A distinct role in breast cancer for two LIV-1 family zinc transporters

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Abstract
Zinc, essential for normal cell growth, is tightly controlled in cells by two families of zinc transporters. The aberrant expression of zinc transporters from the LIV-1 family of ZIP (Zrt/Irt-like protein) transporters is increasingly being implicated in a variety of disease states. In the present paper, I describe a mechanism for the role of ZIP7 in the progression of breast cancer, identifying it as a new target in breast cancer. Furthermore, I document a link between another zinc transporter, LIV-1, and breast cancer metastasis, identifying it as a potential new prognostic indicator of breast cancer spread.

Introduction to zinc transporters
Zinc is essential for life and, as such, is involved in the control of gene transcription, differentiation, development and growth [1]. Zinc deficiency can be detrimental, causing stunted growth and serious metabolic disorders [2], whereas excess zinc can be toxic to cells [3]. It is therefore important that cellular levels of zinc are well controlled. This is achieved primarily by two families of zinc transporters which transport zinc across membranes in opposing directions. The ZnT family (previously termed CDF for cation diffusion facilitator) of zinc transporters [now termed SLC (solute carrier) 30A] are responsible for zinc efflux [4], whereas the ZIP family (for Zrt/Irt-like proteins, now termed SLC39A) of zinc transporters [5] are responsible for zinc influx. The ZnT family contains nine human sequences and the ZIP family contains 14 human sequences [6]. Increasingly, various members of the SLC39A family of ZIP transporters are being implicated in disease states. Since aberrant expression of zinc transporters has been suggested to lead to uncontrolled growth such as cancer, any molecules controlling cellular zinc levels are worthy of investigation.

The LIV-1 family of ZIP transporters
The SLC39A family is divided into four separate groups, nine of which are in the LIV-1 subfamily [6]. All ZIP transporters are predicted to consist of eight TM (transmembrane) domains with conserved histidine residues within TM 4 and 5 (Figure 1) that are believed to be involved with zinc transport [7]. Additionally, they contain a histidine-rich region (of the form HXHXXH where H is histidine and X is any amino acid) on the long cytoplasmic loop between TM 3 and 4 which is also thought to be involved with zinc transport (Figure 1). Importantly, the nine human members of the LIV-1 subfamily contain all these motifs, as well as additional histidine-rich regions on the N-terminus and extracellular loop between TM 2 and 3 [6], with a considerable increase in the number of histidine residues, as indicated in Figure 1, the exact role of which is still uncertain. The LIV-1 family are grouped into a separate subfamily owing to the presence of an additional motif in TM 5 (HEXPHEXGD) which fits the consensus active-site motif of metalloproteinases [6]. The initial histidine residue of this motif matches the position of the conserved histidine residue in the other ZIP sequences, suggesting an important role in zinc transport. Occasionally, some of these family sequences have been predicted to contain fewer than eight TM domains, but this is most likely to be due to the presence of the conserved proline residue within this sequence, preventing certain TM predictive software packages forecasting this region as a TM region [6]. However, the alignment of the LIV-1 family and the other ZIP family members is so robust that it seems appropriate that they all contain eight TM domains [6]. The TM domains are highly conserved within the ZIP transporters, often containing well-conserved interactive residues such as histidine, glycine and aspartate, suggesting an involvement of all TM domains in zinc transport. It is noteworthy that six mutations from within different TM domains in ZIP4 cause acrodermatitis enteropathica, the zinc deficiency disease, by disruption of zinc transport ability and/or regulation [8]. Most of the LIV-1 family proteins are present on the plasma membrane and have been demonstrated to transport zinc into cells [9–12]. However, there is a group designated KE4 proteins within the LIV-1 subfamily which are predicted to reside on intracellular membranes such as the endoplasmic reticulum [6] and therefore would transport zinc into the cytoplasm. We have demonstrated that one of these, ZIP7 (SLC39A7/HKE4), resides on the endoplasmic reticulum membrane [13], the same location as the Arabidopsis homologue, IAR1 [14], and transports zinc into the cytoplasm.

Key words: breast cancer, LIV-1, solute carrier 39A6 (SLC39A6), solute carrier 39A7 (SLC39A7), zinc transporter, Zrt/Irt-like protein 7 (ZIP7).
Abbreviations used: IGFR, epidermal growth factor receptor; IRF-1; IRF-1; insulin-like growth factor 1 receptor; IRS, insulin receptor substrate; PIP1B, protein tyrosine phosphatase 1B; sEFG1, short interfering RNA; SLC, solute carrier; STAT3, signal transducer and activator of transcription 3; TamR, tamoxifen-resistant; TM, transmembrane; ZIP, Zrt/Irt-like protein.
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The predicted tertiary structure of ZIP transporters
ZIP transporters are eight(TM)-domain proteins with extracellular N- and C-termini. Conserved histidine residues in TM 4 and TM 5 are involved in zinc transport as well as a histidine-rich motif on the loop between TM 3 and 4. The LIV-1 subfamily contains an additional histidine in TM 5, part of the conserved potential metalloprotease motif (HEXPHEXGD), which forms the signature sequence for this subfamily, as well as additional histidine-rich motifs as indicated. The star represents a mixed charge region thought to be involved in zinc transport as well as the conserved CPALLY motif. Modified from [6] with permission ©2003 Elsevier.

The role of zinc in anti-oestrogen-resistant breast cancer
The primary clinical treatment for oestrogen-receptor-positive breast cancer is anti-hormones such as tamoxifen. These agents effectively stop the growth of these cancers, although, with time, resistance develops and the cancers can regrow with a more aggressive phenotype [15]. We have developed a unique panel of anti-oestrogen-responsive and -resistant cell lines derived from the oestrogen-receptor-positive human breast cancer cell line MCF-7 [16,17] in an effort to study the mechanisms involved in the development of this type of resistance. Our tamoxifen-resistant (TamR) cells exhibit an increased rate of growth and increased ability to invade in the presence of tamoxifen by efficiently using alternative signalling pathways, such as EGFR (epidermal growth factor receptor) [16,17], Src [18] and IGF-1R (insulin-like growth factor 1 receptor) [19], which allows them to exhibit this more aggressive phenotype [20].

We have additionally observed that these TamR cells have approximately double the level of intracellular zinc of the wild-type MCF-7 cells, when examining fluorescence after loading the cells with the zinc-specific dye Newport Green [21]. Elevated zinc has also been observed in human breast tumours compared with benign breast tissue [22–24], as well as in an N-methyl-N-nitrosourea-induced model of rodent mammary tumorigenesis that has been widely used for investigating breast cancer development [25] which shows up to 19-fold zinc accumulation in mammary tumours compared with their normal counterparts, irrespective of dietary zinc intake [26].

Significantly, the activation of receptor tyrosine kinases has been shown previously to be a result of zinc-induced inhibition of PTP1B (protein tyrosine phosphatase 1B) [27]. Known substrates for PTP1B include EGFR, IRS (insulin receptor substrate) 1/2, IGF-1R and Src [28] and therefore inhibition of a molecule such as PTP1B in our TamR cells might have a significant effect on the activation of signalling pathways by preventing their dephosphorylation and subsequent inactivation. There is little information available in the literature concerning zinc-induced effects on signalling molecules; however, there is some evidence to suggest that zinc can induce gene expression as a downstream effect of activating growth factor signalling pathways [29], including activation of EGFR at Tyr845 by transactivation via c-Src in human epidermoid carcinoma A431 cells [30] and human bronchial epithelial BEAS cells [31].

Additionally, however, we have recently shown that TamR cells harness IGF-1R/IRS-1 signalling to support EGFR activation [19], and a prolongation of such responses would thus also aid aggressive cell behaviour. A role of zinc in insulin and IGF-1R signalling has already been documented [32–34], as well as an ability to induce activation of the non-receptor tyrosine kinase, Src, which will have additional consequences leading to increased proliferation, angiogenesis and survival and increases in motility and invasive capability [35].

The role of ZIP7 in breast cancer
On examination of the expression of all nine human LIV-1 family members in our TamR cells, we observed an increased expression of just one family member, ZIP7 [36]. In an effort to investigate whether this increased expression of ZIP7 was linked to the increased intracellular zinc that we had observed in these cells, we examined whether short (20 min) treatments with 20 µM zinc could also cause stimulation of the various signalling molecules shown previously to be involved in driving the growth of these cells. We were able to demonstrate that such zinc treatment could cause considerable activation of EGFR, Src and IGF-1R signalling molecules as well as increases in growth and invasion [21], all elements observed to be involved in the aggressive phenotype of TamR cells [16–20].

Importantly, we also demonstrated a role for ZIP7 in this aggressive phenotype by removal of ZIP7 using siRNA (short interfering RNA). Excitingly, in the presence of ZIP7 siRNA, the previously observed activation of EGFR, Src, IGF-1R and Akt was not observed [21]. Furthermore, the ability of TamR cells to migrate across Matrigel was considerably reduced in the presence of siRNA for ZIP7. Interestingly, the converse was also true. When wild-type MCF-7 cells were transfected with a construct expressing recombinant ZIP7 for 24 h, there was evidence of activation of EGFR, Src and IGF-1R which was paralleled by an increase in motility.

In order to try to define the mechanism of action of ZIP7, it was necessary to test whether the genetic manipulation of ZIP7 was consistent with a corresponding alteration in intracellular zinc levels. We were able to investigate the intracellular zinc levels under these conditions by loading the cells with zinc-specific dyes and either reading the population
The predicted function of ZIP7

Zinc entering the cell from outside will be highly buffered and absorbed within a ‘muffler’ before transfer to the intracellular store, in this case the endoplasmic reticulum. Any zinc entering the cytoplasm will be transported by ZIP7 and released in the form of a zinc wave, leading to inhibition of phosphatases.

Figure 2 | The predicted function of ZIP7

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The potential role of LIV-1 in breast cancer

LIV-1, the first member of the LIV-1 family to be described, has been known to be associated with oestrogen-receptor-positive breast cancer since 1994 [40] and present in increased amounts in oestrogen-receptor-positive breast cancers with an ability to spread to the lymph nodes [41]. More recently, LIV-1 has become a reliable marker of oestrogen-receptor-positive cancers [42,43] used to distinguish the luminal A type of clinical breast cancer [44,45]. LIV-1 has also been associated with STAT3 (signal transducer and activator of transcription 3), a molecule with a well-defined link to breast cancer progression [46]. This association was discovered in zebrafish embryos where LIV-1 was shown to be the downstream target of STAT3 and essential for the nuclear localization of another transcription factor, Snail, which causes loss of cell adhesion by reducing expression of adherence genes such as E-cadherin [47]. Further evidence for an involvement of LIV-1 with Snail was provided by the observation that siRNA for LIV-1 reduced HeLa cell invasion via a Snail pathway [48].

Expression of LIV-1 in breast cancer samples

In order to dissect the role of LIV-1 in breast cancer, we have examined the expression of LIV-1 mRNA in a small series of breast cancer samples and related LIV-1 levels to prognostic indicators of cancer development. For this analysis, we used a series of tumour samples from 74 patients presenting with primary breast cancer to the Breast Cancer Unit, City Hospital, Nottingham, U.K. [36]. We first observed that expression of LIV-1 in these samples was of a heterogeneous nature, with low expression in some samples and quite high expression in others. Comparative analysis of densitometric data was performed using a statistical analysis package, and statistical significance was assumed if \( P < 0.05 \). We were able to confirm the previous result that LIV-1 was an oestrogen-regulated gene and a prognostic marker of endocrine response by showing a positive correlation to oestrogen receptor status and an inverse relationship to EGFR. We also demonstrated a positive association of LIV-1 with two other erbB receptor tyrosine kinase members, erbB3 and erbB4, and another growth factor receptor, IGF-1R. Interestingly, in the light of both of these models and relies on the ability of ZIP7 to release zinc from intracellular stores. This additionally fits our recent observations [21], provided that the zinc entering the cell is distributed directly to the endoplasmic reticulum, via some as yet unknown mechanism, such as the proposed ‘muffler’ [39], and then released in a wave by a ZIP7-dependent mechanism, causing the zinc-induced inhibition of phosphatases, consistent with [37]. The emergence of zinc dyes that are capable of measuring zinc in stores such as the endoplasmic reticulum will aid confirmation of this mechanism. This is an important finding with implications for treatment of diseases such as cancer, and provides ZIP7 as a potential new and novel target for inhibiting intracellular zinc-dependent mechanisms, such as activation of tyrosine kinases.
the above suggestion that LIV-1 was a downstream target of STAT3, we observed a positive correlation in these breast cancer samples between STAT3 and LIV-1 \( (P = 0.007) \). This is an interesting finding, especially since the level of STAT3 has been well documented to be associated with breast cancer progression \[49\]. LIV-1 expression was also associated with grade as it was highly expressed in low-histological-grade tumours.

**Conclusion**

There is increasing evidence that the LIV-1 family of zinc transporters is important in breast cancer cells and shown how it may be a significant aberrant expression being associated with a variety of disease progression \[49\]. LIV-1 expression was also associated with low-histological-grade breast cancer cells. Breast Cancer Res. Treat. \textbf{97}, 263–274


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