PRECLINICAL STUDY

Prognostic value of LINE-1 retrotransposon expression and its subcellular localization in breast cancer

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Abstract Long interspersed nuclear element 1 (L1) belongs to a family of retrotransposons. Expression of the normally repressed L1 retrotransposons has been shown to induce genome instability by creating DNA double-stranded breaks and chromosomal rearrangements through the process of retrotransposition. At present, little is known about the expression of L1-encoded ORF1p and ORF2p which are indispensable for its retrotransposition activity. Given its potentially harmful effects on the genome, we investigated the implications of both ORF1p and ORF2p expression and their subcellular localization in a range of breast cancer cell lines and breast tumor tissues including 15 normal breast tissues, 25 fibroadenomas, 25 ductal carcinomas in situ (DCIS), and 95 invasive cancers. Clinicopathologic parameters and survival outcomes were investigated in association with the cytoplasmic and nuclear expression of ORF1p and ORF2p using univariate and multivariate analysis. High cytoplasmic expression of ORF1p and ORF2p was seen in DCIS tumors, but they were not related with survival outcome. The majority of invasive cancers were found to express both ORF1p and ORF2p in the cytoplasm, while nuclear expression was also seen in a subclass of those invasive cancers in the range of 28–31 %. Tumors with high nuclear expression of ORF1p and ORF2p were more significantly associated with lymph node metastasis ($p = 0.001$) and the worst patient survival ($p < 0.0001$) than those with cytoplasmic expression. This is the first study examining the effects of both ORF1p and ORF2p expression in breast cancer tissues. Our observation shows altered expression patterns of ORF1p and ORF2p within invasive cancers, which are related to differences in overall patient survival. The differing patterns of both cytoplasmic and nuclear ORF1p and ORF2p expression indicate that further studies of the biology and function of L1 retrotransposons are required in breast cancer.

Keywords Breast cancer · Retrotransposons · LINE-1 · Immunohistochemistry · Subcellular expression · Prognosis

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Introduction

Breast cancer is challenging as it is a group of quite diverse biologies—a great deal of molecular events occur with these carcinomas and differ in profile between subtypes therein. Tumors develop under the influence of both genetic and epigenetic changes. It has been suggested that epigenetic changes are more frequent in cancer cells than genetic mutations [1]. Alterations of DNA methylation patterns are a common feature of epigenetic change that results in aberrant gene expression. During tumorigenesis, cells undergo both genome-wide DNA hypomethylation and local hypermethylation of CpG islands associated with promoters [2]. Hypomethylation of long interspersed nuclear element 1 (LINE-1 or L1) sequences occurs at a

very early stage of malignant transformation, resulting in inappropriate expressions of L1 retrotransposons [3, 4]. Indeed, the majority of DNA methylation studies have used L1 retrotransposon's expression as an indicator of the methylation status of genome [5]. It was recently reported that the hypomethylation of L1 retrotransposons activates an antisense promoter for cancer-specific genes in human cells [6].

About 20 % of the human genomes are made up of L1 retrotransposons, which are capable of reverse transcribing their own mRNAs and inserting DNA copies into new places within the genome [7]. While most of the copies of L1s are defective because of truncations or mutations, the human-specific (L1Hs) subfamily, which contains intact, full-length L1 elements, is still potentially active in the human genome [8]. At present, at least 100 copies of L1Hs have been identified as functional elements, retaining their ability to move about the genome, i.e., retrotransposition competent [9]. An active L1 retrotransposon is composed of the 5′-untranslated region (5′-UTR), which harbors an internal promoter, two open reading frames, and the 3′-UTR which includes a poly-A tail. L1 encodes two proteins: a 40-kDa protein (ORF1p) with RNA-binding activity [10], and a 150-kDa protein (ORF2p) with critical endonuclease and reverse transcriptase activities [11, 12]. ORF2p cuts genomic DNA to provide a 3′-end primer from which the L1 mRNA is copied into DNA. An insertion is then made at this new genomic site, resulting in a newly retrotransposed L1 DNA copy. These L1 insertions are capable of altering the genome in myriad ways by disrupting genes, altering splicing, increasing the frequency of recombination, and negatively affecting the stability and integrity of the genome because of their ability to create breaks in genomic DNA during the process of mobilization or retrotransposition [13–15].

A recent study surveying lung tumors and comparing their genomes against their adjacent normal tissues demonstrated that tumors exhibit high frequencies of L1 retrotransposition activity that are not present in the normal tissues [16]. These findings indicate that, in principle, L1 expression has the potential to contribute to genomic instability. Because of the potential harmful impact of L1 activity, it is believed that L1 expression is held in check by a variety of genome defense mechanisms [17, 18]. L1 expression is undetectable in the majority of normal cells, and thus the detection of overexpression of L1 retrotransposons may serve as an indicator of unstable genome. At present, little is known about the L1 expression pattern. Given that the L1 retrotransposition could induce genome instability, we set out to characterize the expression patterns of both ORF1p and ORF2p at various stages of breast cancer and to determine whether the expression pattern might be of clinical usefulness.

Materials and methods

Cell cultures

Normal human breast epithelial cells (HMEC) and mammary epithelial growth medium (MEGM Bullet kit) were obtained from Lonza (Walkersville, MD). The HMEC (Lonza-CC-2551) and immortalized non-tumorigenic MCF10A cells (ATCC-CRL-10371) were cultured using the MEGM Bullet kit supplemented with 10 μg/ml of insulin. The T47D, SKBR3, BT-20, MDA-MB-361, MCF-7, Hs578T, MDA-MB-231, and MDA-MB-436 cell lines were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium with 2 mM L-glutamine and 10 % FCS at 37 °C under 5 % CO_2.

Protein expression and purification

Codon-optimized synthetic human L1 genes encoding either an ORF1p or ORF2p were synthesized and assembled commercially (GeneART, Regensburg). The sequences encoding ORF1p and ORF2p were cloned separately into the BamHI–XhoI sites of pGEX-4T-1 (GE Healthcare) and the NdeI–XhoI sites of pET32b (Novagen) to create fusion proteins of GST-ORF1p and 6xHis-ORF2p. These constructs were transformed into the E. coli strain BL21-CodonPlus (Stratagene) for the expressions of fusion proteins. The GST-ORF1p purification was performed using Glutathione HiCap column (Qiagen) and the GST tag was removed from the fusion protein by thrombin digestion. To purify the 6xHis-ORF2p, the Ni–NTA affinity column (Invitrogen) was used as described in the user manual. The 6xHis tag was removed by thrombin digestion. New Zealand rabbits were immunized with the purified ORF1p and ORF2p commercially by IMVS, Australia. Antibodies were selectively enriched and purified by two steps: the IgG fractions of antibodies were selectively isolated by saturated ammonium sulfate precipitation (Pierce), followed by antigen-specific affinity purification. The affinity-purified ORF1p and ORF2p were covalently linked to N-hydroxysuccinimide (NHS)-activated HiTrap columns (GE Healthcare), and the column washing and elution of antibodies were performed according to the supplier's instructions.

Validation of antibodies

The construction of the EBNA1-based $L1_{RP}$ expression vector has been described previously [19]. HeLa cells ($\sim 2 \times 10^5$) were transfected with 1 μg of $L1_{RP}$ vector using Lipofectamine-2000 (Invitrogen). At 36 h after transfection, the $L1_{RP}$ transfected cells were enriched by growing in 200 μg/ml hygromycin for 10 days. After

eliminating untransfected cells, cells were fixed in 4 % formaldehyde for 15 min and permeabilized in PBS/1 % BSA/0.25 % Triton X-100 for 10 min, and then blocked in PBS/1 % BSA for 1 h. Cells were incubated overnight at 4 °C with affinity-purified anti-ORF1p or anti-ORF2p antibodies (1:60, 1:80 dilutions, respectively). Bound antibodies were visualized using Texas Red or FITC-labeled secondary antibodies (Jackson Immunoresearch). Between steps, cells were washed with PBS/1 % BSA. Nuclei were stained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). The images were photographed using an Olympus IX81 fluorescence microscope and overlaid using Adobe Photoshop. For immunofluorescence analysis of cancer cell lines, cells were grown on glass coverslips in a 12-well plate at a density of 1×10^3 cells per well. Cells were stained with antibodies and visualized as described above. The specificity of the antibodies was further confirmed by western blotting of whole-cell lysates.

Western blot analysis

Cell lysates were prepared using MPER reagent (Pierce), according to the manufacturer's instructions. Protein samples were separated using a 4–12 % Bis–Tris ployacrylamide gel (Invitrogen) and transferred onto Nylon membranes (GE Healthcare). Western blot analyses were performed with anti-ORF1p and anti-ORF2p antibodies at 1:2,000 and 1: 3,000 dilutions, respectively, followed by the addition of HRP-conjugated secondary antibodies (Dako Cytomation). The resulting signals were visualized using the ECL chemiluminescence system (Pierce). To confirm protein normalization, the membranes were stripped and reprobed with α-tubulin antibodies (Sigma).

Patients and tumor samples

Formalin-fixed paraffin-embedded postsurgical breast specimens were retrieved from the archival material in our department, following ethics approval by the ACT Health Human Research Ethics Committee (no. ETH.8/09.789). These included blocks from 160 randomly selected patients with breast cancer diagnosed between 1997 and 2010 with a mean follow-up of 10.7 years (range 1–14 years). This included 95 patients with invasive cancers (30 grade I, 35 grade II, and 30 grade III) and 25 DCIS (9 low, 8 intermediate, and 8 high grades). Patient demographics, disease pathology, hormone receptor status and treatment information on these patients were obtained from the Australian Capital Territory and South East New South Wales Breast Cancer Treatment Group (ACT & SE NSW BCTG) database. Patients on this database had given informed consent for these details to be recorded and their disease status to be followed annually [20]. Patients were excluded if they were male, had bilateral disease, or had neoadjuvant treatment. We also retrieved blocks of 15 normal breast tissues specimens from women who had reduction mammoplasty. For prognostic evaluation, the following patients' clinico-pathologic features were included into the analysis: patient age (≤50 vs. >50 years), tumor size (≤20 vs. >20 mm), tumor type, histologic grade (1 vs. 2 vs. 3), and tumor stage including lymph node metastases, menopausal status, and hormone receptor status. The outcomes examined were breast cancer recurrence rate, overall survival from the time of primary surgery, breast cancer-specific survival, and aggregate survival from other causes.

Immunohistochemistry

Immunohistochemistry was performed on the Bond automated system (Vision Biosystem), following a standard protocol. In brief, 5-μm tissue sections were dewaxed, rehydrated through graded alcohol, and stained separately with anti-ORF1p and anti-ORF2p antibodies. We used heat retrieval for 28 min at a pH of 8.0. The chromogen Fast Red (Leica Biosystems) and the DAKO Envision kit were used to amplify and visualize the signals. Hematoxylin counterstaining will allow the visualization of cell nuclei. In each run, a positive and a negative isotype-matched control were included on each slide to ensure that there is no false-positive staining. In addition, the normal breast HMEC and breast cancer T47D cell lines were used as an additional negative and positive control, respectively. ORF1p and ORF2p expression was evaluated separately according to the degree and the proportion of staining regardless of location. Tumor having no staining or staining in less than 10 % of tumor cells is considered as negative expression.

Immunohistochemical scoring

Cytoplasmic and nuclear expression levels of each ORF1p and ORF2p were scored individually by one pathologist and one investigator blinded to clinical parameters, using a weighted histocore method [21]. The discrimination of normal and malignant breast tissues was based on morphologic grounds. For each localization, an assessment was made as the degree of staining intensity and the percentage of cells stained with that intensity in the nucleus or in the cytoplasm, which was graded semiquantitatively to produce an intensity distribution score (IDS), with the maximum score 300 (if 100 % cells stain strongly positive) and the minimum score 0 (if 100 % cells stain negative). The IDS was calculated as follows: IDS (maximum score 300) = $1 \times$ percentage of weakly stained cells + $2 \times$ percentage of moderately stained cells + $3 \times$ percentage of strongly stained cells. Average IDS values were determined by examination of 10 fields.

Statistical analysis

Analysis was performed with SPSS, V15.0 software for windows (SPSS Inc., Chicago). The relationships between expressions of ORF1p and ORF2p in tumor cells and non-neoplastic cells were evaluated by Fisher's exact test and χ^2 tests. The same test was used to examine the association between ORF1p and ORF2p expression with prognostic factors such as tumor grade, tumor size, nodal status, and hormone receptor status. Kaplan–Meier survival curves were constructed, and log-rank test was used to assess whether nuclear or cytoplasmic expression of ORF1p and ORF2p had any effect on survival. Multivariate analyses were performed using the Cox regression model. The overall survival and the relapse-free survival were calculated from the date of surgery until the date of death or up to the last follow-up and the date of the relapse or up to the last follow-up, respectively. The statistical significance level was set at $p < 0.05$.

Results

Anti-ORF1p and Anti-ORF2p antibodies

To investigate breast cancers for expression of L1 retrotransposons, we constructed a synthetic L1 gene expressing either an ORF1p or ORF2p. The codon of gene was synonymously optimized for bacterial expression of full-length protein with an apparent molecular mass 43-kDa of ORF1p and 150-kDa of ORF2p (Fig. 1A, B). Antibodies raised against ORF1p and ORF2p were purified and enriched by saturated ammonium sulfate, followed by antigen-specific affinity chromatography. These antibodies recognized ORF1p and ORF2p at the expected band sizes of 43 and 150 kDa, respectively (Fig. 1C, lanes 3 and 6). As a negative control, we used bacterial cell extract because the bacterial genome does not contain L1 sequences. The specificity of antibodies was tested using the bacterially induced expression of the fusion protein tagged with GST-ORF1p and 6xHis-ORF2p. The fusion proteins, with the expected sizes of 69 kDa for GST-ORF1p and 150 kDa for 6xHis-tagged ORF2p, were recognized in the induced bacterial extracts, but not in uninduced extracts (Fig. 1C, lanes 2 and 5).

To test the antibodies for their ability to detect human ORF1p and ORF2p, we transfected HeLa cells with an L1 expression vector containing a full-length $L1_{RP}$ retrotransposon under the control of the CMV promoter [19]. Cells transfected with this vector were initially enriched by applying antibiotic selection for 10 days. After eliminating untransfected cells, cell lysates were subjected to western blot using anti-ORF1p and anti-ORF2p antibodies. In

previous studies, HeLa cells have been shown to express at least a subset of L1 retrotransposons [22]. Human embryonic carcinoma NTera.2D1 cells, which express high levels of L1 mRNA and the encoded proteins as a result of altered transcription start sites [23], were used as a positive control. As shown in Fig. 1D, both ORF1p and ORF2p were significantly overexpressed in cells transfected with $L1_{RP}$ vector compared with weak detection of untransfected cells, which is consistent with previous reports [22, 24]. Next, we performed immunofluorescence assay. Although untransfected HeLa cells (not shown) showed only a weak background staining, which may be due to the endogenous L1 expression [22], the $L1_{RP}$ vector-transfected cells exhibited strong immunostaining in each cell of the culture. Notably, both ORF1p and ORF2p displayed high immunofluorescence signals in the cytoplasm of cells (Fig. 1E, panels a and b). This observation is consistent with previous reports in which strong expressions of ORF1p and ORF2p were demonstrated in the cytoplasm of human cell lines including HeLa [24, 25]. To further confirm that immunoreactivity was a result of the interaction of the antibodies with L1 proteins, we performed immunofluorescence assay with antigen-depleted primary antibodies, which were generated by preabsorption with purified ORF1p and ORF2p immoblized on Sepharose column. Except for a weak background, no immunoreactivity was detected in the antigen-depleted antibodies (Fig. 1E, panels c and d), suggesting that detected fluorescence signals are a result of the specific interaction between L1 proteins and antibodies. Together, these results suggest that the antibodies can recognize human ORF1p and ORF2p with high specificity.

Expressions of ORF1p and ORF2p in breast cancer cell lines and tissues

Earlier studies revealed that the level of ORF1p is significantly elevated in breast cancer cell lines [26, 27]. A recent study of clinical samples has also shown that expression of ORF1p is widespread in breast tumors [25], but the expression levels of ORF2p remain unknown. To gain insight into L1 expression, we first determined the ORF1p and ORF2p expression in breast cancer cell lines by Western blot analysis. Clinicopathologic features of these cells are summarized in Table S1. Human embryonic carcinoma NTera.2D1 cells were used as a positive control. As shown in Fig. 2A, both ORF1p and ORF2p were overexpressed in all tested breast cancer cell lysates (T47D, SKBR3, BT-20, MDA-MB-361, MCF-7, Hs578T, MDA-MB-231, and MDA-MB-436), but not in non-tumorigenic breast epithelial HMECs or its derivative MCF10A cell line. Notably, the relative expression levels of ORF1p and ORF2p were markedly higher in low-invasive cancer cells
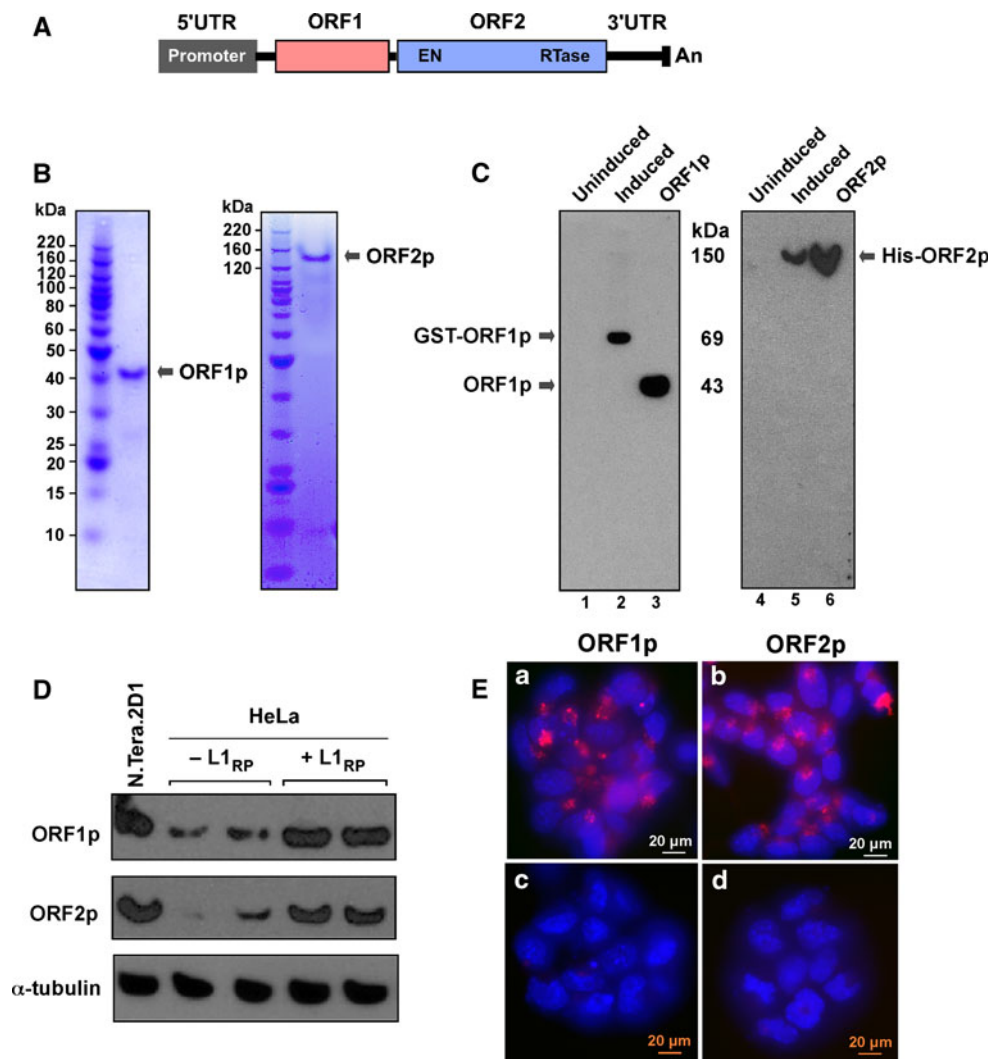
Fig. 1 Anti-ORF1p and Anti-ORF2p antibodies specifically recognize L1-encoded proteins. **A** Structure of a functional human L1 retrotransposon showing the 5′-UTR promoter, ORF1- and ORF2-encoding genes, and the 3′ poly-A tail. *EN* endonuclease, *RTase* reverse transcriptase, *An* poly-A tail. **B** Recombinant 43-kDa ORF1p and 150-kDa ORF2p were used for the generation of antibodies. **C** Western blot analysis examining the specificity of the generated anti-ORF1p and anti-ORF2p antibodies. *Lanes 1* and *4* were loaded with 50 μg of bacterial extracts isolated from uninduced cells, and *lanes 2* and *5* loaded with the induced bacterial extracts of 69 kDa GST-ORF1p (*left panel*) and 150 kDa 6xHis-ORF2p (*right panel*) fusion proteins. No staining was seen in the uninduced bacterial extracts and detection of the fusion proteins of GST-ORF1p and

6xHis-ORF2p in the induced bacterial extracts. *Lanes 3* and *6* were loaded with 25 ng of 43-kDa ORF1p and 150-kDa ORF2p as positive controls. **D** The increased expression levels of ORF1p and ORF2p were detected by western blotting of the L1$_{RP}$-transfected HeLa cells. N.Tera.2D1 cells were used as positive controls. For protein normalization, α-tubulin was used. **E** Immunofluorescence detection of transiently expressed ORF1p and ORF2p in the cytoplasm of HeLa cells using the anti-ORF1p (*panel a*) and anti-ORF2p (*panel b*) antibodies. Nuclei were stained with DAPI (*blue*) and ORF1p and ORF2p (*Texas Red*). As negative controls, the immunofluoresence assays were performed on the L1$_{RP}$-transfected HeLa cells with the antigen-depleted anti-ORF1p (*panel c*) and anti-ORF2p (*panel d*) antibodies

(T47D, SKBR3, and BT20) compared with moderate (MCF7 and Hs578T)-to-highly invasive breast cancer (MDA-MB-231, MDA-MB-436) cell lines. The increased expression of both proteins was further confirmed using immunohistochemistry and immunofluorescence assay (Fig. 2B, C). Interestingly, the relative levels of ORF2p expression were lower in all cell lines compared with ORF1p. While ORF2p shows weak-to-moderate fluorescent staining, ORF1p exhibits high fluorescence signals.

This differential expression is consistent with previous reports from Eugun et al. [24] who detected differential levels of ORF2p expression in various fetal and adult tissues. Lower expression of ORF2p compared with ORF1p was also recently reported in vascular endothelial cells [28].

To further confirm the expression of ORF1p and ORF2p, we performed the Western blot analysis of whole cell lysates from breast tumors and their adjacent normal
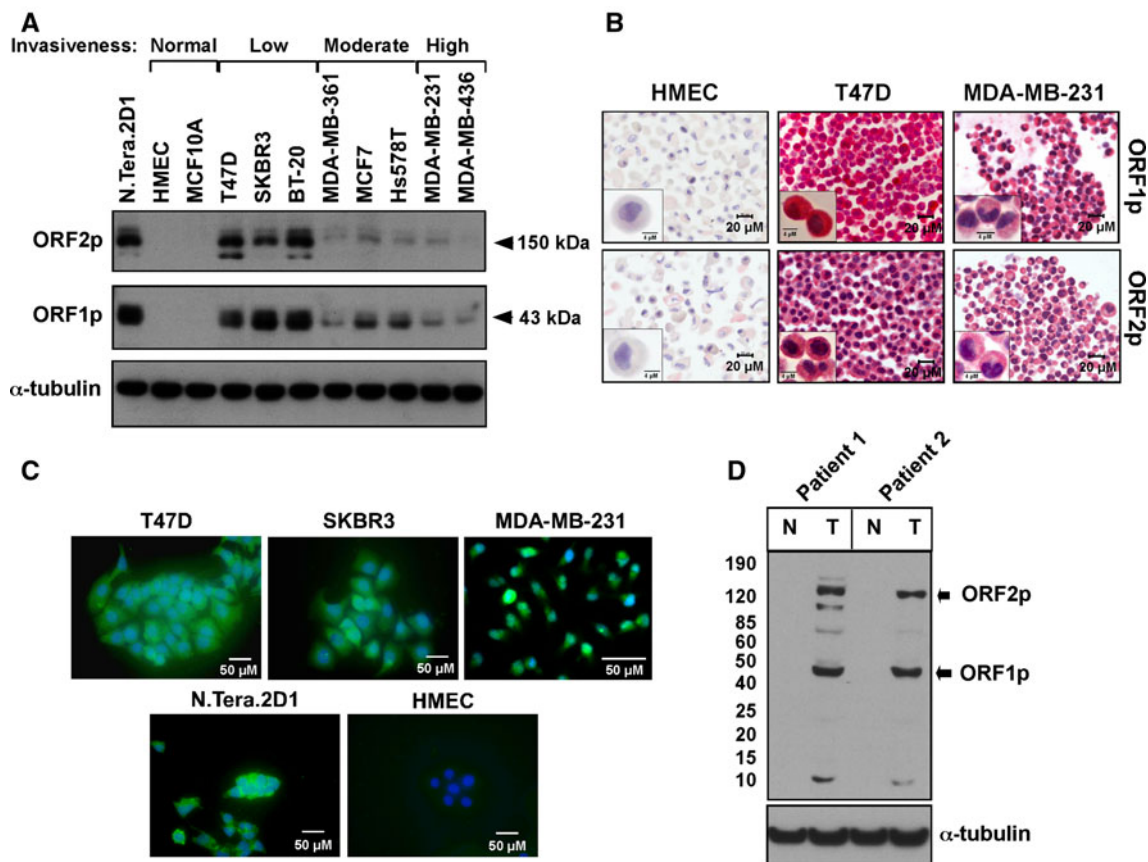
Fig. 2 Aberrant expression of L1 retrotransposons in breast cancer cells. **A** Whole-cell lysates of breast cancer cell lines were analyzed by western blotting with anti-ORF1p and anti-ORF2p antibodies. As a loading control, α-tubulin was used. Cell lysates from N.Tera.2D1 were used as positive controls. Both ORF1p and ORF2p specifically expressed in cancer cell lines but not in non-tumorigenic HMEC and MCF10A cells. **B** Cell blocks taken from the normal HMEC, poorly invasive T47D and highly invasive metastatic MDA-MB-231 cell lines were stained immunohistochemically using the anti-ORF1p and anti-ORF2p antibodies. HMEC cells did not stain, but T47D cells displayed strong cytoplasmic staining, whereas MDA-MB-231 cells showed moderate-to-strong nuclear staining. The merged panel of Hematoxylin (nucleus) and ORF1p or ORF2p are shown. *Bar* represents 20-μm. **C** The immunofluorescence analysis of ORF1p expression. L1-encoded proteins appear to localize in both the cytoplasm and nucleus of cell lines, although the intensity of staining varies between them. Nuclei were stained with DAPI (*blue*) and ORF1p (*green*). NTera.2D1 and HMEC cells were used as positive and negative controls, respectively. **D** Overexpressions of ORF1p and ORF2p in breast tumors. Protein extracts from breast tumors (*T*) and their adjacent normal breast tissues (*N*) were isolated from two different patients and performed western blotting. Using the mixture of both anti-ORF1p and anti-ORF2p antibodies, the expressions of ORF1p and ORF2p were detected only in breast tumors. For protein normalization, α-tubulin was used as a loading control

tissues from two different patients (Fig. 2D). As expected, the antibodies failed to detect any protein in extracts from normal breast tissues even after long exposure of the western blot. In contrast, high expressions of ORF1p and ORF2p were readily detectable in extracts from breast tumor tissues. Together, these results indicate that L1-encoded ORF1p and ORF2p are markedly overexpressed in breast cancer cells. Given that the both ORF1p and ORF2p are required for the process of L1 retrotransposition activity, which could potentially increase genomic instability, characterizing the expression patterns of these proteins may possibly serve as one of the factors responsible for genome instability in these cells.

Subcellular localization of ORF1p and ORF2p in breast tumor tissues

To evaluate a potential link between expression of ORF1p and ORF2p and the various stages of breast cancer, we screened paraffin-embedded breast tumor tissues by immunohistochemistry (Fig. 3). There was little or no expression in normal breast tissues (*n* = 15). In contrast, both ORF1p and ORF2p were differentially expressed in the breast tumors, and expressions were higher and more intense staining in DCIS than in invasive cancers. Table 1 shows the overall presence or the absence of the ORF1p and ORF2p expression regardless of location.
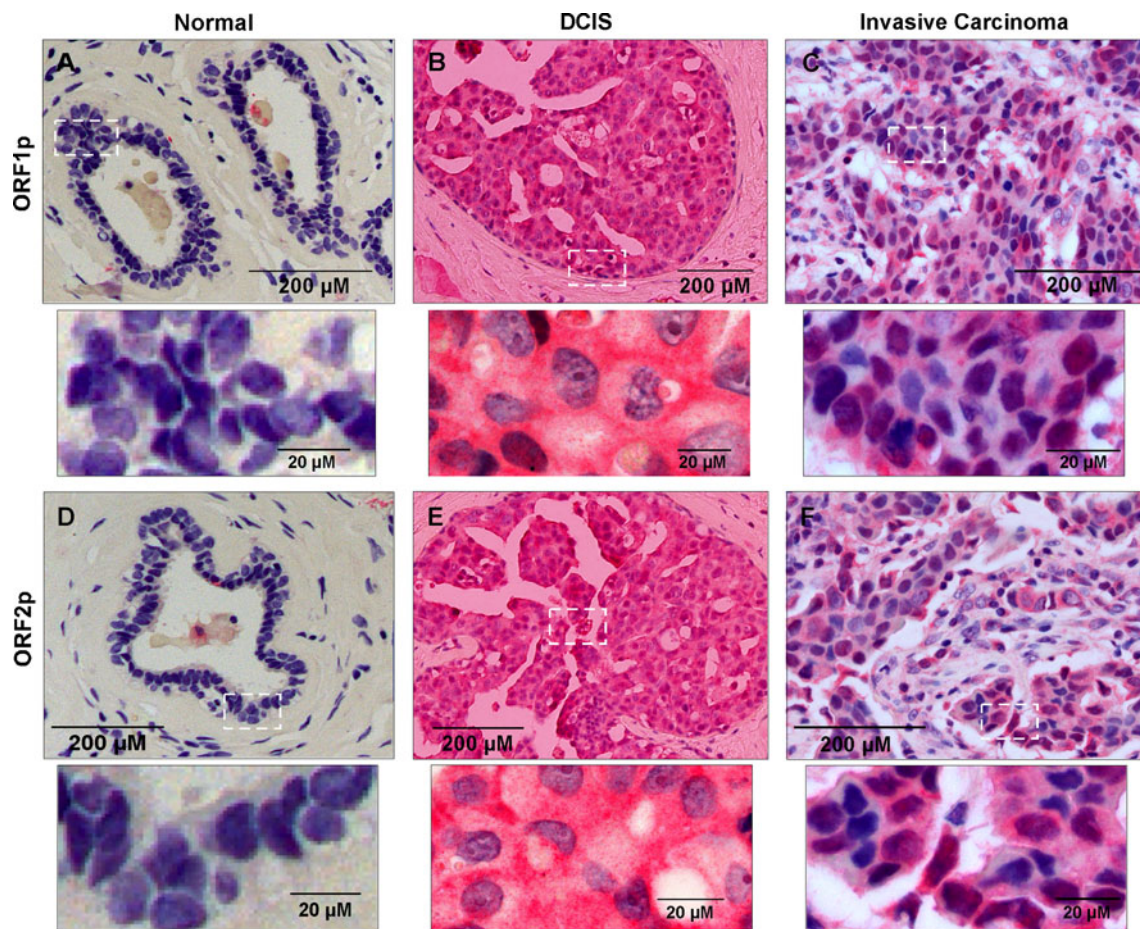
Fig. 3 Immunohistochemistry of ORF1p and ORF2p in patient's tumor tissues. Shown is representative immunohistochemistry of ORF1p (*top panel*) and ORF2p (*bottom panel*) expressions. A higher magnification of insert is shown below. **A**, **D** In normal breast; **B**, **E** showing high cytoplasmic expression in DCIS; and **C**, **F** both nuclear and cytoplasmic expressions of ORF1p and ORF2p. *Bar* represents 200-μm scale. Hematoxylin (*blue*) represents the nuclei of cells and pink highlights ORF1p and ORF2p

Table 1 Differential expression of L1-encoded ORF1p and ORF2p

	ORF1p expression				ORF2p expression			
	Negative (%)	Positive (%)	p value	χ^2	Negative (%)	Positive (%)	p value	χ^2
Normal	15 (100)	0 (0)	<0.001	53.6	15 (100)	0 (0)	<0.001	48.0
Benign	24 (96)	1 (4)			23 (92)	2 (8)		
DCIS	3 (12)	22 (88)			4 (16)	21 (84)		
Invasive	39 (41)	56 (59)			38 (40)	57 (60)		

Tissues having no staining or staining in less than 10 % of cells are considered as negative expression regardless of location

Expression pattern of ORF1p and ORF2p was found to be quite variable, and depending on the tumor subtypes, they can be detected in the nucleus or the cytoplasm. Although being nuclear gene, both ORF1p and ORF2p are exported to the cytoplasm for the formation of a ribonucleoprotein (RNP) intermediate where the proteins are packaged with their own mRNAs, and then enter into the nucleus for the L1 retrotransposition activity [29]. A recent study reported that nuclear localization of ORF1p was significantly associated with aggressive behavior of cancer and poor outcome [25]. Thus, to assess both nuclear and cytoplasmic expressions, we examined the localization of ORF1p and ORF2p in breast cancer cell lines and the various stages and grades of patient samples. Consistent

with previous reports [30], high cytoplasmic expressions were detected in non-invasive (luminal subtype) T47D cells, whereas the invasive (basal subtype) MDA-MB-231 cells exhibited strong nuclear and cytoplasmic stainings (Fig. 2B). None of the normal HMEC cells was stained with any protein except for the occasional faint background staining pattern of which differs considerably from the staining patterns seen in the T47D and MDA-MB-231 cell lines.

In breast tumor samples, the staining intensity and the percentage of cells expressing cytoplasmic and nuclear staining were determined separately using a weighted histocore method [21]. Expression of ORF1p and ORF2p was both categorized into three groups: (i) cytoplasmic expression, weak <29 %, moderate 30–59 %, and high expression >60 %; (ii) nuclear expression, weak < 29 %, moderate 30–59 %, and high > 60 % (Fig. 4). In DCIS tumors ($n = 25$), 88 % and 84 % displayed expression that ranged from weak to high for ORF1p and ORF2p, respectively. There was no significant difference between the pattern of staining between low and high grade DCIS. Notably, both ORF1p and ORF2p exhibited high cytoplasmic expression in 76 % (19 of 25) and 80 % (20 of 25) of cases, respectively. None of the patients with DCIS displayed high nuclear expression. In contrast, 59 % and 60 % of invasive cancers ($n = 95$) showed cytoplasmic and/or nuclear expression for ORF1p and ORF2p, respectively. Notably, 28 % (27 of 95) and 32 % (30 of 95) of

those invasive cancers showed high nuclear expression for both ORF1p and ORF2p and the cytoplasmic staining, respectively, which ranged from weak to moderate was also found in those cases. No significant correlation was observed between the nuclear expression and histologic grades although patients with high nuclear expression of ORF1p and ORF2p showed higher mitotic counts and higher nuclear pleomorphism than patients with cytoplasmic expression. The association between nuclear and cytoplasmic expression was significant for both ORF1p and ORF2p ($p = 0.0001$). Together, these data suggest that L1 retrotransposons are differentially expressed in breast tumors, with the highest expression seen in the cytoplasm of DCIS, whereas the invasive cancers showed expression ranging from weak to high in both the cytoplasm and nucleus of the malignant cells.

Association between ORF1p and ORF2p expression and clinicopathologic characteristics

Tables 2 and 3 summarize the association between cytoplasmic and nuclear expression of both ORF1p and ORF1p and the different clinical features of the patients with DCIS and invasive cancers. There was no significant correlation with cytoplasmic ORF1p and ORF2p expression and the various stages and grades of the breast tumors. Nuclear expression was only found in a subpopulation of the invasive cancers in the range of 28 % for ORF1p and 32 %

Fig. 4 Differential expression of ORF1p and ORF2p. *Boxplots* represent the comparison of histoscores between cytoplasmic and nuclear expression of ORF1p and ORF2p in breast tumors. This revealed a significant increase in the nuclear expression of both ORF1p and ORF2p in invasive cancers compared with DCIS
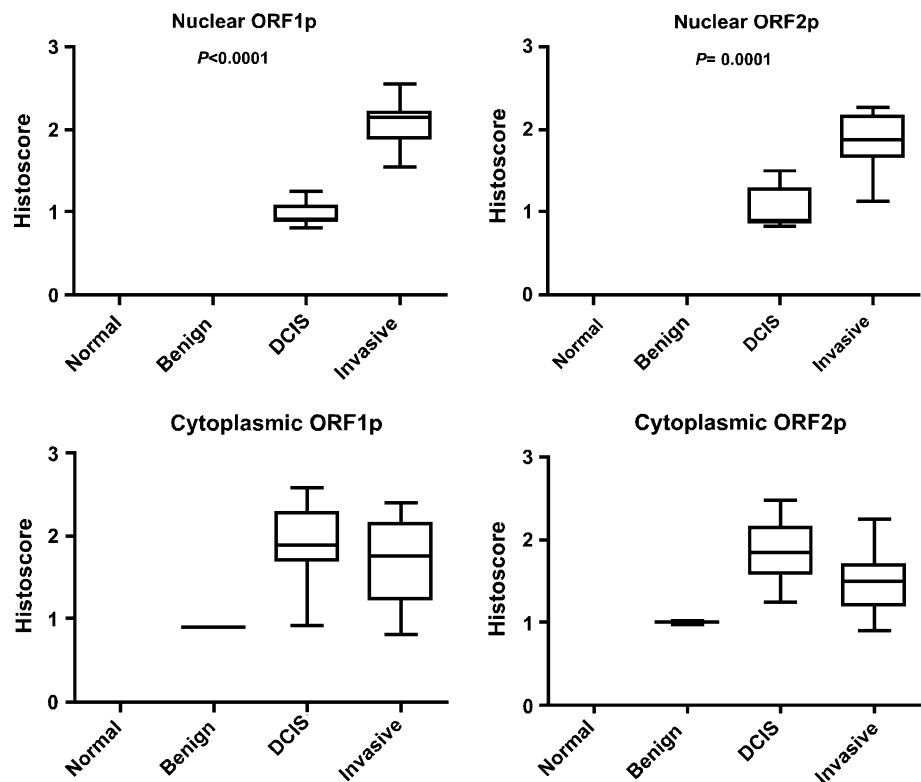
Table 2 Relationships between ORF1p and ORF2p expression and clinicopathologic characteristics of DCIS tumors

Clinicopathologic and biologic characteristics	ORF1p expression						ORF2p expression					
	Cytoplasm			Nucleus			Cytoplasm			Nucleus		
	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value
Age												
≤50	1 (25)	3 (75)	0.383	3 (75)	1 (25)	0.884	0 (0)	4 (100)	0.579	3 (75)	1 (25)	0.504
>50	2 (10)	19 (90)		15 (71)	6 (29)		4 (19)	17 (81)		12 (57)	9 (43)	
Family history												
High/moderate	1 (10)	18 (90)	0.538	13 (65)	7 (35)	0.274	2 (10)	18 (90)	0.166	13 (65)	7 (35)	0.307
Potential/NA	1 (20)	4 (80)		5 (100)	0 (0)		2 (40)	3 (60)		2 (40)	3 (60)	
Menopause												
Pre	0 (0)	3 (100)	0.495	2 (67)	1 (33)	0.826	1 (33)	2 (67)	0.422	1 (33)	2 (67)	0.315
Post	3 (14)	19 (86)		16 (73)	6 (27)		3 (14)	19 (86)		14 (64)	8 (36)	
Size												
≤20 mm	2 (14)	12 (86)	0.692	11 (79)	3 (21)	0.656	3 (21)	11 (79)	0.604	10 (71)	4 (29)	0.188
>20 mm	1 (9)	10 (91)		7 (64)	4 (36)		1 (9)	10 (91)		5 (45)	6 (55)	
ER												
Positive	1 (5)	19 (95)	0.091	13 (65)	7 (35)	0.274	1 (5)	19 (95)	0.016	10 (50)	10 (50)	0.041
Negative	2 (40)	3 (60)		5 (100)	0 (0)		3 (60)	2 (40)		5 (100)	0 (0)	
PR												
Positive	1 (5)	18 (95)	0.133	12 (63)	7 (37)	0.137	1 (5)	18 (95)	0.031	9 (47)	10 (53)	0.022
Negative	2 (33)	4 (67)		6 (100)	0 (0)		3 (50)	3 (50)		6 (100)	0 (0)	
Grade												
Low	2 (22)	7 (78)	0.75	7 (78)	2 (22)	0.295	2 (22)	7 (78)	0.499	7 (78)	2 (22)	0.06
Intermediate	0 (0)	8 (100)		4 (50)	4 (50)		0 (0)	8 (100)		2 (25)	6 (75)	
High	1 (12)	7 (88)		7 (88)	1 (12)		2 (25)	6 (75)		6 (75)	2 (25)	

Table 3 Relationships between ORF1p and ORF2p expression and clinicopathologic characteristics of invasive breast cancer

Clinicopathologic and biologic characteristics	ORF1p expression						ORF2p expression					
	Cytoplasm			Nucleus			Cytoplasm			Nucleus		
	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value
Age												
≤50	9 (35)	17 (65)	0.434	13 (50)	13 (50)	0.004	7 (27)	19 (73)	0.11	13 (50)	13 (50)	0.018
>50	30 (43)	39 (57)		55 (80)	14 (20)		31 (45)	38 (55)		52 (75)	17 (25)	
Family history												
High/moderate	38 (41)	55 (59)	0.795	66 (71)	27 (29)	0.589	37 (40)	56 (60)	0.77	64 (69)	29 (31)	0.571
Potential/NA	1 (50)	1 (50)		2 (100)	0 (0)		1 (50)	1 (50)		1 (50)	1 (50)	
Menopause												
Pre	7 (27)	19 (73)	0.086	14 (54)	12 (46)	0.019	9 (35)	17 (65)	0.511	13 (50)	13 (50)	0.018
Post	32 (46)	37 (54)		54 (78)	15 (22)		29 (42)	40 (58)		52 (75)	17 (25)	
Size												
≤20 mm	23 (42)	32 (58)	0.859	38 (69)	17 (31)	0.528	22 (44)	31 (56)	0.523	37 (67)	18 (33)	0.778
>20 mm	16 (40)	24 (60)		30 (75)	10 (25)		14 (35)	26 (65)		28 (70)	12 (30)	
Vascular invasion												
Positive	16 (50)	16 (50)	0.206	21 (66)	11 (34)	0.359	15 (47)	17 (53)	0.33	23 (72)	9 (28)	0.606
Negative	23 (37)	40 (63)		47 (75)	16 (25)		23 (37)	40 (63)		42 (67)	21 (33)	
ER												
Positive	25 (35)	46 (65)	0.047	46 (65)	25 (35)	0.012	23 (32)	48 (68)	0.009	44 (62)	27 (38)	0.02
Negative	14 (58)	10 (42)		22 (92)	2 (8)		15 (63)	9 (37)		21 (88)	3 (12)	
PR												
Positive	19 (34)	37 (66)	0.091	35 (62)	21 (38)	0.019	17 (30)	39 (70)	0.022	33 (59)	23 (41)	0.017
Negative	20 (51)	19 (49)		33 (85)	6 (15)		21 (54)	18 (46)		32 (82)	7 (18)	
Death												
Due to breast cancer	8 (40)	12 (60)	0.914	9 (45)	11 (55)	0.003	6 (30)	14 (70)	0.304	8 (40)	12 (60)	0.002
No	31 (41)	44 (59)		59 (79)	16 (21)		32 (43)	43 (57)		57 (76)	18 (24)	
Histology												
Ductal	38 (42)	53 (58)	0.642	66 (73)	25 (27)	0.575	35 (38)	56 (62)	0.144	63 (69)	28 (31)	0.418
Lobular	1 (25)	3 (75)		2 (50)	2 (50)		3 (75)	1 (25)		2 (50)	2 (50)	
Grade												
1	17 (57)	13 (43)	0.11	25 (83)	5 (17)	0.111	17 (57)	13 (43)	0.074	22 (73)	8 (27)	0.403
2	12 (34)	23 (66)		21 (60)	14 (40)		12 (34)	23 (66)		21 (60)	14 (40)	
3	10 (33)	20 (67)		22 (73)	8 (27)		9 (30)	21 (70)		22 (73)	8 (27)	
Lymph node metastasis												
Positive	6 (27)	16 (73)	0.134	12 (58)	10 (45)	0.043	5 (23)	17 (77)	0.059	11 (50)	11 (50)	0.034
Negative	33 (45)	40 (55)		56 (77)	17 (23)		33 (45)	40 (55)		54 (74)	19 (26)	

for ORF2p. Patients with high nuclear ORF1p and ORF2p expression showed apparent association with the presence of lymph node metastasis ($p < 0.043$) than did patients with cytoplasmic expression. Although nuclear ORF1p and ORF2p expression was commonly present in ductal tumors rather than in lobular tumors, no significant correlation was observed between the nuclear expression and histologic grades. Interestingly, nuclear ORF1p and ORF2p, but not cytoplasmic, was associated with lymph node metastasis and poorer overall survival ($p < 0.05$).

L1 expression was found to be highly associated with patients whose cancers expressed estrogen (ER) and progesterone (PR). 95 % ER-positive DCIS and 95 % PR-positive DCIS exhibited cytoplasmic expression of both ORF1p and ORF2p. In invasive cancers, 64–69 % of ER-positive and PR-positive cases showed cytoplasmic expression. A similar association was also found with nuclear expression in 35–41 % ER and PR-positive invasive cancers ($p < 0.019$). Unexpectedly, we found the expression of both ORF1p and ORF2p in the cytoplasm of receptors-negative invasive cancers in the range of 37–41 % (9–10 of the 24 patients) ER-negative and 46–48 % (18–19 of 39) of PR-negative tumors, while the nuclear expression was found in 8–12 % (2–3 of 24) of ER-negative and 15–17 % (6–7 of 39) of PR-negative cases. Interestingly, no significant association was observed between ORF1p and ORF2p expression and other pathologic features such as family history, primary size of tumors, and vascular invasion.

In the breast, it has been recently reported that nuclear, but not cytoplasmic ORF1p, is associated with genomic instability of tumors, which are characterized by aggressive behavior and poor outcome [25]. In agreement with this study, no correlation between survival of the patients with DCIS tumors and cytoplasmic expression of ORF1p and ORF2p were observed. There were no breast cancer-related

deaths in patients with DCIS tumors. In the case of invasive cancers, 21 % of patients (20 of 95) died with breast cancer. The mean follow-up time for the patients with invasive cancers was 10.7 years (range 1–14 years). When the localization of the protein was considered, the 10-year survival was 50–51.8 % for overall nuclear expression of ORF1p and ORF2p, while cytoplasmic expression was 75–75.4 %. Analysis of the prognostic significance of expression using the Kaplan–Meier survival curves showed that nuclear expression of ORF1p and ORF2p significantly affected patient outcome ($p < 0.0001$), but not the cytoplasmic expression (Fig. 5). Although nuclear expression of ORF1p and ORF2p was significantly associated with ER- and PR-positive invasive cancers in univariate analysis, the significance of ER and PR disappeared on Cox-regression multivariate analysis. In Cox-regression analysis, nuclear expression of both ORF1p and ORF2p was significantly associated with poor patient outcomes ($p = 0.027$, $p = 0.027$, respectively), while cytoplasmic expression was not (Table 4). In patients with high nuclear ORF1p and ORF2p expression, there was a strong trend for lymph node metastasis ($p = 0.001$).

Discussion

Genomic instability is a major driving force of tumorigenesis that might occur in normal tissues even before morphologic abnormalities are detectable [31]. Although it has not been completely resolved whether genomic instability is a cause or consequence of tumor progression, an unstable genome can be indicative of a poor prognosis in cancer patients [1]. The mechanistic pathways that activate genomic instability are not well understood. The activation of normally repressed L1 retrotransposon has been implicated in a high frequency of DNA breaks and genomic
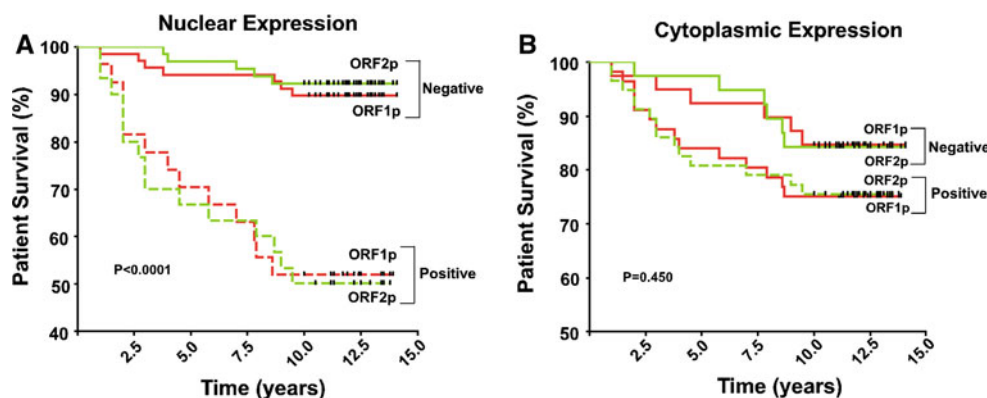

Fig. 5 Effects of subcellular localizations on overall patient survival. Kaplan–Meier analysis performed based on the presence of nuclear or cytoplasmic ORF1p and ORF2p. **A** Nuclear localization of both ORF1p and ORF2p exhibited a significant difference in the overall survival of patients with invasive cancers. **B** Cytoplasmic localization of ORF1p and ORF2p showed no difference in patients' survival. p value was calculated by log-rank test

Table 4 Multivariate Cox-regression analysis of cytoplasmic and nuclear expression of ORF1p and ORF2p

Variable	Hazard ratio	95 % confidence interval	p value
ORF1p			
Cytoplasm	2.159	0.616–7.545	0.228
Nucleus	0.339	0.13–0.886	0.027
ORF2p			
Cytoplasm	0.503	0.141–1.798	0.29
Nucleus	0.239	0.099–0.87	0.027
Age	2.855	0.061–134.132	0.593
Family history	1.32	0.016–110.778	0.902
Menopause	0.383	0.009–16.29	0.616
Size	0.54	0.144–2.021	0.36
Vascular invasion	2.452	0.687–8.754	0.167
ER	1.049	0.199–5.519	0.955
PR	2.499	0.525–11.887	0.25
Histology	1.163	0.137–9.855	0.89
Grade	1.085	0.17–6.905	0.604
Lymph node metastasis	0.182	0.065–0.514	0.001

instability [13, 14]. Several studies have shown that there is a direct correlation between the severity of cancer and the loss of DNA methylation at the L1 promoter [32, 33]. However, little is known about the L1-encoded ORF1p and ORF2p expression. A recent study reported that ORF1p is expressed in a range of cancer tissues including breast and that its expressions is correlated with worst patient survival [25]. However, it remains unclear whether any relationship exists between the various stages and grades of breast cancers and the expression level of ORF2p, which is a key protein involved in creating DNA breaks and inserting L1 copies into genomic DNA during the process of L1 retrotransposition.

In this study, we investigated the expression of both ORF1p and ORF2p in a range of breast cancer cell lines and patient tissues and evaluated whether they could provide any valuable information for cancer prognosis. In in vitro cell line studies, overexpression of ORF1p and ORF2p are found to be colocalized in the cytoplasm of cell lines suggesting that it may be involved in the assembly of RNP intermediates before entering into the nucleus to generate L1 retrotransposition activity [29, 30]. The cytoplasmic expression of L1 is not often related to tumor development, whereas nuclear expression is linked to DNA breaks and genomic instability. In this article, we describe additional data derived from clinical breast cancer tissue samples that support the variation of subcellular localization seen in the studies of cell lines. We found that the expression of ORF1p and ORF2p in the majority of

preinvasive breast cancers (DCIS) was cytoplasmic and not the nucleus, which was not associated with patient survival, but precisely how this occurs is not clear. One possible explanation is that L1 retrotransposons might become active early in cancer development. This early expression is in agreement with DNA methylation studies in which early onset of L1 hypomethylation has been reported to occur in a number of tumors including breast, colon, and lung [32, 33]. Certainly, hypomethylation of L1 promoters, which has been shown to correlate with L1 mRNA expression in breast cancer cell lines [18, 33], would be consistent with the expression of ORF1p and ORF2p that we see in DCIS tumors. While there seem to be some differences in the levels of ORF1p and ORF2p expression depending on the grade of DCIS, the number of DCIS cases was quite small in our studies, and further studies are required to evaluate this.

It is known that depending on microenvironment and genetic background, different tumor subtypes express different protein profiles. Our study shows that overall expression levels of ORF1p and ORF2p were higher in DCIS in the range of 25–28 % than for invasive cancers. It suggests that these tumors do not need as much of this protein once they become invasive. Higher expression levels of other proteins have also been reported in DCIS including ER, PR, and Her-2neu receptors [34, 35], suggesting that some tumors express variable levels depending on which is the critical step(s) in tumorigenesis. Genomic instability tends to correlate with triple negative (ER/PR/ Her-2neu-receptor negative) breast cancer [36]. However, our study shows that nuclear ORF1p and ORF2p expression was found in some cases of ER and PR receptor-positive breast tumors. Although there is known to be cross-talks between ER and growth factors and other signaling pathways, we do not really understand the relation between ER/PR and L1 expression, and so this will require further investigations.

Expression of both ORF1p and ORF2p in the majority of the invasive breast cancers we studied was mainly cytoplasmic while nuclear expression occurs only in a subpopulation of invasive cancers. Changes in subcellular localizations of ORF1p and ORF2p from cytoplasm to nucleus may be a critical step in tumorigenesis. It is known that certain proteins, as we see in ORF1p and ORF2p, change their cellular localization during tumor progression. One well-studied case is ß-catenin which is associated with tumor progression upon nuclear localization [37]. Our results showed that nuclear expression of ORF1p and ORF2p in the invasive cancers and not cytoplasmic, was associated with poorer survival in patients with a more aggressive phenotype of lymph node metastasis. This indicated that different staining locations of ORF1p and ORF2p may have distinct biologic significances for the

metastatic potential of breast cancer, and the result was similar to a recent study in which nuclear ORF1p was found to be associated with increased risk of distant recurrence and poor prognosis [25]. The functional role of nuclear L1 localization and its relationship with tumor development has not been elucidated. In the literature, nuclear L1 expression is linked to genomic instability and DNA breaks. Sciamanna et al. [38] have shown that the inhibition of reverse transcriptase activity of ORF2p reduces cell proliferation and promotes differentiation by reprogramming gene expression in tumorigenic cell lines. Recently, it has been postulated that the L1 retrotransposition activity in the nucleus might interfere with the transcription machinery of cells, and thereby it may be involved in the development and progression of cancer [39]. Thus, identifying the mechanisms by which nuclear localization of ORF1p and ORF2p occur may help us understand the consequences of L1 expression in breast cancer cells.

Conclusion

This study has presented the relationships and relevance with respect to biologic and clinical usefulness of expression and subcellular localization of L1-encoded ORF1p and ORF2p in breast cancer. In this study, we have shown, first, high cytoplasmic expression of L1 retrotransposon in the DCIS. In the majority of invasive cancers, L1 expression is found mainly in the cytoplasm, while nuclear expression occurs only in a subclass of invasive cancers. Second, only nuclear expression of ORF1p and ORF2p is associated with poorer survival in patients with invasive cancers and it is not related to tumor size and family history. Third, tumors expressing estrogen or progesterone receptors are more likely to show L1 expression than that of receptor negative. This study is the first to observe the effects of both ORF1p and ORF2p expression in human breast cancer. Our observations show that considerable heterogeneity exists in the expression pattern of ORF1p and ORF2p in breast tumors, which relate to biologic and clinical differences in overall patient survival. The associations found for both cytoplasmic and nuclear expression of ORF1p and ORF2p suggest that further studies of the biology and function of L1 retrotransposons are warranted in breast cancer.

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