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Cytoplasmic L1 Levels in Cancer

Eleana Hatzidaki[1], Panagiotis Apostolou[1], Ioannis Papasotiriou[2*]

[1]Research Genetic Cancer Centre SA, Florina, Greece
[2]Research Genetic Cancer Centre International GmbH, Zug, Switzerland
Email: *office@rgcc-genlab.com

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Abstract

In addition to shaping genome diversification over evolutionary time, L1 retrotransposition alters gene expression as well. The most notable gene altering process involves insertional mutagenesis. The aim of the study was the examination of both nuclear L1 expression levels and cellular localization in cancer cell lines, PBMCs from healthy volunteers and PBMCs from cancer patients. L1 was detected by FISH in chromosome preparations. L1 probe was custom-made using end-point PCR against L1-ORF2 and conjugated with FITC. It was found that cancer cell lines and clinical samples from cancer patients contained significantly elevated levels of L1 per nucleus compared to healthy volunteers. Cytoplasmic L1 was also increased in the above mentioned samples denoting that cancer could be associated with increased L1 activation and mobility. Our results may provide a novel cancer diagnostic marker and highlight the possibility of cytoplasmic L1 inhibition as a therapeutic intervention for cancer.

Keywords

L1, Cancer, Retrotransposition, FISH, Cytoplasmic

1. Introduction

Transposable elements (TE) account for half to two-thirds of the human genome [1]. They are able to move from one locus to another via a copy-paste mechanism using either DNA or RNA-mediated transposition. Transposable elements were once thought to be junk or parasitic DNA, however it is now considered to be contributing to shaping the genome over evolutionary time and altering gene expression patterns [2] [3]. Among them, LINE-1 (long interspersed nuclear element-1, L1), the only autonomous TE, comprises 17% of human DNA [4]. Active, full-length copies contain a 5'-untranslated region (UTR), two open

reading frames (ORF) and a 3' UTR. ORF1 and ORF2 encode the p40 protein and a protein with endonuclease and reverse transcriptase activity that are necessary for retrotransposition [5].

The life cycle of L1 starts with the transcription of its DNA sequence in the nucleus. The resulting mRNA codes for 2 proteins, ORF1 and ORF2. ORF1 is an RNA binding protein with a nucleic acid chaperone activity [6] whereas ORF2 has both endonuclease and reverse transcriptase activity. L1 mRNA exits the nucleus into the cytoplasm where the 2 proteins are translated and bound to L1 mRNA, forming a L1 ribonucleoprotein particle complex (RNP) which is then imported back into the nucleus. Inside the nucleus, L1 RNP inserts a DNA copy into a new genomic target locus using the ORF2p endonuclease followed by the synthesis of a DNA that is complementary to L1 mRNA using the ORF2p reverse transcriptase. A second strand of cDNA is then synthetized and joined to adjacent genomic DNA [7].

L1 insertions can be potentially mutagenic. Their effect on gene expression however, depends on the locus per se. Insertions in exons or regulatory sequences have the ability to cause insertional mutagenesis [8] [9]. The insertion of L1 sequences into introns on the other hand, can reduce transcriptional elongation of target genes [10] or may have no detectable effects.

There have been 124 L1 insertions linked with genetic diseases [11]. Since genetic rearrangements are the hallmark of cancer, the association of L1 with tumorigenesis is an attractive concept. L1 insertions have been found in a colon cancer [12] and an endometrial carcinoma [13] patient as well as in gastrointestinal [14] and pancreatic [15] cancers.

In a previous study using molecular assays, we have found that circulating tumor cells from cancer patients have higher ORF2 gene expression compared to healthy individuals [16]. In this study using cytogenetic assays, we explored the phenomenon further and examined not only ORF2 protein expression levels but also ORF2 protein cellular localization in cancer cell lines, PBMCs from healthy volunteers and PBMCs from cancer patients.

2. Materials and Methods

2.1. L1 Probe Construction

The L1 probe was produced using fluorescent dUTPs (NU-803-FAMX-L; Jena Bioscience) in endpoint PCR. The reaction included labeled dUTPs: dTTPs at a ratio 20:80 and the program was as follows: Initial denaturation 94˚C for 5 minutes, followed by 40 cycles of denaturation, annealing and extension at 94˚C for 15 sec, 60˚C for 15 sec and 72˚C for 30 sec, respectively and a final extension at 72˚C for 5 minutes. The probe was validated using a spectrophotometer and agarose gel electrophoresis. The primer sequence used can be found in **Table 1**.

2.2. Commercial Cancer Cell Lines, Patients and Control Subjects

Samples studied included 9 commercial cell lines (CACO2, CALU1, COLO684,

Table 1. The table represents the sequence of the primers that were used in cytogenetic assays.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
L1-ORF2 (Probe)	AAACCCATCTCATGTGCAGAGACA	TTCTGTGGGATCGGTGGTGATA

COLO699N, HCT15, HELA, MCF7, MDAMB231, SK MES-1), 8 healthy volunteers and 7 cancer patients with various types of cancer. Blood samples from volunteers and patients were collected after written informed consent was obtained. Cancer cells were grown in the suitable media according to depositor's instructions. More specifically, CACO2, MCF7 and HELA were cultured in RPMI 1640 supplemented with 2 mM Glutamine, 1% Non-Essential Amino Acids (NEAA) and 10% FBS; CALU1 was cultured in RPMI 1640 supplemented with 2 mM Glutamine, 1% NEAA, 1 mM Sodium Pyruvate and 10% FBS; COLO684 and COLO699N were cultured in RPMI 1640 supplemented with 2 mM Glutamine and 10% FBS; HCT15 were cultured in RPMI 1640 supplemented with 2 mM Glutamine and 20% FBS; MDAMB231 were cultured in RPMI 1640 supplemented with 2 mM Glutamine and 15% FBS, MES-1 were cultured in RPMI 1640 supplemented with 10% FBS.

2.3. Chromosome Preparation

Chromosome preparations were obtained for all the above samples using 0.075 M KCl, at 37˚C added to the cell pellet drop by drop. Cells were incubated at 37˚C for 20 minutes and then centrifuged for 5 minutes at 500 g. Supernatant was discarded and pellet was re-suspended by gentle tapping. 5 ml ice cold fixative solution (Methanol-Acetic acid, 3:1) was added drop by drop. Cells were incubated at −20˚C for 1 hour and then centrifuged for 5 minutes at 1000 g. Supernatant was discarded and cells were washed again with ice cold fixative solution 2 more times. Finally, cells were re-suspended in 500 ul fixer solution and kept at −20˚C for further experiments.

2.4. Slide Preparation

Slides were cleaned with distilled water and kept in absolute ethanol at −70˚C. For slide preparation, metaphases kept at −20˚C were washed and re-suspended in fresh ice cold fixative solution. Three drops from the sample solution was added on the slide and then passed through water vapors. Slides were then left to dry overnight and dehydrated in 70%, 80%, 90% and 100% ice cold ethanol for 2 minutes each. Slides were kept in absolute ethanol at −70˚C until used.

2.5. Hybridization

Slides were removed from −70˚C and left to dry at room temperature. Subsequently they were digested with 5 ug/ml Proteinase K, for 10 minutes at RT and washed in 2× SSC buffer for 5 minutes. Slides were then incubated with 0.5 mg/ml RNase in 2× SSC for 1 hour at 37˚C and then washed in 2× SSC buffer for 5 minutes. Finally, slides were treated with 0.05 mg/ml Pepsin in 10 mM HCl

for 10 minutes at 37˚C and then washed in 2× SSC buffer for 5 minutes. Slides were then denatured in 70% Formamide at 78˚C for 2 minutes and then blocked for 1 hour at 37˚C. Blocking buffer consisted of 10% dextran sulfate, 50% formamide, 50 mM PBS and 0.1 mg/ml Solomon sperm. After blocking, 20 ul of hybridization solution was added in each slide, covered with a glass coverslip and sealed with rubber cement. Slides were left for hybridization at 37˚C, overnight. Hybridization solution for one slide consisted of 0.3 ul of 1:4000 L1 diluted in PBS plus 10 ul formamide and 0.1 ug/ul COT-1. The solution was denatured for 5 minutes at 80˚C and then placed on ice immediately. Subsequently 10 ul hybridization buffer was added as well.

2.6. Post-Hybridization

After hybridization, coverslips were removed and the slides were washed in 0.25× SSC buffer for 2 minutes at 72˚C, 0.5× SSC buffer for 10 minutes at RT and 2× SSX buffer for 10 minutes at RT. Five μl DAPI was added as a counter-stain and coverslips were applied and sealed with rubber cement.

2.7. Image Acquisition and Analysis

Slides were mounted on a Nikon eclipse microscope and visualized using Cytovision. Results were analyzed using ImageJ Particle Analysis. For total L1, signal counts were divided by the number of nuclei present. For cytoplasmic L1, nuclei were first digitally deleted using Paint 3D program and then analyzed using ImageJ Particle Analysis. Signal counts were evaluated using student's two tailed t-test. The level of significance was chosen as $p < 0.05$.

2.8. Ethical Approval

This study was not a clinical trial and did not include any interventions. The study was reviewed and approved by the Bioethical Committee of the Research Genetic Cancer Centre Group. All patients provided written consent for the use of their sample in the present study. The patients retained the right to withdraw their sample until time of test.

3. Results

It was found that cancer cell lines and clinical samples contained significantly elevated levels of total L1 per nucleus compared to healthy volunteers (Healthy PBMCs vs Cancer Cell Lines $p = 0.0007$; Healthy PBMCs vs Cancer PBMCs $p = 0.005$; Cancer Cell Lines vs Cancer PBMCs p = NS) (**Figure 1**).

The same trend applies to cytoplasmic only L1 (Healthy PBMC vs Cancer Cell Line $p = 0.01$; Healthy PBMC vs Cancer PBMC $p = 0.005$; Cancer Cell Lines vs Cancer PBMC p = NS) (**Figure 2**).

Representative FISH and ImageJ images ready for analysis for cancer cell lines, healthy PBMCs and cancer PBMCs can be seen in **Figure 3**. According to our results, healthy PBMCs fraction exhibit L1 signal inside the nucleus, but no

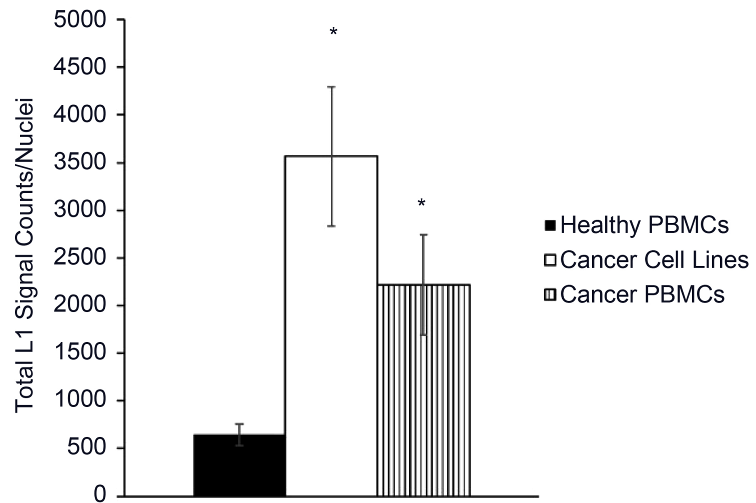

Figure 1. Total L1 signal counts per nuclei in PBMCs from healthy volunteers, commercial cancer cell lines and PBMCs from cancer patients. Healthy PBMCs vs Cancer Cell Lines p = 0.0007; Healthy PBMCs vs Cancer PBMCs p = 0.005; Cancer Cell Lines vs Cancer PBMCs p = NS. * denotes statistical significance compared to healthy group.

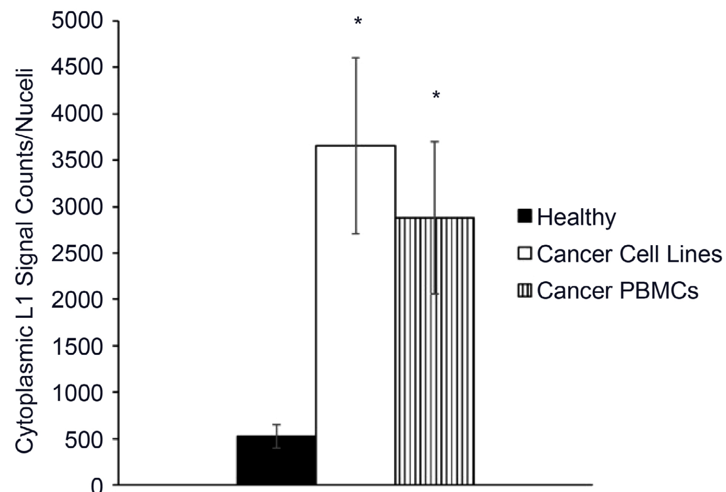

Figure 2. Cytoplasmic only L1 signal counts per nuclei in PBMCs from healthy volunteers, commercial cancer cell lines and PBMCs from cancer patients. (Healthy PBMC vs Cancer Cell Line p = 0.01; Healthy PBMC vs Cancer PBMC p = 0.005; Cancer Cell Lines vs Cancer PBMC p = NS). * denotes statistical significance compared to healthy group.

apparent signal is detected in the cytoplasm. In contrast, both commercial cell lines and PBMCs from cancer patients, have increased signal both inside the nucleus and in the cytoplasm, denoting the presence of L1 in both cellular compartments.

4. Discussion

Retrotransposons have long puzzled scientists and their role is still up for debate. Until recently, they were considered to be junk or parasitic DNA. This view is

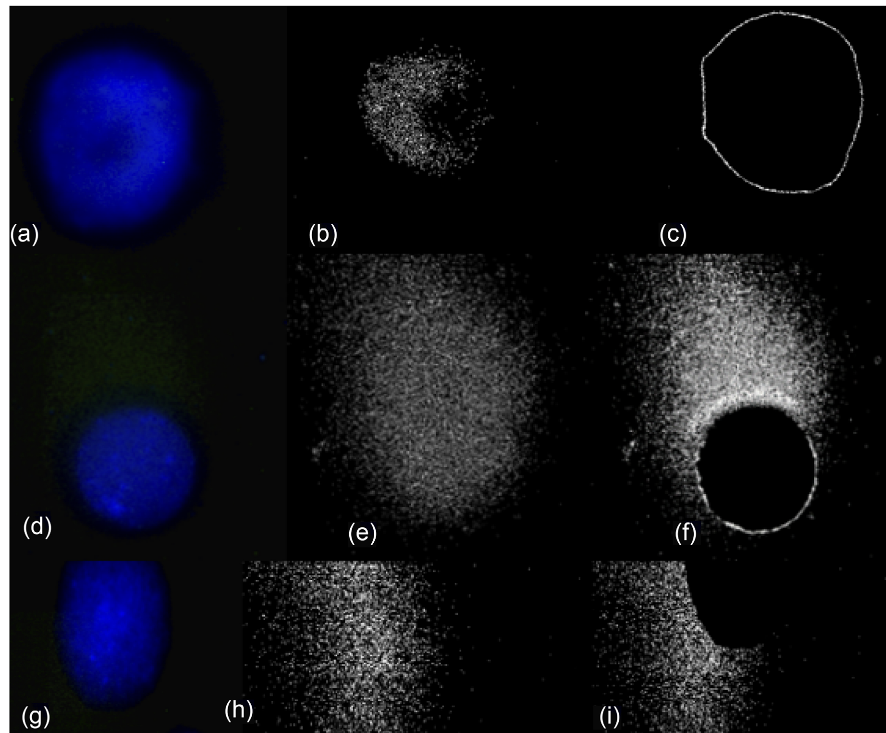

Figure 3. Healthy PBMC L1 FISH image as acquired using Cytovision (a); Healthy PBMC total L1 signal after particle analysis using ImageJ (b); Healthy PBMC cytoplasmic only particle analysis using ImageJ (c); Commercial Cancer Cell Line HCT15 L1 FISH image as acquired using Cytovision (d); Commercial Cancer Cell Line total L1 signal after particle analysis using ImageJ (e); Commercial Cancer Cell Line cytoplasmic only particle analysis using ImageJ (f); Cancer PBMC L1 FISH image as acquired using Cytovision (g); Cancer PBMC total L1 signal after particle analysis using ImageJ (h); Cancer PBMC cytoplasmic only particle analysis using ImageJ (i).

however challenged and retrotransposons are now though to be associated with physiological and pathological processes. The Cancer Genome Atlas project has unveiled 183 L1 insertions in colorectal, prostatic and ovarian carcinomas [17].

L1 life cycle begins in the nucleus with the transcription of a bicistronic mRNA, continues to the cytoplasm where L1 RNA translates ORF1 and ORF2 proteins forming L1 ribonucleoprotein particles (RNPs) and enters the nucleus again where it exerts its effects.

Mita *et al.* [18] have demonstrated that L1 RNPs enter the nucleus during mitosis, where the nuclear envelope is broken down and thus it is easier to penetrate. Moreover, most of the insertions take place during the S phase where cells are replicating. Therefore, L1 RNPs need to survive in the cytoplasm until cell division causes the nuclear membrane to disappear and once inside the nucleus the complex must survive until the S phase where replication proteins and nucleotides become available [19].

It has been demonstrated that transposable element over-amplification is associated with hypomethylation [20]. Since DNA methylation is a major epigenetic feature for the control of transposable elements it is fair to assume that

there is a relationship between transposable element amplification, genetic rearrangement and methylation abnormalities. Interestingly enough changes in methylation status has been associated with tumorigenesis as well [21].

In this project we manufactured a new ORF2 probe for the subsequent detection of L1. There have been alternative protocols for labeling the probes tested (nick-translation), however the signal on FISH experiments was not satisfactory. This could be due to the temperature sensitivity of the reaction, or the low specific activity of the probes generated. The use of endpoint PCR with fluorescent nucleotides is widely used as a probe labeling technique [22]. Since L1 is present many times on the genome, the probe length was much shorter than other regular probes. There have been different probe lengths and different dUTP:dTTP ratios tested, and we ended up with a probe of 1093 bp and a ratio of 20:80, which provided signals that were measurable, eliminating background signals at the same time. The probe sequence was based on ORF2 region of L1.

Using this probe we found increased amounts of ORF2p in cancer cell lines and cancer clinical samples compared to healthy volunteers. Cytoplasmic ORF2p was also increased in the above mentioned samples denoting that cancer could be associated with increased L1 activation and mobility. Clinical samples had greater variability than cell lines that could be due to the heterogeneity of cancer. Increase in L1 activation could be due to the higher proliferation rates or due to demethylation of cancer cells.

Whether L1 is the driver or the passenger in tumorigenesis remains to be elucidated. Although it was found that tumor cells have frequent L1 mobilizations [23] it is not known whether L1 activation is the cause or the result. In fact, it has been found that L1 insertions can occur after tumorigenesis [24].

Also in this study ORF2p was used as a means of L1 detection. ORF2p however by itself can have an effect on the genome not only due to L1 associated reprotransposition but also due to DNA breaks and genomic deletions [25] [26], ORF2p has also been implicated in DNA translocations that take place with other DNA-binding proteins and may regulate its endonuclease activity [27]. Therefore, ORF2p expression alone may have important roles in cancer onset and progression through perturbation of regulatory networks.

The present study was a proof-of-concept study, and therefore the number of the samples was low. For achieving significance, more samples and various cancer types need to be analyzed. In this study, we demonstrated that cancer cells both from cell lines and patients have an increased expression of cytoplasmic ORF2 protein. Whether ORF2p activation is correlated with L1 activation and whether it is the result or the cause of cancer remains to be elucidated. Finally, it should be noted that the probe detects both RNA and DNA and a positive signal can be obtained regardless of ORF2 protein binding. Therefore, the signals detected could represent both active and inactive L1. However, the fact that ORF2 protein expression in highly elevated in cancer samples may prove useful as diagnostic marker in cancer detection. Furthermore, it highlights the possible usefulness of cytoplasmic inhibition of reptrotransposition as a therapeutic intervention.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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