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Immediate early response gene X-1, a potential prognostic biomarker in cancers

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Abstract

Introduction—The immediate early response gene X-1 (IEX-1) plays a pivotal role in the regulation of cell apoptosis, proliferation, differentiation and metabolism. Deregulation of IEX-1 expression has been confirmed in multiple cancers in humans, in association with either poor or better prognosis depending on the type and progression stages of the cancer.

Areas covered—This review summarizes clinical studies of altered IEX-1 expression in ovarian, pancreatic, blood, breast and colorectal cancers, lymphoma and myeloma. The authors also outline the current understandings of the complex functions of IEX-1 gained from studies with animal models and tumor cell lines so as to help us comprehend the significance of the clinical findings.

Expert opinion—IEX-1 holds great promise to be a valuable biomarker, either alone or in combination with other genes, for monitoring progression of some cancers. IEX-1 expression is highly sensitive to environmental cues and distinct between normal and cancer cells. However, use of IEX-1 as a biomarker remains a significant challenge because too little is understood about the mechanism underlying the diverse activities of IEX-1 and a standardized clinical assay for IEX-1 detection and validation of clinical results across different studies are still critically lacking.

Declaration of interest

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Keywords

apoptosis; biomarkers; cancer prognosis; IF1; immediate early response gene X-1

1. Introduction

The immediate early response gene X-1 (IEX-1), also known as *IER3*, p22/PRG1, Dif-2, gly96, belongs to the family of the immediate early response genes that include the well characterized *c-fos*, *c-jun*, and *c-myc* [1,2]. This family of genes can be transcriptionally activated within minutes without the need of *de novo* protein synthesis and their expression can reach a peak level by 15–20 min following stimulation. Many members of this family are transcription factors that can rapidly activate transcription of genes critical for a cell to respond to a stressor in a timely fashion. But, IEX-1 is not a transcriptional factor and it lacks a DNA-binding domain. It may, however, act as a coactivator or corepressor sometimes [2,3].

IEX-1 is a stress-inducible gene and exerts divergent effects on cellular responses in a cell under stress. It is, thus, not surprising that IEX-1 frequently comes out as an outlier in global gene expression microarray studies in cancers, because cancer cells commonly originate from survival advantages in a harsh condition [4–6]. These gene expression profiles not only confer unbiased recognition of an importance of IEX-1 in the prognosis and pathogenesis of these cancers but also raise an urgent need for a better understanding of the cellular functions of this gene. This review summarizes recent clinical studies obtained from global gene expression microarray studies and links the clinical findings to the mechanistic insights made in cell lines and genetically engineered mice to help us comprehend how IEX-1 may contribute to the pathogenesis of cancers and its deregulation may serve as a prognosis for cancers.

2. Complex cellular functions of IEX-1

To determine clinical significance of IEX-1, it is essential to understand its cellular function, which turns out to be very difficult, because IEX-1 displays a complex and sometimes contradictory role in cell cycle, differentiation and survival in a cell-type and stimulus-dependent manner. Induction of IEX-1 expression confers survival advantages for some cells, but promotes apoptosis or impedes cell differentiation in other cells in a poorly defined manner [1,2,7–9]. Structurally, IEX-1 is a protein of 156 amino residues in length and contains three potential extracellular signal-regulated kinase (ERK) phosphorylation sites at positions 18 (Threonine or T), 123 (T) and 126 (Serine or S), a PEST [proline (P), glutamic acid (E), S, and T]-rich sequence that makes it prone for degradation [10], a nuclear location sequence (NLS), a potential transmembrane domain (TM), an N-linked glycosylation site (NG) (Figure 1) and more than 10 O-linked glycosylation sites as predicted by the NetOGlyc or the YinOYang program [11]. The TM region spanning from 86 to 101 amino residues can potentially target the protein to endoplasmic reticulum, Golgi and mitochondria. Indeed, IEX-1 is found at these cellular organelles in addition to nuclei [12]. Sequence-targeting mutagenesis analysis revealed that the TM region was important for both its proapoptotic and antiapoptotic functions [11]. Substitution of three hydrophobic residues

with hydrophilic ones within the TM region abolished its proapoptotic and antiapoptotic effects. Mutation of the ERK phosphorylation site at T18, N-glycosylation or truncation of 18 amino residues at the C-terminus all resulted in a loss of antiapoptotic activity of IEX-1, but fully preserved its proapoptotic function. On the other hand, NLS mutation abrogated proapoptosis of IEX-1, without incurring any significant effect on its antiapoptotic activity [11,13].

IEX-1 appears as multiple isoforms in SDS-PAGE analysis, with molecular masses ranging from 17 to 32 kDa, which probably result from glycosylation at different sites to varying degrees or other posttranslational modifications [11,14]. Notably, removal of the NG site yielded two protein bands of ~ 29 and 31 kDa, larger than the two major wild-type (WT) isoforms of 20 and 28 kDa, presumably by *O*-linked glycosylation of IEX-1 protein [11]. The posttranslational modification may be necessary for IEX-1 stability or its divergent functions in response to different stimuli. However, no study has been conducted yet to address these possibilities so far.

2.1 Antiapoptotic activity of IEX-1

Discernible regions of IEX-1 responsible for its antiapoptotic and proapoptotic activities argue strongly about nonoverlapping mechanisms involved in these two opposing activities of IEX-1. While proapoptosis of IEX-1 seems to associate with its nuclear localization, IEX-1-mediated cell survival is apparently related to its ability to control the production of reactive oxygen species (ROS) at mitochondria in the basis of our *in vitro* and *in vivo* studies. As depicted in Figure 2, the mitochondrial respiratory chain consists of complexes I – IV and an ATP synthase/ATPase that is composed of two functional distinct parts, F1 and Fo [15,16]. Under a physiological condition, the inner membrane potential ψ_m is about 80–140 mV. Protons H^+ generated by oxidation of NADH in complexes I, III and IV are pumped to the cytosolic side and then returned to the matrix via the Fo proton channel in coupling with generation of ATP from ADP by the F1 catalytic sector. Inhibition of the ATP synthase due to stress-induced high membrane potential, a high ATP:ADP ratio, or accumulation of IF1, a natural inhibitor for the F1 complex, would slow down the proton flux. Electrons will then accumulate around complex I and, perhaps, complex III, where they would be available to be donated to O_2 to give $\cdot O_2^-$, producing ROS [16]. As part of the mechanism to counteract excessive ROS production, in cells under stress the F1Fo-ATP synthase switches into an ATPase, hydrolyzing ATP (red dash line), promoting the proton flux through Fo channels, and relieving the potential of the mitochondrial inner membrane from a stress status to a phosphorylating one. This switch from F1Fo-ATP synthase to ATPase activity is partially controlled by IF1 that binds to F1 complex and inhibits its ATPase activity [17–19]. IF1 expression was increased in cancers, thus, playing a crucial role in elevated levels of aerobic glycolysis in the cells [20,21]. IEX-1 may be one of the keys to a linkage of environmental cues to energy metabolic regulation at mitochondria by the control of IF1 protein level in cancer cells.

In this regard, our investigation shows that IEX-1 targets IF1 to degradation via a yet unidentified mitochondrial protease [12]. Immunoprecipitation in conjunction with DNA mutagenesis studies revealed that IEX-1 physically interacted with IF1 protein at

mitochondria via its C-terminus, which might expose its cleavage site to an unknown mitochondrial protease [12]. IEX-1-facilitated IF1 degradation could be sufficiently blocked by *o*-phenanthroline, an inhibitor of mitochondrial metalloproteases [12]. In accordance with this, over-expression of IEX-1 reduced the level of IF1 protein expression, increased ATPase activity and sustained the mitochondrial inner membrane ψ_m at a phosphorylating status, resulting in diminished ROS generation and protection of the cells against mitochondria-dependent apoptosis [12]. Our investigations further showed that all the mutants that lost their antiapoptotic effect were unable to prevent increases in ROS production at an onset of apoptosis, whereas those mutants that could sustain anti-death function also controlled ROS production as sufficiently as WT IEX-1 [11].

IEX-1-regulated ROS production appears to be delicate and mainly confined at mitochondria. Hence, a lack of IEX-1 causes little oxidative stress but activates specific redox-sensitive signaling pathways leading to altered proliferation and differentiation in one type of the cells while increasing their apoptotic susceptibility in another closely related cell type, probably because the cells significantly differ in their sensitivity to ROS. For instance, IEX-1 null mutation specifically renders T helper 1 (Th1) cells to apoptosis, while augmenting the differentiation of Th17 cells, giving rise to Th17-biased inflammatory responses [22] and aggravated arthritis in IEX-1 knockout (KO) mice immunized with collagen I, in sharp contrast to Th1-skewed immune responses and T-cell lymphoma observed in IEX-1-transgenic mice [23–25]. The increases in Th17 differentiation and Th1 apoptosis were reversed by treatment of the mice with antioxidant *N*-acetylcysteine or mitochondrial-targeted antioxidant mitoquinone [23,26]. Moreover, in obesity, macrophages in adipose tissues elicit a strong inflammatory reaction by changing their phenotype from an anti-inflammatory M2 state to a pro-inflammatory M1 state. M2 macrophages are the major resident macrophages in lean adipose tissue and produce a high level of anti-inflammatory cytokine IL-10, protecting adipocytes from inflammation [27,28]. In contrast, M1 macrophages release an excessive amount of inflammatory cytokines, such as TNF- α , IL-6, IL-12, etc., and generate ROS [27,29]. Our investigation showed that IEX-1 null mutation sensitized M1 but not M2 macrophages to apoptosis, by which a reduced inflammation was maintained in the adipose tissue and IEX-1-deficient mice were protected from obesity induced by high fat diet (manuscript in submission).

Apart from inflammatory cells, null mutation of IEX-1 enhanced apoptosis in hematopoietic stem cells (HSCs). When HSC-enriched lineage-negative, Sca-1-positive, c-Kit-positive cells or Lin⁻Sca⁺1c-Kit⁺ (LSK) cells were examined in bone marrow in the mice [30], an approximate 40% decrease in the frequency of LSK population was seen in the absence as against the presence of IEX-1 ($p < 0.01$) (Figure 3A). Consistent with this was a 50 or 150% increase in apoptosis of bone marrow cells or LSK cells, respectively, in IEX-1^{-/-} mice over WT control mice as measured by apoptosis-specific annexin V staining (Figure 3B). Moreover, analysis of RNA content with a fluorescent dye Pyronin Y and DNA content with Hoescht 34222 showed a significant reduction in the proportion of IEX-1^{-/-} LSK cells at G₀ phase (52.4 ± 4.2 vs 69.7 ± 5.5 , $p < 0.01$), concurrent with a proportional increase in IEX-1^{-/-} cells at G₁ phase compared to WT counterparts (41.1 ± 1.9 vs 29.4 ± 3.1) (Figure 3C; $p < 0.01$). Hence, IEX-1 deficiency appears to increase both apoptosis and proliferation

of LSK cells, which may be ascribed to a significant increase in ROS formation in IEX-1^{-/-} LSK cells compared to their WT counterparts, measured by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate staining followed by flow cytometry [12]. These observations highlight complex effects of IEX-1 on cell survival, apoptosis and proliferation, presumably in association with a delicate balance between oxidative phosphorylation and ROS production in the cells.

2.2 Proapoptotic activity of IEX-1

Our investigations, along with others, have demonstrated requirement of the NLS domain for the proapoptotic activity of IEX-1. Mutation of the NLS domain blunted its proapoptotic but not its antiapoptotic function [11]. Kruse *et al.* have also shown that mutation of the NLS domain abolished both nuclear translocation of IEX-1 as well as its proapoptotic activity in HeLa cells treated with DNA-damaging agent etoposide or UV-B radiation [13]. In the nucleus, IEX-1 was found to associate with RelA/p65 transcription factors and inhibit the transcription, by which the expression of antiapoptotic genes Bcl-2, Bcl-xL, cIAP1 and cIAP2, all targets of NF-κB, were hindered, rendering the cells susceptible to apoptosis [3]. This is also considered to be a feedback response to keep activation of NF-κB under check after stress. There is some discrepancy with respect to function of the C-terminus between our study and the study by Arlt and Schafer [2]. Our mutation study showed that the last 18 (1–138) amino residues were not required for its proapoptotic function [11], whereas the last 25 amino residues (1–131) proved to be a key for its interaction with Rel/p65 in the study by Arlt and Schafer [2]. It is possible that sequence from 131 to 138 amino residues may be needed for its apoptotic function. Alternatively, the discrepancy is ascribed to different cells and/or stimuli used in these two studies. Nevertheless, it is clear that IEX-1 executes its proapoptotic and antiapoptotic functions by very different mechanisms. How its proapoptotic and antiapoptotic activities are determined in a cell remains completely unknown.

2.3 IEX-1-mediated signaling

IEX-1 has been noted to play a pivotal role in the control of the duration of MAPK/ERK activation in response to thrombopoietin (TPO) in megakaryocytes [31]. Binding of TPO to its receptor leads to the activation of MAPK/ERK1/2, which triggers IEX-1 expression and phosphorylation that in turn augments the phosphorylation of ERK and sustains the ERK signaling [31,32]. It is also shown that ERK activation results in IEX-1 phosphorylation that then interacts with the PP2A regulatory subunit B56 [33], thereby inactivating PP2A and preventing dephosphorylation of ERK1/2 and other ERK1/2 substrates, such as Akt1 [34]. Prolonged ERK signaling is necessary for marker induction and cell growth arrest in megakaryocytes and IEX-1 may be one of the substrates that control one of the two events [31]. In support, IEX-1-deficient mice, and not WT control mice, developed irreversible thrombocytopenia after being subjected to a single dose of 300 rad (3 Gy), a non-myeloablative level of radiation (our unpublished data). Besides, IEX-1 transcription was found to be upregulated by ERK-dependent phosphorylation of acute myeloid leukemia 1 (AML1) [31]. AML1 was originally identified as one of the most frequent targets of leukemia-associated gene aberrations. Point mutations that impair AML1 function are found in patients with myelodysplastic syndromes (MDS), a disease manifested by anemia,

thrombocytopenia and/or bone marrow dysplasia [35,36]. Mutation of AML1 may be one of the mechanisms behind the well-described deregulation of IEX-1 in the majority of patients with MDS [36], since multiple consensus-binding sites for AML1 are found in IEX-1 promoter [31].

In another study, IEX-1 has been shown responsible for translocation of myeloid cell leukemia-1 (Mcl-1) from mitochondria to nuclei upon stress that potentially causes DNA damage such as radiation. Nuclear translocation of IEX-1/Mcl-1 complex leads to activation of checkpoint kinase 1 and G₂ arrest, permitting an extra time for cell cycle repair of DNA damage, which may account, at least in part, for tumor resistance to some chemotherapeutic agents [37]. The nuclear translocation is associated with a protective activity of IEX-1, rather than its proapoptosis. Additionally, IEX-1 may interfere with 26S proteasome activity by reducing the expression of some proteasomal components [38]. The ubiquitin–proteasome pathway takes part in the orchestration of multiple cellular processes, such as differentiation, proliferation and apoptosis by selective degradation of specific kinases, phosphatases or transcription factors in cells under stress [39]. IEX-1-mediated regulation of ubiquitin–proteasome activity may be another contributable factor for the complex and contradictory role of IEX-1 in these cellular processes. The complex cellular activities of IEX-1 are summarized in Figure 4, in which IEX-1 inhibits the production of ROS that can promote mitochondria-dependent apoptosis or act as second messenger molecules regulating the expression of multiple genes critical for cell differentiation and proliferation. Cell differentiation and proliferation can be also influenced by IEX-1 through its ability to regulate MAPK/ERK signaling pathways and the ubiquitin–proteasome activity. Under stress that may cause DNA damage, IEX-1 could enhance DNA repair or apoptosis if the damage is too severe to repair by inhibition of NF- κ B transcriptional activity in the nucleus. However, how IEX-1 switches from antiapoptosis to proapoptosis is still a mystery. Because IEX-1 participates in the regulation of multiple central pathways critical for maintenance of cell homeostasis, metabolism, inflammation, DNA stability and stress responses, it can significantly contribute to cancer development and progression in many different ways.

3. IEX-1 as a prognostic biomarker in cancers

IEX-1 is constitutively expressed in epithelia of the skin, trachea, gastrointestinal and genitourinary systems that have direct contact with external environment [14]. A difference in its expression in cancer cells as compared to normal cells in the same anatomic position and cell types may reflect how a cancer cell copes with the relevant stress and, thus, can be very informative in both the prediction of cancer outcomes and in the clinical management of the cancer. As summarized in Table 1, current data collected from global gene expression profile studies suggest that positive IEX-1 expression is associated with a good prognosis in ovarian cancer, because IEX-1 functions as a proapoptotic factor and restrains tumor growth. On the contrary, a high level of IEX-1 expression appears to link poor survival of patients with AML, myeloma, Sézary syndrome and breast cancer, in part, owing to IEX-1–mediated survival of cancer cells. These clinical findings are discussed in the following.

3.1 Ovarian cancer

Ovarian cancer is the most common cause of mortality from gynecological cancers. Owing to atypical clinical symptoms and insufficient screening of available biomarkers, most patients have wide metastasis at stages III – IV at the time of diagnosis. The current treatment is mainly surgical debulking in combination with chemoradiotherapy. Five-year survival rates are considerably low, only at 25–30%, in spite of recent improvement [40]. Immunohistochemical study of 56 patients with epithelial ovarian carcinoma and 21 patients with benign tumors showed an inverse correlation of IEX-1 expression with advanced FIGO stages ($p < 0.001$) and pathological grades ($p < 0.001$), with IEX-1 decreasing as epithelial ovarian tumors progressing from benign to malignancy (Table 2) [5]. Decreased IEX-1 expression was also concurrent with diminished apoptosis measured by terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL). Apoptotic index in groups with negative, weak and strong expression of IEX-1 was 18, 40 or 50 on average, respectively, in 56 patients with ovarian cancer. A positive relationship between IEX-1 expression and apoptosis index highlighted a tumor-suppressive potential of IEX-1 in ovarian cancer via its proapoptotic activity [5]. A lack of IEX-1 expression was also correlated with a shortened survival time: the median survival time for the group with positive IEX-1 expression was about 30 months that was significantly longer than the survival time of 15 months in IEX-1 negative group ($p < 0.01$). The authors believe that IEX-1 expression and FIGO stages are independent survival predictors for ovarian cancer patients [5]. Moreover, Lee *et al.* evaluated transcriptomic biomarkers in salivary collected from 11 ovarian cancer patients and 11 matched controls, followed by RT-qPCR validation in another cohort of 21 ovarian cancer patients and 35 healthy controls [41]. Their investigation identifies that IEX-1, in combination with other four genes can discriminate ovarian cancer patients from the healthy control with 86% sensitivity and 91% specificity [41]. This noninvasive and convenient approach holds great promise for early or initial screening of signs of ovarian cancer, a key for further diagnosis and treatment of this disease. IEX-1 can be one of the biomarkers for initial screening as well as progression of ovarian cancers from benign to malignancy. In future study, we should also investigate how IEX-1 expression is downregulated or lost in a subgroup of ovarian cancer patients.

3.2 Pancreatic cancer

Similar to ovarian cancer, pancreatic cancer is often diagnosed at a later stage when tumor has invaded nearby tissues and has produced distant metastasis. The median survival time for patients with advanced disease is 6 months, and only 1–4% patients survive 5 years after being diagnosed, stressing an urgent need of finding useful molecular markers for early diagnosis and progression of the disease [42]. An early study recognized IEX-1 to be a growth-associated early responsive gene in several pancreatic carcinoma cell lines [43]. In the study of 78 patients with pancreatic cancer by immunohistochemical evaluation, 41 patients (53%) had a positive staining for IEX-1 expression in pancreatic ductal adenocarcinoma and 37 (47%) patients stained negatively for IEX-1 [6]. Similar to what had been described with ovarian cancers, patients positive for IEX-1 expression had a significantly better survival time than those stained negatively, with median survival of 540 as against 207 days, respectively. In a subgroup of 48 patients undergoing a macroscopically

curative surgery, diminished IEX-1 expression was correlated with local invasion of pancreatic tumors to adjacent arteries and serosa [6]. Although not studied yet, IEX-1 appears to promote apoptosis of pancreatic cancer cells and a loss of IEX-1 may result in the growth and invasion of cancer cells. The study concludes that a limited local invasion, negative lymph node involvement and positive IEX-1 expression are significantly favorable factors for survival of patients with pancreatic cancers. The authors suggest that immunohistochemical staining for IEX-1 expression can be used as a useful and independent prognostic factor during preoperative biopsy to estimate the aggressiveness of the tumor and patient prognosis before surgery [6]. However, an opposite was recently reported showing that expression of IEX-1 was associated with a poor prognosis in patients with pancreatic cancers [4]. From a study of 34 patients with a follow-up of 24 months each, the authors found that IEX-1 was coexpressed with nuclear protein 1 (Nupr1) in pancreatic cancer cells and the coexpression was inversely correlated with a survival time of the patients. Moreover, authors demonstrated that IEX-1 was the mechanism underlying Nupr1-mediated protection against stress-induced cell death in pancreatic cancer cells in the mouse model [4]. Additional studies in large samples should clarify whether the discrepancy between the two studies results from different stages or subgroups of the cancer, such as Nupr1⁺ versus Nupr1⁻ pancreatic cancers, and thus establish the potential of IEX-1 as a prognostic biomarker for a subset of pancreatic cancers.

3.3 Blood cancer

MDS are characterized by an inability of bone marrow HSCs to differentiate into one or multiple myeloid lineages of blood cells. HSCs in patients exhibit a high rate of apoptosis in the early stage of the disease, followed by diminished apoptosis at more advanced stages progressing toward AML at a high rate (> 23%) [44,45]. Hofmann *et al.* compared gene expression of bone marrow-derived CD34⁺ hematopoietic progenitor cells of seven patients with low-risk MDS, four patients with high-risk with four healthy control subjects [46]. In low-risk patients, IEX-1 was found to decrease by sevenfold as compared to healthy controls. Similar data was obtained by Prall *et al.* in which CD34⁺ cells from 6 healthy donors and 16 MDS patients were analyzed by cDNA microarray [44]. IEX-1 was the most downregulated gene out of 1,185 tested (a 37-fold decrease) and it was even more drastically reduced in MDS patients with short survival (a 58-fold decrease). The reduced expression was confirmed by Western blotting analysis with IEX-1-specific antibody or quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Steensma *et al.* further showed deregulated IEX-1 expression in more than 60% of MDS patients [47]. Diminished IEX-1 expression was concurrent with high apoptotic rates in bone marrow cells in the early stage of MDS [47]. When the disease progressed into AML, IEX-1 was upregulated at a high level, in parallel to low apoptotic rates of bone marrow cells, consistent with antiapoptosis of IEX-1 in the disease [48]. A crucial role of IEX-1 in HSC homeostasis was highlighted by increased apoptosis of IEX-1^{-/-} HSCs as shown in Figure 3. IEX-1-deficient mice developed irreversible thrombocytopenia, a hallmark of MDS, after a single dose of a non-myeloablative level of radiation. These observations clearly implicate that a decrease in IEX-1 expression is a cause, rather than result, of the pathogenesis of MDS, which lays a foundation for IEX-1 to be a biomarker in monitoring MDS progression to AML.

Multiple myeloma is another type of blood cancer characterized by an uncontrolled proliferation of plasma cells with increased angiogenesis in bone marrow. Gene expression profile of endothelial cells in bone marrow has been used to track multiple myeloma progression from monoclonal gammopathy to multiple myeloma. A high level of IEX-1 expression was detected in five patients with an advanced stage of the disease compared to five monoclonal gammopathy patients [49]. IEX-1 expression was correlated with a high level of angiogenesis and the correlation was corroborated by the small interfering RNA knockdown of IEX-1, qRT-PCR and Western blotting analyses [49]. The study demonstrates a potential for IEX-1 as a biomarker for increasing angiogenesis and tumor progression in this type of blood cancer. Apparently, more case studies across different clinical laboratories are required to validate the finding.

3.4 Cutaneous T cell lymphomas

Cutaneous T cell lymphoma (CTCL) is the second most common form of extranodal lymphomas and one of the most frequent T-cell malignancies. Sézary syndrome and mycosis fungoides, comprising 55% of CTCL, are indolent tumors of mature CD4⁺CD26⁻ T cells [50]. Resistance to apoptosis plays a critical role in the pathogenesis of the early CTCL as opposed to uncontrolled proliferation accounting for the tumor expansion [51]. One of the key inducers of apoptosis in activated T cells is TNF- α . *In vivo* studies using IEX-1-transgenic mice demonstrated that targeted IEX-1 expression in T cells resulted in splenomegaly, lymphadenopathy and T-cell lymphoma due to insufficient apoptosis of activated T cells [52]. A high level of IEX-1 expression was found in CD4⁺CD26⁻ cells in the blood of patients with Sézary syndrome as compared to nonmalignant CD4⁺CD26⁺ cells in the same patients or normal CD4⁺CD26⁺ or CD4⁺CD26⁻ lymphocytes [53]. These malignant T cells were characterized with convoluted cerebriform nuclei and responded poorly to TNF- α -induced apoptosis. The resistance to TNF- α -induced apoptosis in the cells may be attributed to both an elevated level of IEX-1 expression and concomitant reduction in TNF receptor 1 (TNFR1) expression on the cells. In support, we found significant abnormalities in TNF- α signaling pathway, including, among others, a significant reduction of the TNFR1 and ROS production in Sézary cells when compared to normal T cells in response to TNF- α challenge [53].

The findings raise an intriguing possibility that therapeutic targeting IEX-1 may render malignant lymphocytes susceptible to TNF- α -induced apoptosis. Current therapies for patients with advanced CTCL are palliative and durable long-term remissions are rare. The poor 5-year survival of these patients using existing therapies clearly emphasizes an importance of developing new targeted therapies to treat this fatal disease [54]. Upregulation of TNFR1, reduction of IEX-1 expression and/or enhancement of ROS production might sensitize CTCL cells to TNF- α -induced apoptosis, on the basis of our investigation [53]. Several agents such as vitamin D3, dopamine and serine protease inhibitors have been proposed as potential therapeutic agents. Among them, vitamin D3 has been reported to stimulate the expression of TNFRs [55–57] but suppress IEX-1 expression [58]. Chemotherapeutic agent doxorubicin can enhance ROS production within malignant lymphocytes [59]. Moreover, bortezomib (Velcade/PS341; Millennium Pharmaceuticals) is a reversible 26S proteasome inhibitor that has recently been approved by the US Food and

Drug Administration for the treatment of multiple myeloma [60,61]. The Phase II clinical trials have shown considerable clinical efficacy of bortezomib as a single agent in patients with relapsed or refractory cutaneous T-cell lymphoma, with an overall response rate of 67% [62]. The inhibitor hinders NF- κ B activation, and thus IEX-1 expression, because IEX-1 is a transcriptional target of NF- κ B [63,64]. We can, thus, expect therapeutic effects of bortezomib in the setting of IEX-1 overexpression in CTCL patients. Moreover, it has been recently shown that bortezomib activates the mitochondrial pathway of apoptosis in activated CD4⁺ T cells by altering the equilibrium of proapoptotic and antiapoptotic proteins at the outer mitochondrial membrane and by inducing ROS production [65]. Possible involvement of IEX-1 in bortezomib-induced therapeutics merits further investigation.

3.5 Breast cancer

Successful breast cancer treatment depends on the stage of diagnosis and invasiveness of the tumor. Preinvasive and invasive mammary tumors from transgenic ErbB2, Ras and cyclin D1 mice were compared by qRT-PCR for gene expression profiles. A fourfold increase in IEX-1 expression was detected in invasive mouse tumors compared to preinvasive lesions [66]. The finding was subsequently confirmed in human samples in which IEX-1 upregulation was detected in tumors progressing from ductal carcinoma *in situ* to invasive ductal carcinoma. Samples from 42 patients with atypical ductal hyperplasia or ductal carcinoma and from 27 patients with invasive carcinoma of the breast demonstrated a three- to four-fold increase in IEX-1 expression in invasive tumors compared with preinvasive tumor or healthy tissue [66]. The finding of IEX-1 expression correlating with progression of breast cancer to invasive phenotype suggests its potential as a biomarker for a poor prognosis for the disease. IF1 expression was also increased in invasive ductal breast cancer [20,21], arguing against a role for IEX-1 in IF1 degradation. Perhaps, a counterbalance between IF1 and IEX-1 participates in the regulation of a ratio between aerobic glycolysis and oxidative phosphorylation in invasive breast cancer. Conceivably, increased IF1 expression can lead to more ROS formation that augments activation of NF- κ B [20,21]. Activation of NF- κ B then triggers IEX-1 expression that in turn promotes IF1 degradation by which IF1 expression is controlled at a certain threshold. Indeed, cancer cells use both oxidative phosphorylation and aerobic glycolysis with an enhanced ratio of the latter compared to normal cells [67]. An understanding of how oxidative phosphorylation and aerobic glycolysis are altered in cancer cells is of high significance in the treatment and prognosis of cancers.

Several human mammary cell lines were also studied to elucidate the mechanism whereby IEX-1 contributed to mammary tumorigenesis. Homeobox A1 (HOXA1) is a transcription factor playing an important role during normal growth and differentiation in mammalian tissues. Its expression was hardly detected in normal tissues but was clearly presented in human breast tumors [68]. Overexpression of IEX-1 in immortalized human mammary epithelial cell line MCF-10A resulted in abnormal acinar morphogenesis and oncogenic transformation *in vivo* [69]. Microarray analysis of MCF-7 mammary carcinoma cell line transfected with HOXA1 showed a significant increase in IEX-1 expression that conferred apoptosis resistance in the cells and was directly involved in mammary tumorigenesis. Another report demonstrated one- to three-fold upregulation of IEX-1 expression in

MCF-10A or MCF-7 cell lines after exposure to environmental carcinogen, benzo(a)pyrene [70,71].

In contrast to the aforementioned results where IEX-1 upregulation was associated with tumor progression, Hu *et al.* reported downregulation of IEX-1 expression in p53-null model of mammary carcinogenesis using serial analysis of gene expression (SAGE) [72]. A difference obtained from this study might arise from a particular mouse model used, where lack of p53 could potentially downregulate IEX-1 expression. In this study, transplantation of epithelial cells from p53-null mice into cleared mammary fat pad of syngenic mice yielded 60% mammary adenocarcinomas of intraductal origin. SAGE interspecies (mouse–human) analysis, where mouse data and known human breast cancer libraries were compared, demonstrated downregulation of IEX-1 gene in both mouse and human tumors. Further investigation of these opposite profiles of IEX-1 gene expression should be undertaken to evaluate whether IEX-1 expression differs in different subgroups of breast cancers if we are to use IEX-1 as a biomarker for breast cancer progression.

3.6 Colorectal cancer

Colorectal cancer progression is characterized by several stages, including preneoplastic aberrant crypt formation (ACF), preinvasive carcinoma and malignant carcinomas [73]. The ACF is further divided into two classes, hyperplastic (low risk) and dysplastic (high risk) lesions, but these two lesions are difficult to discriminate with conventional immunohistochemistry. Nambiar *et al.* used microarray analysis of two ACF-prone mouse lines in an attempt to identify molecular markers allowing prediction of a tumorigenic potential of ACF [74]. They found increased IEX-1 expression in AKR/J mice that developed low risk lesions as compared to high cancer risk A/J mice after both mice were treated with azoxymethane to induce ACF development. An association of a high level of IEX-1 expression with a low risk of colorectal cancer was later corroborated by immunohistochemical staining of 10 human tumors matched with adjacent normal epithelium [74]. In the same patient, IEX-1 immunohistological staining was markedly reduced in tumor crypts compared to adjacent normal colonic crypts. In accordance to a role for IEX-1 in facilitation of IF1 degradation, IF1 was upregulated in human colon cancer in favor of aerobic glycolysis [21]. The study hints that positive IEX-1 expression, concurrent with diminished IF1 expression, could be good for outcome prediction and prognosis in patients with ACF, an early stage of colorectal cancer, which, however, may not be the case with late stages of colorectal cancers.

In this regard, IEX-1-deficient mice showed a reduced level of inflammatory responses and colorectal cancer after exposure to dextran sulfate sodium (DSS) and azoxymethane. The reduction was attributed to an increase in both proliferation of intestinal epithelium and differentiation of IL-17-producing T cells [75]. Absence of IEX-1 increased the proliferating rate of intestinal epithelial cells by twofold when compared to WT controls after DSS treatment, suggesting accelerated repair of the intestinal barrier in the mice. Consistent with our findings in IEX-1 KO mice, Segditsas *et al.* demonstrated an association of increased IEX-1 expression with colon adenocarcinoma [76]. In that study, Segditsas *et al.* analyzed more homogenous pool of samples in which all human patients had a germline mutation in

APC gene, and all mice had *APC*^{Min (R850X)} mutation [76]. *APC* or adenomatous polyposis coli is a tumor suppressive gene and its mutation has been linked to colorectal cancer both in humans and mice. Microarray analysis followed by qRT-PCR of 16 human and 63 mouse adenomas demonstrated a more than 2-fold or 10-fold increase in IEX-1 expression in human or mouse colon adenoma tumors, respectively [76]. The studies suggest that IEX-1 may play completely different roles in different subsets or stages of colon cancers and a careful study is required to understand the complex before we can determine its potential as a biomarker for monitoring progression of the disease.

4. Conclusion

In the past decade, the mass of data generated by cDNA microarray not only help us to understand the molecular basis for individual subsets of cancers but also offer ample opportunities for new molecular classification of cancers, for their early diagnosis and prognosis, and for developing rational combinatorial therapies of cancers. Current clinical studies clearly show that IEX-1 expression is distinct not only in tumors versus normal cells but also in the same tumors with different disease stages. Its positive expression could indicate a lesion conversion to a metastatic type in AML, breast cancer, myeloma, and conversely, a lack of IEX-1 expression may be a poor indicator of ovarian cancer. Among the cancers analyzed, IEX-1 shows promise for monitoring progression of ovarian cancers from benign to malignant stages. IEX-1 may be also useful for determining a relative lifespan of patients with MDS at early stages, but whether it can be a good biomarker for MDS progression into AML remains to be determined. Controversial conclusions are made with breast, colorectal and pancreatic cancers, to which we do not have good answers yet. The controversial data collected from these cancers may be associated with IEX-1 being both proapoptotic and antiapoptotic accelerators. IEX-1 can be rapidly and transiently induced by a variety of environmental cues allowing us to assess dynamic cancer responses in different individuals at different times, which can be very informative in monitoring cancer progression. IEX-1 may have very different cellular effects on tumor cells as opposed to the surrounding normal cells. These unique attributes of IEX-1 make it a potentially useful biomarker, either alone or in combination with other genes, for cancer prognosis.

5. Expert opinion

Cancers were classified and treated as a disease based on their cell and organ originals in the past. It is now realized that cancers are heterogeneous with diverse etiologies and can be further categorized into subgroups. Even in the same subgroup cancer may be different from one individual to the other and can be discriminated by molecular fingerprints. The cancer should be, thus, treated differently in different patients. This so-called personalized medicine may be fundamental for cancer treatment in the future. The current clinical data suggest that IEX-1 can potentially be a good biomarker for monitoring progression of ovarian cancers from benign to malignant stages. However, because of the heterogeneous nature of the cancer, a combination panel of IEX-1 with other biomarkers is necessary to offer accurate prognosis, probably, in a subgroup of the patients [77], which should be a major focus in the future.

It is also essential to investigate how IEX-1 is diverted from one cellular function to the other. One of the possible underlying the functional diversion mechanisms is via posttranslational modifications. IEX-1 can be phosphorylated at T18 as well as at positions T123 and S126. While phosphorylation of T18 is required for its antiapoptotic activity, it is dispensable for its proapoptotic activity. Whether phosphorylation of IEX-1 at a different site dictates its proapoptotic function in response to a given stressor warrants an investigation. IEX-1 can be glycosylated at multiple sites to varying degrees in different cells [11,14]. Unfortunately, specific measurement of these individual isoforms is not possible at present, hampering the investigation of a significance of each of these isoforms in the complex cellular activities. Alternatively, energy provision in a cell provides a cue for the function of IEX-1 in light of its importance in the regulation of oxidative phosphorylation and ROS production at mitochondria [12]. The current data hint that IEX-1 may promote apoptosis in cells with energy supply mainly via aerobic glycolysis, such as tumor cells and cell lines upon starvation. In contrast, the primary function of IEX-1 is to control ROS production in primary cells in which oxidative phosphorylation is a major source of ATP. Another possible mechanism would be that a counterbalance between IEX-1 and IF1 may determine a relative level of aerobic glycolysis over oxidative phosphorylation, contributing to tumorigenesis. An outcome of IEX-1's effects on individual cancers may depend on the level of IF1 expression. A decrease in IEX-1 expression, concomitant with an increase in IF1 expression may be linked to poor prognosis of colorectal cancer, whereas an increase in both IEX-1 and IF1 expression may be related to invasive ductal carcinoma in breast cancers. Simultaneous investigations of both IF1 and IEX-1 expressions with the same human cancer samples are required for us to establish a ratio of IEX-1 to IF1 and its relevance to energy metabolism in cancer cells in the future.

Third, clinical validation and standardization with large cohorts of patients in both retrospective and prospective studies are a prerequisite for IEX-1 to be used in the clinics. To accomplish this, standardization of detection methods must be first established. Spatial patterns of IEX-1 expression by IEX-1 specific immunohistological staining are more useful for diagnosis and prognosis in cancers than qRT-PCR that gives overall expression levels and may not be representative for the cancer. A readily available method of detection and a standardized antibody for IEX-1 immunohistological staining are crucial, a lack of which impedes comparison and analysis of results across different studies. Polyclonal antibodies against IEX-1 peptides at different regions are available and can specifically recognize IEX-1 in a standard immunohistochemical staining for paraffin-embedded tissues after immunogen-affinity purification. But the antibodies may vary from batch to batch in specificity and affinity due to varying purification and production procedures from time to time. Monoclonal antibody specific for IEX-1 that is suitable for immunohistochemical staining is not available yet, otherwise, it would provide much more consistent results in different clinical studies. Moreover, a standardized method of scoring IEX-1 expression in tissues is also needed to adequately analyze results across different studies. In brief, development of standardized IEX-1 specific antibody and clinical assays to detect IEX-1 in tumor samples is of high priority for IEX-1 clinical study.

Finally, few studies have been conducted in targeting IEX-1 for cancer therapy, but IEX-1 has a potential to serve as a therapeutic target for chemosensitization and effective tumor

treatment. The main goal of cancer therapy is to selectively destroy tumor cells while sparing normal cells to a maximal extent. Differential expression of IEX-1 and its distinct functions in cancer cells versus surrounding normal tissues opens a vital avenue for novel anticancer strategies in patients whose tumors express IEX-1. For instance, most of ovarian cancer patients at the time of diagnosis cannot undergo a curative radical operation of the tumor due to advanced-stage disease with local invasion to nearby organs and/or distant metastasis. Radiotherapy and chemotherapy are the significant choices in treatment of terminal ovarian cancers. Unfortunately, the tumor cells are usually resistant to irradiation or chemotherapy. Overexpression of IEX-1 in HeLa cells enhanced their sensitivity to apoptosis induced by chemotherapeutic agent [78] and overexpression of IEX-1 also sensitized human glioma cell lines to apoptosis induced by irradiation [79]. Thus, IEX-1 may be a candidate gene for enhancing the efficacy of radiotherapy or chemotherapy. Similarly, sensitization of CTCL cells to apoptosis may be achieved by suppressing IEX-1 expression and/or increasing ROS production within malignant lymphocytes using vitamin D3 analogs or chemotherapeutic agent such as doxorubicin [57–59]. Undoubtedly, this strategy requires extensive preclinical and clinical studies as a subtle difference of IEX-1 in the functionality between cancer and surrounding cells is yet to be explored.

IEX-1 is an important but truly understudied gene. Its deregulation in cancer cells, in conjunction with its critical role in cell apoptosis, survival and growth stresses its role in the pathogenesis of cancers. However, use of IEX-1 as a biomarker for cancer prognosis remains a significant challenge because the precise molecular action of IEX-1 is still elusive; there is no a standardized detection assay for clinical screenings of IEX-1 expression and we understand too little about the mechanism underlying its complex cellular functions.

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Article highlights

- Clinical application of IEX-1 as a prognostic biomarker is promising but still at an early stage of the investigation.
- IEX-1 can be rapidly and transiently induced in response to a variety of stimuli, by which it may link environmental cues to the energy metabolic regulation at mitochondria in cancer cells via the control of IF1 expression level.
- IEX-1 differently affects the survival, apoptosis, proliferation and differentiation in different cells, which can directly restrain or promote cancer development.
- Distinct IEX-1 expression in normal versus cancer cells and in the same tumor with different stages makes it a potentially valuable biomarker for cancer diagnosis and prognosis.
- Further clinical validation and standardization of clinical IEX-1 detection across different studies are urgently needed for IEX-1 to gain clinical application.
- Different effects of IEX-1 on the survival of tumor cells versus normal cells may confer sufficient strategies to destroy tumor cells while maximally sparing normal cells in cancer treatment.

This box summarizes key points contained in the article.

Figure 1. A schematic diagram of IEX-1 protein and functional domains
ERK-p, a phosphorylation site for extracellular signal-regulated kinases; PEST, a proline (P), glutamic acid (E), serine, and threonine-rich sequence; NLS, nuclear localization sequence; TM, transmembrane and NG, *N*-linked glycosylation. The numbers indicate amino residue positions in IEX-1 polypeptide. C₁₈ and C₂₅ are truncated for 18 or 25 amino residues at the C-terminus, respectively. Each functional domain is denoted by proapoptotic or antiapoptotic activity of IEX-1 underneath the diagram based on DNA mutagenesis study [11].

Figure 2. IEX-1's role in regulation of mitochondrial respiration and ROS production

Respiration chain in the inner mitochondrial membrane consists of complexes I – IV and Fo/F1 ATP synthase/ATPase that produces ATP from protons H⁺. IF1 can bind to the F1 complex and blocks its ATPase activity. IEX-1 induced by stress can bind to IF1, making it prone for degradation.

Figure 3. Aberrations in IEX-1-deficient bone marrow stem cells

Bone marrow cells were isolated from IEX-1 KO and WT mice and analyzed for a frequency of LSK cells (**A**), apoptosis in bone marrow and LSK cells (**B**) and cell cycle (**C**). The numbers in the profiles are the mean frequency $\pm$ standard derivation (SD) relative to total bone marrow cells (**A** and **B** upper panel) or LSK cells (**B** low panel and **C**).

Figure 4. Complex cellular functions of IEX-1. IEX-1 has been shown to regulate multiple cellular processes including apoptosis, proliferation and differentiation and DNA repair. These diverse functionalities may be achieved by its ability to control ROS production at mitochondria, to modulate the ubiquitin–proteasome activity, to sustain the duration of MAPK/ERK signaling and to inhibit NF-κB-mediated transcription.

Table 1

Summary of IEX-1 expression in various cancers.

Cancer	IEX-1	Possible function	Prognosis	Refs.
Ovarian	+	pro-Apo	Longer survival	[5]
Pancreatic	↑	anti-Apo	Poor	[4]
Pancreatic	+	pro-Apo	Longer survival	[6]
MDS early	↓	anti-Apo	Low risk	[44,46]
MDS early	↓↓	anti-Apo	High risk	[46,47]
AML	↑	anti-Apo	Poor	[47]
Myeloma	↑	anti-Apo	Poor	[49]
Breast	↑	anti-Apo	Poor	[66]
Colorectal	↓	pro-Apo	High risk	[74]
Sézary syndrome	↑	pro-Apo	Poor	[53]

+, positive; ↑, increase or ↓, decrease in IEX-1 expression compared to normal counterparts; pro-Apo, proapoptosis; and anti-Apo, antiapoptosis.

MDS: Myelodysplastic syndromes.

Table 2

Inverse correlation of IEX-1 expression with progression of ovarian cancer (n = 56).

	Patients	IEX-1 staining cores				p value
		0	1	2	3	
Age group						
60 years	30	16	3	2	9	0.241
> 60 years	26	19	1	3	3	
* FIGO stage						
I – II	7	3	3	1	0	<0.001
III – IV	49	32	1	4	12	
Grade of differentiation						
G1 – G2	14	5	3	4	2	<0.001
G3	42	30	1	1	10	
Median survival (months)						
IEX-1-positive	24			30.1		<0.01
IEX-1-negative	32			15.5		

* FIGO, a standard International Federation of Gynecology and Obstetrics (FIGO) classifications of ovarian cancer.