Published in final edited form as:

Clin Chem. 2017 April ; 63(4): 816–822. doi:10.1373/clinchem.2016.257444.

The human LINE-1 retrotransposon: an emerging biomarker of neoplasia

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Abstract

Background—A large portion of intronic and intergenic space in our genome consists of repeated sequences. One of the most prevalent is the Long INterspersed Element-1 (LINE-1, L1) mobile DNA. LINE-1 is rightly receiving increasing interest as a cancer biomarker.

Content—Intact LINE-1 elements are self-propagating. They code for RNA and proteins which function to make more copies of the genomic element. Our current understanding is that this process is repressed in most normal cells, but that LINE-1 expression is a hallmark of many types of malignancy. Here, we will consider features of cancer cells when cellular defense mechanisms repressing LINE-1 go awry. We will review evidence that genomic LINE-1 methylation, LINE-1-encoded RNAs, and LINE-1 open reading frame 1 protein (ORF1p) may be useful in cancer diagnosis.

Summary—The repetitive and variable nature of LINE-1 DNA sequences pose unique challenges to studying them, but recent advances in reagents and next generation sequencing present opportunities to characterize LINE-1 expression and activity in cancers, and identify clinical applications.

Introduction

A very small portion - about one percent - of our DNA is recognizable as protein-coding gene exons. Much of the intervening sequence is intronic and intergenic space littered with the remains of mobile DNAs. These are known as transposable elements (TEs), and their ability to copy themselves over time has shaped much of the modern human genome. We

Conflict of Interest

D.T.T. receives sponsored research from Affymetrix, Inc. related to RNA *in situ* hybridization technologies. M.S.T. and K.H.B. license a mouse monoclonal antibody against LINE-1 ORF1p through the Johns Hopkins University School of Medicine Technology Transfer Office. This is sold by EMD Millipore (Billerica, Massachusetts, catalog #MABC1152).

carry hundreds of thousands of copies of these sequences scattered as interspersed repeats. Collectively, they make up half of our DNA (1, 2).

TEs exist as transposons, which operate via a “cut-and-paste” mechanism, or retrotransposons, which propagate by a “copy-and-paste” mechanism known as retrotransposition. Retrotransposons use an RNA intermediate expressed from a genomic locus which is then reverse transcribed by retrotransposon-encoded proteins to make a new genomic insertion. Retrotransposons are classified as long terminal repeat (LTR) or non-LTR elements. They can furthermore be described as autonomous or non-autonomous depending on whether they encode the protein machinery necessary for retrotransposition. The only autonomous, active elements in humans are non-LTR retrotransposons known as Long INterspersed Elements (LINEs). LINEs have an evolutionary history that predates humans by hundreds of millions of years. In aggregate, the human genome is 17% LINE-1 sequence and 5–6% LINE-2 and LINE-3 sequences (1, 3). All retrotransposition today is driven by LINE-1 (L1), the only autonomous element in humans, which remains the focus of this review (4–7).

An intact LINE-1 sequence measures approximately 6 kilobases in length and encodes two well-recognized proteins, open reading frame 1 protein (ORF1p) and open reading frame 2 protein (ORF2p)(Figure 1). LINE-1 also has an antisense promoter (ASP) activity that can initiate fusion transcripts (8–10) and aberrant coding sequence (ORF0) (11) in the opposing direction.

ORF1p trimerizes to form an RNA binding complex required for LINE-1 transposition (12–14). ORF2p encodes two enzymatic activities also essential for retrotransposition, an endonuclease and a reverse transcriptase (15–17). ORF2p reverse transcribes new genomic DNA copies of LINE-1 from its RNA and is co-opted to copy other repeats, namely the *Alu* Short INterspered Element (SINE)(18), and the SVA (SINE, VNTR, *Alu*) composite elements (19, 20).

Each individual inherits a small complement of full-length, retrotransposition-active or ‘hot’ LINE-1 loci (21–24). The specific loci vary from person to person. The prevailing hypothesis in the field is that these potentially protein-coding LINE-1 are then kept in check by a series of host defenses to maintain genome integrity. In this article, we will briefly reference aspects of normal LINE-1 control and contrast this with LINE-1 expression in malignancy.

Mechanisms of LINE-1 Repression

Expression of LINE-1 sequences and subsequent steps in retrotransposition are repressed by host factors. In the germ line, the piRNA pathway is critical in establishing repressive DNA methylation patterns (reviewed in (25)). In somatic cells, the SWI/SNF2-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 6 (SMARCA6) helicase (26, 27) and p53 (28) feature prominently.

Once expressed, LINE-1 transcripts and proteins can be altered in their activities. Mechanisms include RNAi pathways (29, 30), nonsense mediated decay via up-frame shift 1

(UPF1) (31), and antiviral proteins like apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3) cytidine deaminase (32), Moloney leukemia virus 10 (MOV10) (33, 34), and zinc-finger antiviral protein (ZAP) (35, 36).

LINE-1 Hypomethylation in Cancer

Full-length LINE-1 transcription is driven by a CpG dinucleotide-rich internal promoter. Many genomic LINE-1 sequences are 5' truncated at the time of genomic integration, and so have lost their promoter. Many intact promoter sequences are found throughout the genome, however, and CpG methylation of LINE-1 is used by many as a surrogate marker of whole genome methylation levels (37). It is important to note that while many tumor suppressor gene promoters are methylated in tumors, whole genome methylation and LINE-1 methylation specifically tend to be reduced in malignancies.

LINE-1 methylation studies have been conducted in many of the most common lethal cancers, including (in order of mortality) lung cancer, colon and rectal cancers, breast cancer, prostate cancer, liver cancer, ovarian cancer, and esophageal cancer (Figure 2). In non-small cell lung cancer, LINE-1 promoter hypomethylation is common (38) and is associated with genomic instability (39) and poor prognosis (40). In colon cancer, LINE-1 hypomethylation appears to be an early event (41) also associated with poor outcomes (42, 43). It appears inversely correlated with microsatellite instability (44, 45). Interestingly, colon cancer patients whose tumors exhibit extremely low levels of LINE-1 methylation may be a clinically distinct group, with a tendency to present at a younger age (46). LINE-1 hypomethylation is more pronounced in colon cancer liver metastases compared to matched primary tumors (47). In breast cancer, LINE-1 hypomethylation has been reported in preneoplastic phases of epithelial atypia with persistently low LINE-1 promoter methylation seen in *in situ* and invasive lesions (48). It has also been associated with decreased overall survival and drug resistance in younger patients (49). In prostate cancers, LINE-1 hypomethylation is also reported, particularly in association with chromosome 8 abnormalities (50); it appears more pronounced in metastatic lesions than in primary tumors (51). In hepatocellular carcinoma, several groups have associated LINE-1 hypomethylation with poor clinical outcomes, including disease recurrence after resection (52–54). In epithelial ovarian cancers, LINE-1 hypomethylation is correlated with more aggressive histology, poorer progression-free intervals, and poorer survival (55). Finally, in esophageal squamous cell carcinomas, LINE-1 hypomethylation is also recognized and associated with poorer survival (56). Evidence of aberrant LINE-1 hypomethylation is less frequently reported for hematolymphoid neoplasias.

A meta-analysis of LINE-1 hypomethylation as a marker for cancer risk revealed that tissue-based DNA assays fairly consistently reveal LINE-1 hypomethylation in cancers compared to controls (57). In contrast, LINE-1 methylation status in blood is apparently not a marker of cancer risk across 19 studies included in the meta-analysis, suggesting that direct assays of malignant tissues are more sensitive to these changes (57).

Many of the studies cited above compare aggregate normal and tumor LINE-1 methylation levels without delineating the specific LINE-1 loci driving these changes or accounting for differences in the complement of inherited LINE-1 sequences between individuals.

However, very recent work suggests that more personalized approaches are likely to yield new insight, and add nuance to the overly simplistic model that relating cancerous histopathology or poor outcomes with LINE-1 hypomethylation. Emma Scott and her colleagues in Scott Devine's laboratory recently traced somatic retrotransposition events in a case of colon cancer to three inherited, full-length LINE-1 elements. One of these hot elements, a LINE-1 on chromosome 17, appears responsible for the cancer as it generated a 1.4 kb insertion in the adenomatous polyposis coli (*APC*) gene (58). This is a classic 'driver mutation' positioned to inactivate the tumor suppressor. The 'parent' or 'source' element is a polymorphic variant in human populations, which can be methylated appropriately – it is methylated in reference DNA from lymphoblastoid cell lines. However, its promoter sequence was largely unmethylated in both normal colon and cancerous tissue from this patient. So, while some LINE-1 may acquire hypomethylation in the permissive environment of a cancer cell, others appear to escape silencing in normal tissues and be poised to play important inciting roles in tumor pathogenesis.

LINE-1 RNA

LINE-1 and other interspersed repeat RNAs have been less well characterized than gene messenger RNAs (mRNAs) in cancer. They pose a challenge because there are hundreds of thousands of LINE-1 genomic loci that have the potential to be incorporated into larger transcripts. Indeed, most of these genomic copies are 5' truncated, and it follows that their transcription will not be directed by the LINE-1 promoter, but rather exclusively by 'read-through' transcription. Most LINE-1 do not have intact ORFs, and roles of their RNAs are not well understood. LINE-1 RNA has long been recognized as a component of heterogeneous nuclear RNAs (59), and recent *in situ* hybridization studies demonstrate that repetitive RNAs, and 3' LINE-1 RNA in particular, are long-lived components of chromatin (60). The abundant expression of these fragments of LINE-1 RNA has been seen across cancers (61).

LINE-1 RNA that is the intermediate for retrotransposition is encoded by the LINE-1 promoter and is the same length as a full-length genomic element, 6 kilobases (kb) (i.e., the so-called *unit* LINE-1 transcript (62)). Before advances in next generation sequencing, RNA expression directed specifically from the LINE-1 promoter could be most reliably detected by Northern blots so that the size of the resulting RNA could be assessed. This was how LINE-1 RNA was first identified in cytoplasmic fractions of Ntera2D1 teratocarcinoma cells (63), and it remains a valuable approach for experimentalists today (64). Despite caveats for interpreting their results, RNAse protection assays, RT-PCRs, and *in situ* hybridizations have also been used to infer unit LINE-1 expression. When these assays target the 5' end of LINE-1, they are expected to be relatively more specific than when 3' positioned probes and primers are used. Similarly, selecting for polyadenylated, cytoplasmic RNA and/or using assays specific for the sense strand of LINE-1 can further promote specificity for unit transcripts.

There is recent evidence reported by Claude Philippe and colleagues with Gaël Cristofari's laboratory that active LINE-1 RNAs [L1Hs or L1(Ta)] can be traced to specific templating loci using next generation sequencing data. Their analysis involves integrating a

combination of data types, including a genomic LINE-1 insertion map, RNA-seq reads, and chromatin immunoprecipitation (ChIP)-seq data (65). Actively transcribed LINE-1 loci have a two-part 'signature' : (i.) RNA-seq reads corresponding to 'read-through' transcription downstream (3') of the polyA tail, as well as (ii.) histone H3K4 trimethylation, H3K27 acetylation, and RNA polymerase II ChIP-seq reads extending into upstream (5') sequence. With these as proxies for LINE-1 expression, the group was able to identify a small number of transcribed LINE-1 loci in human cancer cell lines – about 5 to 15 elements appear responsible for most unit LINE-1 RNA expression in cells. These data are consistent with a model wherein cancer cells maintain LINE-1 repression at most full length loci, with only a handful of escaping elements with the capability of retrotransposition.

Long read sequencing may be useful for detecting unit LINE-1 RNAs as well as resolving RNA species transcribed from other genomic repeats. These long reads may incorporate unique flanking sequence, may also enable unequivocal mapping of full-length transcripts. Being able to accurately phase internal sequence variants of unit LINE-1 RNAs and relate these variants back to individual genomic loci (58) will represent an important advance.

ORF1p expression

Intact, full length LINE-1 sequences code for two proteins, ORF1p and ORF2p. Of these, expression of the first has been best characterized in human cancers. LINE-1 expression constructs in *in vitro* transfected cells produce ORF1p at 1,000- to 10,000-fold higher levels than ORF2p (31).

ORF1p (p40) is an RNA binding protein essential for retrotransposition (12–14, 66). Its crystal structure has been solved (67). Three ORF1p protein molecules intertwine throughout the length of their N-terminal coiled coil to form a homotrimeric complex. The central RNA recognition motifs (RRMs) (68) and C-terminal domains (CTDs) project outward from the coiled coil axis to form deep intervening clefts. These clefts are highly positively charged surfaces that likely interact with the backbone of single-stranded RNA.

At least two studies have sequenced the RNAs that interact with LINE-1 protein in HEK293T cells overexpressing exogenous LINE-1. Taylor *et al.* immunoprecipitated LINE-1 ribonucleoproteins using FLAG-tagged ORF1p or ORF2p and found that L1 RNA represented 8.3–10.3% and 18.0–28.2% of reads associated with ORF1p pulldown or ORF2p pulldown, respectively (31). There was also enrichment of U6 snRNA. Mandal and colleagues used photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) followed by sequencing to discover that 22% of all ORF1p-associated RNAs were mRNAs with known pseudogenes (69). ORF1p also associated with small structured RNAs included spliceosomal and hY RNAs, in addition to LINE-1, *Alu*, and SVA RNA. It is not known whether ORF1p sequesters cellular RNAs within tumors or what impacts this may have on cancer cell biology.

The first antibody developed against ORF1p was a rabbit polyclonal reagent described in 1990 by Debra Leibold and colleagues in Thomas Fanning's laboratory (70). The group reported detecting LINE-1 ORF1p in embryonal carcinoma cells (Ntera2), teratocarcinoma cells (2102Ep), and choriocarcinoma cells (JEG-3). Many of the first studies of ORF1p

expression then focused on these highly expressing germ cell tumors. Gary Bratthauer also with Thomas Fanning surveyed primary adult testicular germ cell tumors and pediatric germ cell tumors by immunohistochemistry to find ~10% positive for L1Hs expression. All were epithelial with typical embryonal carcinoma or yolk sac tumor appearances (71, 72).

A larger study of pediatric malignant germ cell tumors (MGCT) was next conducted by the Children's Oncology Group (COG), also using the same Fanning laboratory reagent, but with a more sensitive immunohistochemistry protocol. Using this method, the group, led by Xiao-Ou Shu found evidence for expression in all of the 162 MGCT cases assessed. They stratified these into cases that were strongly, moderately, and weakly immunoreactive for LINE-1 ORF1p and reported that strong expression of ORF1p was associated with poor differentiation, extragonadal sites of disease, and yolk sac tumor histologies (73).

Breast malignancies also received early recognition as LINE-1 ORF1p-expressing cancers. Bonnie and Harold Asch in collaboration with the Fanning laboratory reported that ORF1p expression could be detected by Western blot in both malignant and nonmalignant breast epithelium. Most (4/5) normal tissue samples recovered from reduction mammoplasties had low levels as compared with tumors. However, their work suggested that malignant cells produce more of the protein. They suggest that immunostaining intensity can serve as an indicator of malignancy; they report ORF1p reactivity in all cases of invasive cancer examined (12), whereas benign proliferative disease and normal tissues were weakly reactive and negative (74).

In 2010, Chris Harris and colleagues prepared rabbit polyclonal antibody against ORF1p and described a broader expression pattern in human tumors (75). The group reported positivity in 99% of breast cancers, but as well, expression in a significant proportion of bladder cancers, prostate cancers, colorectal cancers, ileal carcinoids, and pancreatic neuroendocrine tumors. Much of their work focused on breast cancers, where they reported nuclear localization of ORF1p in a subset of cases. Nuclear immunoreactivity was associated with increased incidence of local recurrence, distant metastases, and poorer overall survival.

We have since developed a mouse monoclonal antibody against ORF1p (76, 77). The reagent was raised against amino acids 35–44 of ORF1p (AAB60344.1, MENDFDELRE). This is a region close to the N-terminus of the protein, before the coiled-coil domain begins and where mouse and human LINE-1 sequences diverge. Using a combination of rabbit polyclonal antibody from Chris Harris and this mouse monoclonal antibody, Nemanja Rodić surveyed human cancers using tissue microarray immunostaining. We did not see nuclear staining in this study, but cytoplasmic immunoreactivity was very common, and either specific for malignant tissue or overexpressed in malignant cells as compared to adjacent normal. LINE-1 ORF1p immunoreactivity was seen in significant proportions of lung cancers (51%), esophageal cancers (64%), breast cancers (97%), liver cancers (19%), colon cancers (50%), ovarian cancers (92%), and prostate cancers (41%). These data are summarized in Figure 2.

There is no direct way to trace ORF1p protein expression back to individual LINE-1 genomic loci. Within an individual, multiple LINE-1 loci may contribute to protein

expression, and between individuals, the LINE-1 loci contributing to protein expression need not be shared.

Several types of tumors have been shown to have genomic evidence of LINE-1 retrotransposition, implying that in addition to ORF1p, LINE-1 unit-length RNA and ORF2p are also expressed. In partnership with the Kazazian laboratory, we have demonstrated expression of ORF1p by immunohistochemistry in specific cases of pancreatic ductal adenocarcinomas (78), esophageal adenocarcinoma (79) and esophageal squamous cell carcinoma (80) with somatically-acquired genomic LINE-1 insertions.

Concluding Remarks

Once dismissed as obscure junk DNA, LINE-1 is now the focus of increasing studies in cancer biology and in the search for cancer markers. Genomic LINE-1 hypomethylation, increases in LINE-1 RNA transcription, and ORF1p accumulation seem to be features of malignant cells more so than adjacent normal tissues. Going forward, a better understanding of consequences of LINE-1 expression in cancer biology is critical in our view.

We note that the phenomenon is not uniform across tumor types or individual patients. This may reflect features inherent to the pathogenesis of each disease, p53 function for example, raising the possibility that LINE-1 expression will be useful for subclassification or prognosis. For instance, ORF1p expression positively correlates with *TP53* deficiency and higher-grade lesions (76). Tumor cell type also matters. Aberrant LINE-1 ORF1p expression is a hallmark of epithelial tumors more than, for example, hematolymphoid malignancies. Finally, personalized approaches that interpret LINE-1 expression markers in light of the inherited complement of LINE-1 loci may become important.

Due to the complexity of LINE-1 genomic sequences and RNA species, we remind readers that biomarkers related to these should be regarded as uncoupled from LINE-1 protein expression. Depending on assay design, LINE-1 methylation or RNA expression may serve as surrogates of global or local chromatin status, whereas protein may be a more specific indicator of *bona fide* expression of the unit LINE-1 element.

Acknowledgments

The authors thank Alex Forrest-Hay for discussions. This work has been supported by the Burroughs Wellcome Trust (D.T.T.), K12CA087723 (D.T.T.), Affymetrix, Inc. (D.T.T.), the Warshaw Institute for Pancreatic Cancer Research (D.T.T.), and the Verville Family Pancreatic Cancer Research Fund (D.T.T.) as well as the Burroughs Wellcome Fund (K.H.B.), R01CA163705 (K.H.B.), R01GM103999 (K.H.B.), and the Systems Biology of Retrotransposition P50GM107632 (K.H.B.).

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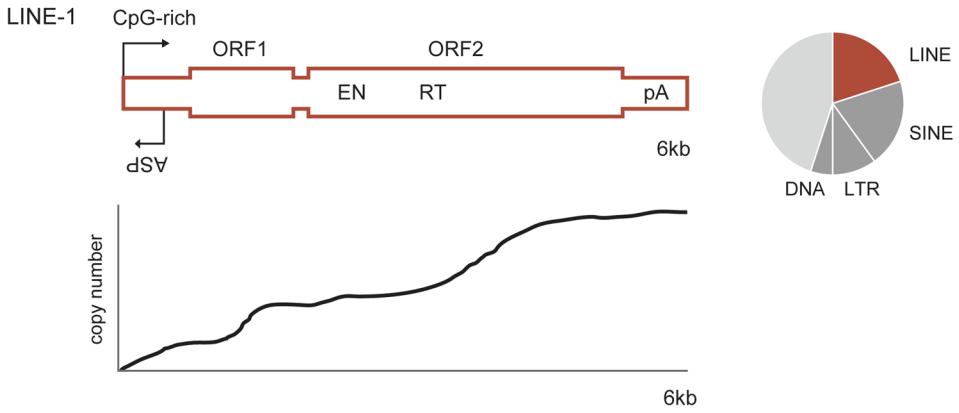

Figure 1.

A schematic of a LINE-1 element. A full-length LINE-1 is 6 kilobases (kb) in length. It includes a CpG-rich bidirectional promoter and two open reading frames for ORF1p and ORF2p proteins. The element is illustrated as a block with widened open reading frames. ASP=antisense promoter; EN=endonuclease, RT=reverse transcriptase, pA=polyA tail. Beneath this schematic is a plot showing the relative genomic copy number of L1Hs sequences in the human genome as a function of position along the length of the 6kb consensus sequence. There are relatively more copies of the 3' end of the element because many copies are 5' truncated at the time of their integration. The length of a LINE-1 is stable after insertion with the exception of the polyA portion. The pie chart to the right illustrates the percentage of the human genome comprised of repetitive elements. LINE=Long INterspersed Element; SINE=Short INterspersed Element; LTR=Long Terminal Repeat; DNA=DNA transposons ('cut-and-paste' transposons).

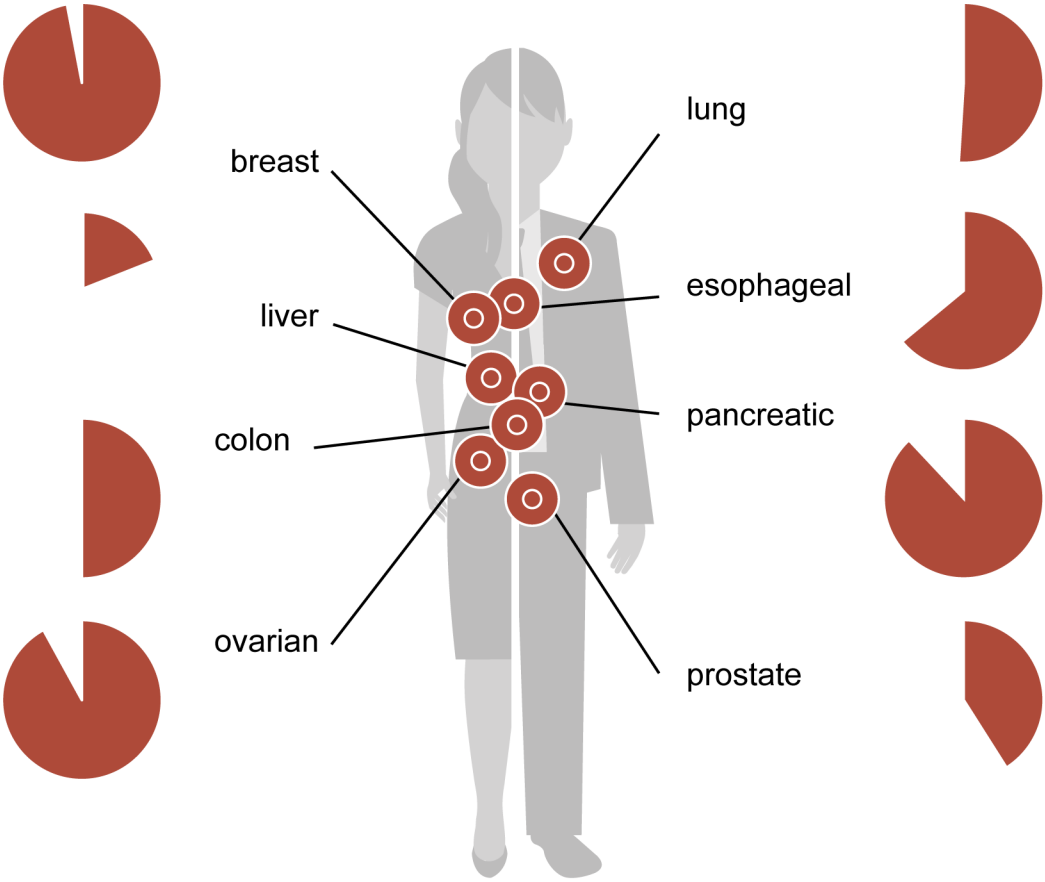

Figure 2.
LINE-1 ORF1p expression in cancer. Diagram of tissue types with known LINE-1 positive cancers. Hypomethylation of LINE-1 promoters has been described for tumors originating from all of these sites. The proportion of cases with LINE-1 ORF1p expression detectable by sensitive immunohistochemistry (IHC) is shown next to each tissue of origin.