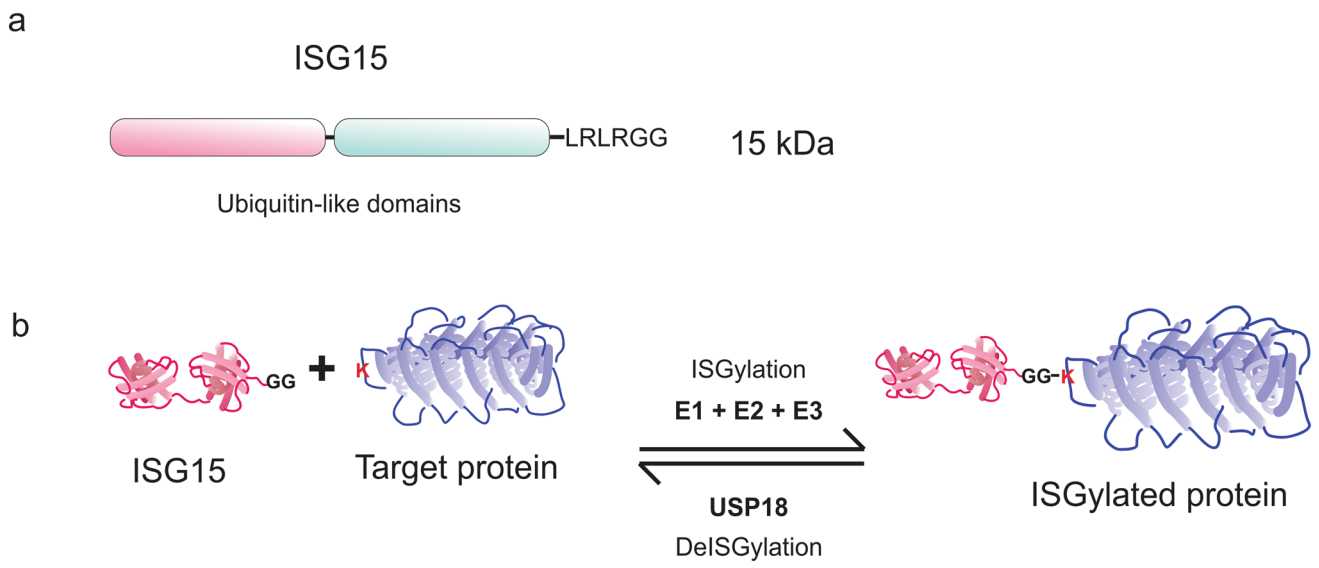

Fig. 1 **a** ISG15 protein structure. ISG15 is formed by two ubiquitin-like domains and the same motif of ubiquitin protein to covalently associate with proteins to modify them. **b** ISGylation and de-ISGylation systems. E: enzyme; E1: UBE1L; E2: UBCH8; E3: HERC5, HHARI, or EFP. USP18: De-ISGylation enzyme

Free ISG15 and protein ISGylation in breast cancer cells

Free ISG15 levels and protein ISGylation are cell type-specific, and both seem to play a critical role in breast cancer cells. For instance, a study indicated that MCF-7 ERα+ breast cancer cells had the highest levels of free ISG15 protein in comparison with other cell types, and a specific profile of ISGylation marks [34]. More recently, free ISG15 and ISGylation were also detected in triple-negative breast cancer cells (ERα-, PR-, HER2-), showing numerous ISGylation marks and low levels of free ISG15 protein, a pattern contrary to that observed in MCF-7 ERα + breast cancer cells [34, 35]. Together, these data suggest that ISGylated proteins may be distinct between ERα+ and ERα− (like triple-negative) breast cancers. In addition, the differences between free ISG15 and ISGylation profiles may be associated with the ISGylation system expression (*HERC5* and *EFP* E3 ligases expression) in these breast cancer types (Fig. 2).

Subcellular distribution of ISG15 and protein ISGylation in breast cancer cells

It has been suggested that ISG15 protein is distributed mainly in the cytoplasm, followed by the nucleus, mitochondria, plasma membrane, and cytoskeleton [34]. Experimentally, through immunofluorescence assays, it has been shown that ISG15 is localized in the cytoplasm and nucleus of breast cancer cells. Through cellular fractionation assays, a differential compartmentalization of free and conjugated ISG15 bands between the cytoplasmic and nuclear compartments has been shown. Free ISG15 protein is mainly distributed in the cytoplasm of MCF-7 and MDA-MB-231 breast cancer cells. However, ISGylated protein marks were mainly detected in the nucleus of MCF-7 cells, whereas ISGylation marks were detected mainly in the cytoplasm of MDA-MB-231 cells, suggesting that ISGylated proteins can be cytoplasmic and nuclear, having a specific ISGylation profile for ERα+ and ERα− breast cancer cells [35]. Thus, ISG15 may function in multiple ways as free or conjugated ISG15 in several intracellular compartments in addition to the extracellular actions of free ISG15 in breast cancer cells (Figs. 3, 4).

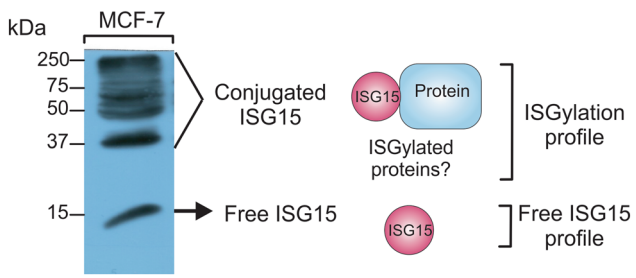

Fig. 2 Detection of free and conjugated ISG15 by immunoblot assay using the total extract from MCF-7 ERα+ breast cancer cells

Association of ISG15 with the IFN-γ pathway in breast cancer

ISG15 is induced by interferons (IFNs) α and β, and recently, IFN-γ has appeared as one of the most important inductors of *ISG15* in breast cancer contexts. The IFN-γ canonical pathway contains a specific heterotetrameric receptor complex (composed of IFNGR1 and IFNGR2 subunits) and the JAK-STAT1 system. IFN-γ binds to its receptor complex, and through the triggering of JAKs1/2 promotes the activation of STAT1 via its phosphorylation, followed by its homodimerization and its binding to the gamma interferon-activated site (GAS) on the regulatory sequences of IFN-γ target genes to regulate its expression [36].

Specifically, in breast cancer, IFN-γ seems to mediate apoptosis and cell cycle arrest. Moreover, IFN–γ autocrine signaling may be generated in breast cancer cells [37, 38]. An initial study showed that IFN–γ increases the mRNA levels of *ISG15* in MCF-7 cells [39]. More recently, it was demonstrated that IFN-γ also increases the mRNA levels of *ISG15* in MDA-MB-231 breast cancer cells [40]. Likewise, IFN-γ enhances free ISG15 protein levels and protein ISGylation marks, and this upregulation occurs in parallel with changes in the morphology of breast cancer cells [40, 41]. Furthermore, it has not been observed to affect the subcellular transport dynamic of free and conjugated ISG15 induced by IFN-γ. However, IFN-γ differentially increased ISGylation patterns between the nucleus and the cytoplasm of MCF-7 and MDA-MB-231 cells, whereas IFN-γ enhances the levels of intracellular free ISG15 in the cytoplasm of both cell types [34, 35].

The IFN–γ pathway increases sensitivity to tamoxifen and restores sensitivity to fulvestrant in breast cancer cell lines. For this reason, IFN-γ-induced genes, including *ISG15,* may also affect the response to endocrine therapy with fulvestrant or tamoxifen in these cells [38, 42, 43]. In addition, patient-derived ERα− mammary tumor xenografts (PDXs) in athymic mice can secrete IFN-γ. In this study, residual tumor cells of chemo-responder PDXs displayed strong overexpression of IFN-target genes after adjuvant chemotherapy. One of the IFN-inducible genes associated with chemotherapy response in ERα- breast cancer was *ISG15.* In this process, the upregulation of *ISG15* correlated with an increase in STAT1 phosphorylation levels, as well as DNA damage and apoptosis [44].

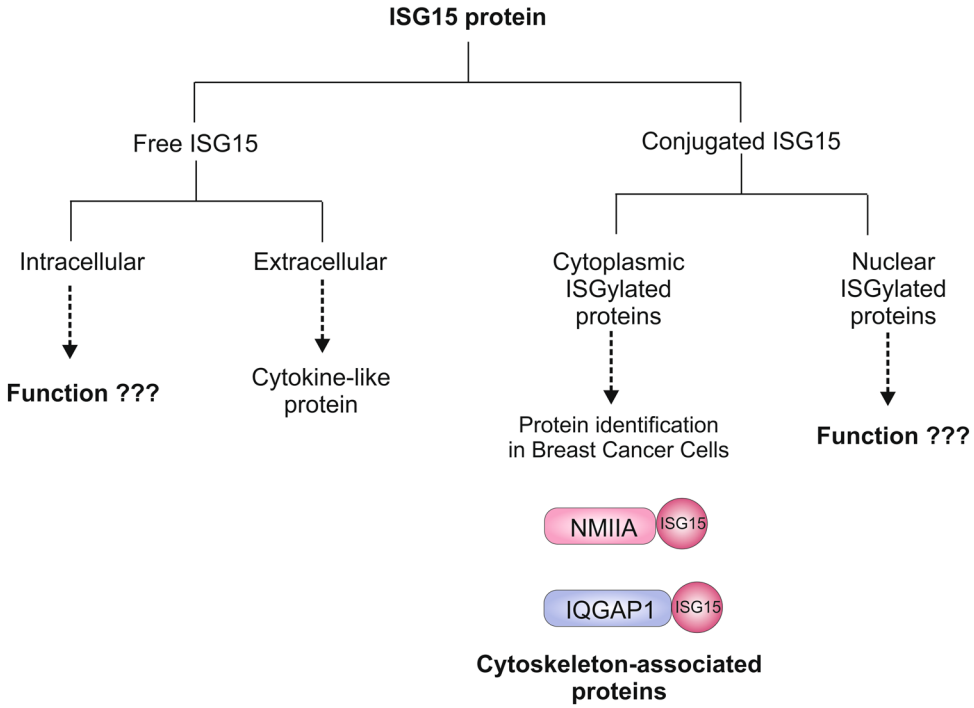

Fig. 3 Forms of ISG15 protein and its subcellular distribution

It has been suggested that IFNs-responsive cells, expressing genes like *ISG15,* are sensitive to DNA damage and consequently are responsive to chemotherapy [44]. However, another study demonstrated that experimental resistance to DNA damage is associated with the expression of IFN-related DNA damage resistance signature (IRDS), which includes *ISG15*. The authors propose that chronic stimulation of the interferons pathway could lead to constitutive IRDS expression, promoting DNA damage resistance, cytotoxic signal failure, and pro-survival signals [45]. Thus, the IFN-γ pathway and its target genes seem to be involved in the sensitivity to DNA damage and in the resistance to DNA damage in the chronic activation condition of IFN-γ signaling.

On the other hand, NMIIA and IQGAP1 are two cytoplasmic proteins that have been identified as ISGylation targets in breast cancer cells, and IFN-γ increases their ISGylation [41, 46] NMIIA is a 230 kDa protein that associates with the filaments of actin, generating the actomyosin complex for cytoskeletal remodeling and cellular spreading, which is implicated in cell motility [47]. Both NMIIA and ISG15 proteins co-localize in the marginal spreading lamellar region and in the cytoplasm of MDA-MB-231 cells during the spreading of these cells induced by fibronectin (FN) substrate, suggesting that ISGylation of NMIIA is important in this process [41]. In addition, IQGAP1 is also associated with cytoskeletal remodeling [40, 48]. Thus, IFN-γ-induced cytoplasmic protein ISGylation in breast cancer may be relevant to the reorganization of the cytoskeleton of these cells [40, 41]. To date, few proteins have been identified as ISGylation targets in breast cancer induced by IFN-γ. Many other cytoplasmic and nuclear proteins modified by ISGylation remain to be elucidated. Together, these data suggest that the IFN-γ pathway could activate ISGylation of proteins related to cytoskeletal changes, and these changes are important for processes, such as invasion involved in cancer progression.

Association of ISG15 with other signaling pathways in breast cancer cells

Other signaling pathways can also modulate ISG15/ISGylation in breast cancer cells. It has been demonstrated that the reduction of *Ki-Ras* expression by interfering RNA in MDA-MB-231, ZR-75-1, and MCF-7 breast cancer cells leads to a decrease in the levels of free ISG15 and protein ISGylation, suggesting that oncogenic Ki-Ras pathways may regulate the expression of *ISG15* and the ISGylation system [49]. In addition, a positive correlation between *ISG15* and *KSRI* (kinase suppressor of Ras 1) expression has been reported in breast cancer [50]. Moreover, long-term estrogen exposure increases *ISG15* expression in MCF-7 cells, and its expression is not affected by RAL (raloxifene, an anti-estrogen SERM) [51]. In triple-negative breast cancer, low levels of p53 and ARF (ADP-ribosylation factor) correlated with high levels of STAT1 and ISG15, leading to proliferation and tumorigenicity. This is important, because some malignant mammary tumors have a co-inactivation of p53 and ARF. Thus, the tumor suppressor pathways of ARF and p53 may

be affected in breast cancers, facilitating the upregulation of STAT1–ISG15 signaling [52]. In addition, it has been reported that fibronectin (FN), an extracellular matrix protein, may induce *ISG15* expression and protein ISGylation via integrin-dependent signaling in breast cancer cells [53]. Likewise, a connection between ISGylation, FN, and IFN-γ may exist in breast cancer cells, modulating changes in cell morphology and cell spreading [41] (Table 1).

Effects of *ISG15* expression in vivo and in vitro studies in a breast cancer context

To evaluate the significance of *ISG15* expression in breast cancer, cell lines derived from this cancer have been studied. The reduction of *ISG15* expression using specific interfering RNA decreases the proliferation and migration of breast cancer cells. Using the same experimental strategy, the reduction of *UBCH8* (E2-enzyme of the ISGylation system) decreases the levels of protein ISGylation, showing similar effects to the reduction of *ISG15*. Both the reduction of *ISG15* expression and the reduction of *UBCH8* expression reverse the epithelial–mesenchymal transition (EMT) of MDA-MB-231 breast cancer cells [49]. Furthermore, ISG15 affects the cytoskeleton and enhances cellular motility in ZR-75-1 breast cancer cells. *ISG15* and *UBCH8* expression disrupts F-actin architecture and the formation of focal adhesions, and enhances the migration of these cells [54]. Some studies have proposed that protein ISGylation confers oncological abilities to breast cancer cells [49, 54, 55]. In this context, ISGylation has been suggested as a mechanism for interfering with the ubiquitination pattern in breast cancer cell lines [54]. Therefore, *ISG15* expression seems to have pro-tumor activity in in vitro studies.

Intriguingly, the expression of *ISG15* in vivo using athymic mouse models seems to have a contrary effect to the results shown by in vitro studies. For example, xenotransplantation of ZR-75-1 and MDA-MB-231 breast cancer cells treated with interfering RNA for ISG15 into athymic mice promoted tumor development and decreased NK cell filtration in xenograft tumors in comparison with the controls. Hence, *ISG15* expression seems to have anti-tumor activity for in vivo studies. The use of these animal models suggests that some elements of the in vivo tumor microenvironment may be critical to modulate or coordinate the actions of ISG15 in mammary tumor progression, which was not evidenced by in vitro studies. Most importantly, in the same study, recombinant purified free ISG15 was injected subcutaneously near the site of tumor implantation with MDA-MB-231 cells with interfering RNA control. Suppression of xenograft tumor growth in nude mice injected with free ISG15 was clearly shown, along with an increase in NK cell infiltration in tumor sections and an increase in major histocompatibility complex (MHC II) surface expression [56]. These data are critical, showing an antitumor function promoted by extracellular free ISG15. Therefore, a possible protumor action for protein ISGylation and anti-tumor for extracellular free ISG15 has been suggested [56] (Fig. 4).

ISG15 as a potential biomarker

Some studies have detected an upregulation of *ISG15* expression and protein levels in breast cancer compared with normal breast tissue [34, 40, 57]. For instance, both the mRNA and protein levels of ISG15 were upregulated in breast carcinoma cells compared with normal mammary tissue, evidencing that *ISG15* overexpression significantly correlated with unfavorable prognosis of breast cancer [57]. Furthermore, using Cancer Genome Atlas Program (TCGA, 450 patients) and Curtis (1700 patients) data sets from Oncomine, the mRNA levels for *ISG15* were analyzed in tumors of patients diagnosed with invasive ductal breast cancer and compared with normal mammary tissue. *ISG15* expression was upregulated in breast cancer, and the highest mRNA levels of this gene were detected in grade 3 tumors, suggesting that its expression may be associated with the progression of mammary tumors [40]. At the protein level, an analysis to detect ISG15 through immunohistochemistry (IHC) was performed using a breast cancer microarray that contained 16 cases of invasive breast cancer in duplicate, as well as adjacent normal tissue from the same patients. ISG15 protein was strongly detected in all cases of breast cancer

Table 1 Pathways associated with the regulation of ISG15 and ISGylation in breast cancer cells

Signaling/Protein	*ISG15* mRNA	ISG15 protein	Protein ISGylation	References
IFN-γ	Increase	Increase	Increase	[34, 35, 39–41]
IFN-α/β	–	Increase	Increase	[54, 56]
Estradiol (long term)	Increase	–	–	[51]
Fibronectin	Increase	–	–	[53]
Ki-Ras depletion	–	Decrease	Decrease	[49]
KSR1	Positive correlation	–	–	[50]
LIPG	Increase	–	–	[60]

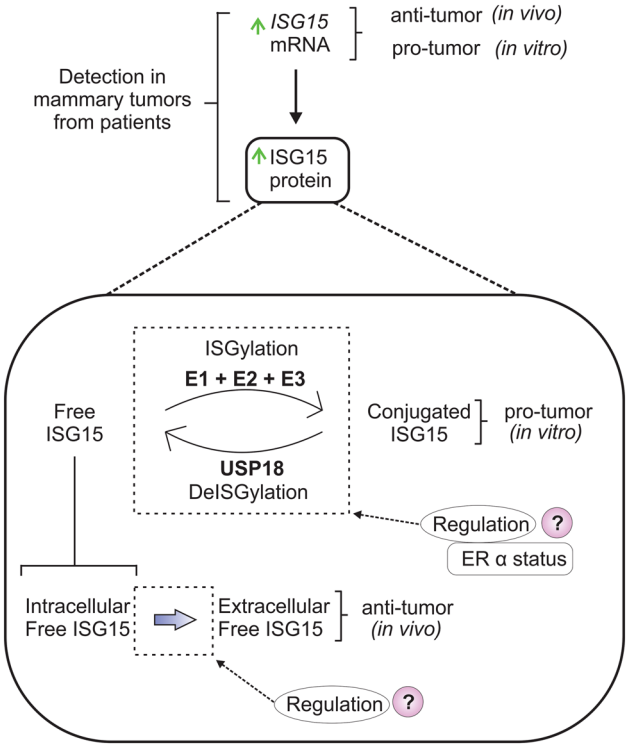

Fig. 4 Contribution of several studies regarding the effects of the *ISG15* expression and ISG15 protein forms in breast cancer cells

in comparison with the adjacent normal mammary tissue [40]. All these data indicated that ISG15 might be useful as a potential biomarker for breast cancer.

It is also known that mammary tumors can invade tissues such as the lung, bone, liver, and brain. However, the role of ISG15 in distal metastasis from mammary tumors has not been completely defined. Nonetheless, a retrospective study reported that *THBS1* (Thrombospondin 1), *AP1M1* (Adaptor-Related Protein Complex 1 Subunit Mu 1), and *ISG15* genes can be potential biomarkers for central nervous system metastasis from triple-negative tumors [58]. Similarly, through a meta-analysis of three microarray data sets, it was proposed that the overexpression of *CD80* and *ISG15* was a biomarker for the progression and metastasis of mammary tumors [59].

Furthermore, in vitro and in vivo studies demonstrated that the signaling triggered by LIPG (endothelial lipase) stimulates tumor initiation, as well as metastasis in triple-negative breast cancer and one of the mediators seems to be ISG15, since LIPG protein increases the expression of *ISG15* in breast cancer cells. Moreover, DTX3L protein inhibits proteasome-mediated LIPG degradation, leading to LIPG accumulation, and consequently increases *ISG15* expression, promoting metastasis of triple-negative tumors [60]. These studies also indicate that ISG15 may be useful as a metastasis biomarker in breast cancer, but more studies are needed to evaluate the functional role of ISG15 in cancer dissemination.

ISG15 as a therapeutic strategy for breast cancer

Thus far, it would be difficult to state predictive values of *ISG15* gene expression or free ISG15/ISGylation for resistance to treatments, including chemotherapy and radiation, for breast cancer. However, some studies have analyzed the role of *ISG15* expression in breast cancer treatments. For instance, one study determined that the sensitivity of breast cancer cells to camptothecin (a DNA topoisomerase inhibitor) is dependent on *ISG15* expression. Camptothecin and its derivatives are used as second- or third-line treatments for breast cancer resistant to endocrine therapy [61]. In this study, it was observed that when cells express high levels of *ISG15*, they are more sensitive to this drug compared with control cells. However, the depletion of *UBCH8* (E2-conjugating enzyme for ISG15) decreases the sensitivity to camptothecin, suggesting that alterations in the ISGylation system can affect the sensitivity of breast cancer cells to this drug [62] (Table 2).

Another case is ΔNp63 alpha (a variant of p63), which suppresses the transcriptional activity of p53 family members. ISGylation may act as a tumor suppressor, since

Table 2 ISG15 and ISGyltion in therapeutic studies of breast cancer

	References
ISG15 expression associated with DNA damage resistance and resistance to chemotherapy and radiation in breast cancer (in vitro studies)	[45]
Chemo-responder patient-derived xenografts (PDXs) overexpress *ISG15,* along with DNA damage and apoptosis in comparison with chemo-resistant patient-derived xenografts (PDXs) upon chemotherapy	[44]
The sensitivity of breast cancer cells to camptothecin used in chemotherapy depends on *ISG15* expression. The silence of *UBCH8* (element for ISGylation) decreases the sensitivity to camptothecin (in vitro studies)	[62]
ISGylation mediates the effects of doxorubicin through the ISGylation of ΔNp63 alpha, blocking its activity in mammary epithelial cells treated with this drug (in vitro studies)	[63]
ISG15 is proposed as a tumor-associated antigen for cancer immunotherapy	[64]

ΔNp63 alpha is ISGylated to block its activity when the mammary epithelial cells are treated with doxorubicin. Hence, this modification may mediate some of the anticancer effects of doxorubicin [63]. More studies are required to determine how *ISG15* expression and activity may affect the response to chemotherapy in breast cancer cells. In addition, ISG15 is being considered as a tumor-associated antigen for cancer immunotherapy [64].

Future challenges

In breast cancer, extracellular free ISG15 seems to have anti-tumor activity in vivo, whereas ISGylation has been associated in vitro with pro-tumor activity. Therefore, the regulation of *ISG15* expression at all levels and the elements of ISGylation and de-ISGylation systems are critical, because the molecular mechanisms involved in this regulation could determine the proportion of free and conjugated ISG15 levels, leading to anti-tumor or pro-tumor effects in breast cancer cells. Furthermore, the microenvironment of breast tumors may affect the free ISG15/ISGylation activity; for example, extracellular matrix proteins such as FN can induce *ISG15* expression in breast cancer cells [53]. In addition, ERα status appears to have an influence on the profile of free ISG15 and ISGylation marks by mechanisms not yet determined. Hence, a better understanding of free or conjugated ISG15 and its regulation is still required in mammary tumors classified as ERα– and ERα+. The analysis of patient samples shows that ISG15 may be a potential biomarker for breast cancer, but it is necessary to investigate whether ISG15 can be used as an indicator for the selection of the therapy and/or as a prognostic marker. Furthermore, the potential of ISGylation system elements and USP18 as biomarkers and target therapeutics should also be explored. ISGylation targets in breast cancer cells are pending identification, but many of them may be central in molecular pathways for promoting or controlling the development of mammary tumors. To date, the identification of IQGAP1 and NMIIA as ISGylated proteins indicates the implications of ISGylation and its regulatory factors (IFN-γ, integrins, FN) in changes of the cytoskeletal structure, cell morphology, and probably migration and invasion of these cells. The identification of new ISGylation targets in breast cancer would help to understand the role of ISG15/ISGylation pathways in this pathology. The potential of free ISG15 as a therapeutic molecule to control tumor development demands further studies to support this possible action and generate novel strategies for the treatment of this cancer. Thus, the ISGylation system and free ISG15 are emergent factors in the understanding of breast cancer and open new study lines to explore the basic molecular aspects, biological and functional transcendence, and medical implications in breast cancer.

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