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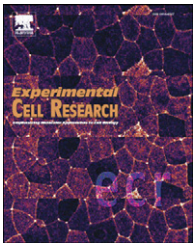

Research Article

Evaluation of LINE-1 mobility in neuroblastoma cells by in vitro retrotransposition reporter assay: FACS analysis can detect only the tip of the iceberg of the inserted L1 elements

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ABSTRACT

Long Interspersed Nuclear Elements (L1) are retroelements generally repressed in most differentiated somatic cells. Their activity has been observed in some undifferentiated and tumour cells and could be involved in tumour onset and progression. Growing evidences show that the L1 activation can occur in neuronal precursor cells during differentiation process. Neuroblastoma is a tumour originating from neuronal precursor cells, and, although the molecular basis of its progression is still poorly understood, the implication of L1 activation has not yet been investigated. In this study L1 mobility in neuroblastoma BE(2)C cells was assessed using the in vitro retrotransposition assay consisting in an episomal EGFP-tagged $L1_{RP}$ element, whose mobility can be evaluated by cytofluorimetric analysis (FACS) of EGFP expression. FACS results have shown a low retrotransposition activity. To detect $L1_{RP}$ integrated in transcriptionally repressed genomic sites, both a cell treatment with a stimulator of reporter gene promoter, and a quantitative Real-Time PCR analysis were performed. A retrotransposition activity ten and one thousand times that of FACS was found, respectively. These results point out that the real rate of L1 retrotransposition events in tumour cells might be considerably higher than that reported so far by evaluating only the reporter gene expression.

Introduction

Long Interspersed Nuclear Elements (LINE-1, L1) are transposable elements so abundant to represent large portions of the human genome (about 20%). They belong to the non-LTR retrotransposon class and amplify by retrotransposition process that consists of several steps: transcription of L1 RNA, transport to the cytoplasm, translation of ORF1 (40-kDa nucleic acid binding protein) and ORF2 (150-kDa protein with endonuclease and reverse transcriptase activities), formation of ribonucleic particle, return to nucleus, reverse transcription and integration of retrotranscripts in a new genomic location by a molecular mechanism termed target-site primed reverse transcription (TPRT) [1].

The majority of human L1 are defective, but about 100 elements have been estimated functional and their mobility resulted to be involved in a variety of diseases [2,3]. L1 mobility can modify genome structure and function by causing insertional mutagenesis, transduction, genomic deletions, alteration of gene expression. However each molecular step of the retrotransposition process is so strictly regulated that L1 activity is generally repressed in most differentiated somatic cells, with the exception of some embryonic cells [4–8]. Some of these suppressive

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mechanisms are compromised in tumours cells [9], and L1 activity has been found in some tumours and in a variety of human cell lines by using a retrotransposition reporter assay [10–19].

Moreover an increased LINE-1 mobility has been observed as a consequence of exposure to some well-known carcinogens such as heavy metals [20–22], benzo(a)pyrene [23] and γ-radiation [24].

Results from some studies have suggested that L1s can play a role in tumorigenesis [9,25] and also in the genomic instability observed during the tumour progression of some tumours such as melanomas [26], haematological cancers [27,28], germ cell tumours [29]. This issue has to be meaningfully addressed because genome instability due to retrotransposition could be regulated by therapeutic drug [25,30] and therefore new findings could be of clinical relevance.

Neuroblastoma is a childhood tumour originating from precursor cells of the sympathetic neuronal system, the molecular basis of its tumorigenesis and progression is still poorly understood [31]. Until now neuroblastoma cells have never been studied in relation to their ability to support retrotransposition activity. However the observation that L1 mobility has been observed in neuronal precursor cells during neuronal differentiation process [5,8] induce to hypothesize that retrotranspositions could occur in neuroblastoma cells affecting tumour progression.

The aim of the present study is to verify whether the L1 retrotransposition events can occur in the human neuroblastoma BE (2)C cells, which are thought to represent embryonic precursors of sympathetic neurons [32,33]. To this purpose, the in vitro retrotransposition assay developed by Kazazian laboratory [34], consisting in an episomal EGFP-tagged L1, was used. In order to detect and quantify the inserted L1 that have been transcriptionally silenced, cells were treated with a stimulator of the reporter gene promoter, moreover a quantitative Real-Time PCR analysis on genomic DNA was carried out.

Materials and methods

Plasmids

$pL1_{RP}$-EGFP, containing $L1_{RP}$ full-length retrotransposon, and $pL1_{RP}$ (JM111)-EGFP, containing a non-functional L1 mutant, have been previously described [34] and were a generous gift from Dr. John Moran (University of Michigan, Ann Arber, MI). Both constructs contain the Cytomegalovirus immediate-early promoter (CMV)-EGFP retrotransposition cassette. $L1_{RP}$ expression is driven by its native promoter. A draft of $pL1_{RP}$-EGFP is shown in Fig. 1. pCEP4 and pEGFP-C1 were kindly provided by Dr. R. Bernardoni (University of Bologna, Italy). Plasmid DNAs were extracted using Plasmid Midi Kit (Qiagen) according to the manufacturer's protocol.

Cell culture and treatment with all-trans-retinoic acid

Neuroblastoma BE(2)C cells, a generous gift from Prof. Della Valle (Bologna, Italy), were grown in DMEM medium, containing 10% heat-inactivated fetal bovine serum (Euroclone) and 100 UI /ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma), in 5% CO_2 humified atmosphere at 37 °C. In some experiments, BE(2)C cells, 48 h after the transfection, were treated with all-trans-retinoic acid (ATRA, Sigma). Cells were re-fed every 48 h with fresh medium containing ATRA. Since ATRA was dissolved in ethanol (vehicle), cells treated with ethanol were used as a control.

Transfection

Transfections were carried out using Lipofectamine™ LTX and Plus™ Reagent (Invitrogen) according to manufacturer's instructions. Cells were seeded at about 70% confluence and transfected 24 h later with $pL1_{RP}$-EGFP, or $pL1_{RP}$ (JM111)-EGFP or pCEP4 plasmid. Cells were split 1 day after the transfection and plated in 6 well plates at a density of 50,000 cells/well. Transfection efficiency was determined by co-transfecting the cells with an EGFP-expression plasmid (pEGFP-C1) and analysing them by FACS analysis 48 h after the transfection (efficiency: 40%).

Cells were selected for transfection with 0.8 μg/ml of puromycin (Sigma) on days 2–3 [24].

Fluorescence microscopy

The appearance of EGFP positive cells was monitored using fluorescence microscopy (Nikon, Eclipse 901). One day after transfection, BE(2)C cells were detached and plated in a 6-well culture plate on tail rat collagen (Sigma) coated glass cover-slips at a density of 50,000 cells/well and after 72 h ATRA or ethanol (vehicle) was added. After various time (from 5 to 12 days after transfection) cultures were fixed with 4% paraformaldehyde (Sigma) and each coverslip was entirely examined by fluorescence microscopy counting the overall single EGFP-positive cells. In order to evaluate the single retrotransposition event, when a group of green fluorescent cells clearly arising from a single retrotransposition event was observed, a single EGFP positive cell was counted.

Flow cytometry

At various time points after the transfection, cells were detached, centrifuged, resuspended in 0.5 ml of phosphate saline buffer containing propidium iodide (PI) dye (5 μg/ml; Sigma, Milan Italy), kept on ice and analysed by means of a flow cytometer (FACScalibur, Becton Dickinson, San Josè, Ca, USA). Gating was determined comparing and analyzing cells transfected with a negative control plasmid (pCEP4) and cells transfected with a positive one (pEGFP-C1). 30,000 cells per sample were analysed by CellQuest Software®.

Cells were tested for transfection by green fluorescence (FL-1) and for viability by exclusion of PI dye or red fluorescence (FL-2). Transfection efficiency was determined taking into account the absolute number of cells which had FL-1 but not FL-2.

DNA extraction and PCR analysis

At various time points after the transfection, genomic DNA was extracted from cells using GenElute™ (Qiagen), treated with Dnase free Rnase (Qiagen), and quantified by Nanodrop fluorometer (Thermo scientific, Wilmington, DE 19810 USA). DNA was subjected to PCR using Ready Mix Taq PCR (Sigma) with primers specific for EGFP cassette listed in Table 1. The primers anneal after stop codon of ORF2 and to the first exon of EGFP cassette flanking γ-globin intron.