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Aurelien J Doucet, Gael Cristofari

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1 **Noninvasive and multicancer biomarkers: the promise of LINE-1**
2 **retrotransposons**

3 Aurelien J. Doucet* and Gael Cristofari*

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5 University Cote d'Azur, Inserm, CNRS, Institute for Research on Cancer and Aging of Nice (IRCAN), Nice,
6 France

7 * Co-corresponding authors. Correspondence should be addressed to:

8 Aurelien.Doucet@univ-cotedazur.fr or Gael.Cristofari@univ-cotedazur.fr

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10 **RUNNING TITLE**

11 Circulating retrotransposon protein as cancer biomarker

12 **SUMMARY**

13 LINE-1 retrotransposons are frequently active in epithelial tumors. In a new study, Taylor,
14 Wu *et al.* now describe that one of the proteins encoded by LINE-1 elements, ORF1p, is
15 detected in the bloodstream of cancer patients, and can be used as a noninvasive and
16 multicancer biomarker for diagnosis or treatment monitoring.

17

18 Although almost half of our genome is made up of repeated DNA sequences, these are not
19 routinely exploited as cancer markers, with the notable exception of microsatellite instability
20 (MSI). Forming a distinct class of repeats, transposable elements are highly dispersed and
21 abundant in the human genome. Among them, the Long INterspersed Element-1 (LINE-1 or
22 L1) is the only element encoding a functional machinery and capable of autonomous
23 mobilization in humans. L1 replication is based on a copy-and-paste mechanism known as
24 retrotransposition, involving an RNA intermediate and a reverse transcription step (1)
25 (**Figure 1**, top). Active L1 copies are 6kb-long expression units, containing an internal
26 promoter as well as two open reading frames, ORF1 and ORF2. The first encodes an RNA-
27 binding protein (ORF1p, also known as p40), while the second encodes an enzyme with
28 endonuclease and reverse transcriptase activities (ORF2p), both essential for
29 retrotransposition (1). Upon transcription and translation of the bicistronic L1 mRNA, ORF1p
30 and ORF2p proteins assemble with the L1 mRNA to form L1 ribonucleoprotein particles
31 (RNPs) that mediate reverse transcription in the nucleus at sites of integration through a
32 process called target-primed reverse transcription (1).

33 In most tissues, L1 expression is repressed, preventing or limiting L1-mediated mutagenic
34 activity. However, specific physiological or pathological situations are associated with a
35 profound remodeling of the cellular epigenetic landscape, opening windows of opportunity
36 for L1 expression and even mobilization (2). For instance, the germline and early embryo
37 can harbor high levels of L1 activity. In these territories, new insertions create inheritable
38 polymorphisms and thereby contribute to the genetic diversity of the human population.
39 Occasionally they can also cause genetic diseases (1). L1 expression was also reported in a
40 few somatic tissues, such as the brain or senescent cells thereby contributing to age-
41 associated inflammation (2,3).

42 As early as the 90's, the L1 ORF1p was found as specifically expressed in some tumors but
43 not in adjacent normal tissues, and L1-mediated somatic mutations could be identified in

44 colon cancer. More recently, the advent of high-throughput sequencing technologies and the
45 development of monoclonal antibodies against ORF1p have extended these initial
46 observations (4,5). A large survey suggested that nearly half of human epithelial tumors
47 show L1 ORF1p expression (5). Moreover, L1 somatic retrotransposition, as well as other
48 forms of L1-mediated genomic alterations, are detected in several epithelial cancer types
49 such as lung, colorectal, liver, breast, uterine, ovarian, prostate, and head-and-neck
50 cancers, but are virtually undetectable in leukemia or brain tumors (6). Even if examples of
51 L1 insertions acting as drivers of tumorigenesis are limited, L1 promoter hypomethylation
52 and ORF1p levels often correlate with poor prognosis in many cancer types. Moreover, the
53 accumulation of ORF1p in tumors is associated with *TP53* loss-of-function, consistent with
54 the notion that high level of L1 activity is associated with genomic instability (5). Altogether,
55 these observations have led to the notion that ORF1p expression in tumors could serve as a
56 biomarker for cancer diagnosis or treatment outcome, but the translation of this idea into
57 clinically relevant assays has been limited so far.

58 In this issue of *Cancer Discovery*, Taylor, Wu *et al.* (7) develop several ultrasensitive
59 immunoassays to detect L1 ORF1p, and they identify circulating ORF1p as a highly specific
60 cancer biomarker, that is thus amenable to liquid biopsy (**Figure 1**).

61 The authors first produced and characterized a new set of monoclonal antibodies and
62 nanobodies targeting ORF1p with high specificity and affinity, which they use to implement
63 digital immunoassays (**Figure 1**, bottom). These single-molecule approaches are based on
64 a “sandwich” immunocomplex similar to Enzyme Linked Immunosorbent Assays (ELISAs).
65 Beads coated with a first antibody capture the target protein, while a second antibody allows
66 the detection of the first immunocomplex through an enzymatic reaction. However, unlike
67 ELISA, quantification is digital, achieved by recording the number of negative and positive
68 beads (like the 0 and 1 in binary coding) rather than measuring signal intensity, enabling
69 highly sensitive detection of low-concentration targets. These technical developments have

70 led to a remarkable sensitivity, reaching the attomolar range in the most sensitive versions of
71 their assays, and to the discovery that free circulating ORF1p can be detected in the
72 bloodstream.

73 By comparing healthy donors with patients diagnosed with various forms of cancers of
74 epithelial origin, Taylor, Wu *et al.* revealed that circulating ORF1p is a highly specific
75 multicancer biomarker (7). Patients affected by breast, ovarian, uterine, colorectal,
76 gastroesophageal, pancreatic, liver, lung, head and neck, or prostate cancers display
77 detectable levels of circulating ORF1p, in the range of 10 to 75% of patients depending on
78 the cancer type, while it was only detectable in around 1% of the healthy donors. With a
79 second-generation assay, circulating L1 ORF1p could be detected in as much as 75% of
80 colorectal cancer patients and 90% of ovarian cancer patients. Combining the detection of
81 circulating ORF1p with other currently available biomarkers, such as CA125 and HE4 in
82 ovarian cancer, improved the performance of the diagnosis. Anecdotally, one of the healthy
83 donors tested had a higher level of circulating ORF1p than any of the cancer patients in the
84 study. Although the patient was considered healthy at the time of blood donation, he was in
85 fact subsequently diagnosed with cancer. While most of the ORF1p-positive patients were at
86 a late stage, the sensitivity of the assay also allowed identification of patients with early
87 stages of ovarian and lung cancers. Circulating ORF1p could thus become a powerful
88 multicancer and noninvasive biomarker for early diagnosis and early treatment at a stage
89 when patients are often asymptomatic.

90 Finally, Taylor, Wu *et al.* (7) investigated the levels of circulating ORF1p in the plasma of
91 patients that underwent therapy. In a group of gastroesophageal cancer patients that
92 received systemic therapy, circulating ORF1p levels are correlated with the clinical response
93 to treatment. Patients with low ORF1p levels prior to treatment showed a better clinical
94 response than patients with high levels. In this cohort, high levels of circulating ORF1p

95 before therapy were also associated with reduced survival. Thus, circulating ORF1p levels
96 could also serve as an early predictor of response to therapy.

97 Understanding why some tumors express ORF1p and other not is a key question as ORF1p-
98 negative tumors represent a potential source of false negatives. Some cancer types, not
99 studied in the present work, such as neurologic or hematological cancers, appear to
100 reactivate L1 elements to a very limited extent (6). However, even within cancer types of
101 epithelial origin, some patients do not show ORF1p-positive tumors and have undetectable
102 circulating ORF1p (5,7). ORF1p positivity was found to be strongly associated with *TP53*
103 loss of function (5,7). By identifying other factors influencing L1 expression in tumors, it may
104 be possible to rationally design panels of complementary circulating biomarkers. In addition,
105 individual human genomes contain a unique combination of inherited L1 loci, some being
106 fixed in the human population, but others being polymorphic with respect to their presence or
107 absence, or to their internal sequence (4,8,9). Among these loci, only a small subset has the
108 potential to express a functional ORF1p and an even more restricted subset is capable of
109 somatic retrotransposition. Thus, it is conceivable that the ability of tumors to express
110 ORF1p or not depends on the ancestry of the patient. Being able to identify which loci
111 contribute the most to circulating ORF1p could help improve and personalize the accuracy of
112 the diagnosis.

113 Defining the baseline level of circulating ORF1p in a large cohort representing the general
114 population will be essential in the future. What are the tissues of origin of the low ORF1p
115 levels detected in healthy individuals? As mentioned above, L1 expression can occur in
116 cancer cells but also in germ cells and senescent cells. The work by Taylor, Wu *et al.* (7)
117 suggests that circulating ORF1p is not correlated with age or sex. It is possible that, in some
118 cases, circulating ORF1p in healthy individuals originate from microtumors that would never
119 grow and lead to disease, or would be naturally eliminated by the immune system, raising a
120 risk of over-diagnosis. The question of how ORF1p is specifically released into the

121 bloodstream by cancer cells and not by other cell types expressing L1 remains open. The
122 level of expression could simply be higher in cancer cells, but cancer cells may also exhibit
123 unique release pathways. Size exclusion chromatography experiments suggest at least that
124 the process does not involve extracellular vesicles (7).

125 Retrotransposon-derived antigens have recently emerged as potential targets for antitumoral
126 immunotherapy (10). In the present study, Taylor, Wu *et al.* (7) extend the use of
127 retrotransposon proteins to cancer diagnostics. They provide a proof-of-concept that the
128 early detection of circulating ORF1p by digital immunoassays is a promising strategy to
129 diagnose cancer and monitor the early-response to treatment in a noninvasive manner,
130 through a simple and cost-effective blood test. Such a noninvasive follow-up could help to
131 dynamically adjust treatment of cancer patients. Its adoption in the clinical practice will
132 depend on automation for implementation in clinical laboratories, and on future clinical trials
133 on larger cohorts to define the practical use of this new biomarker.

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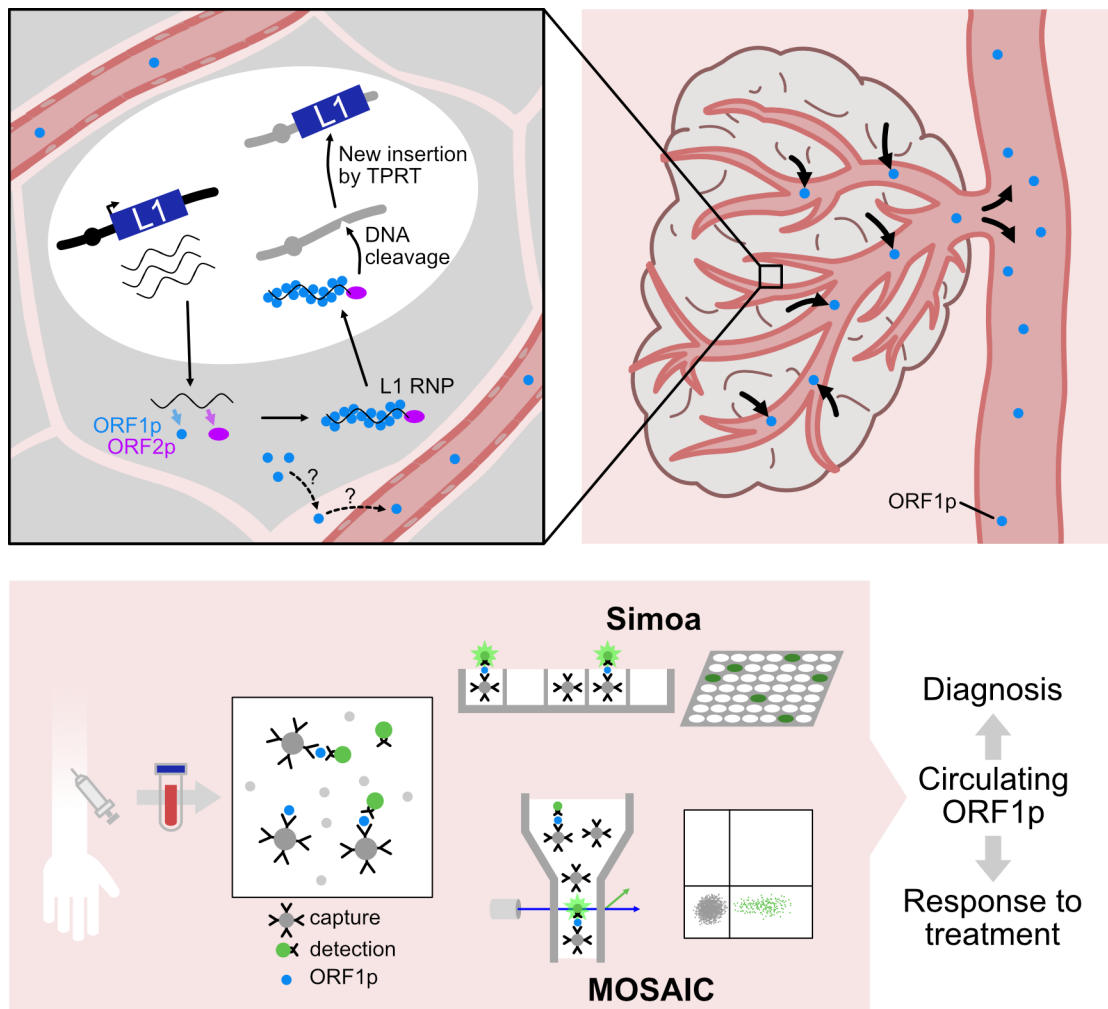
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180



182

183 **Figure 1 – A circulating L1 retrotransposon protein, ORF1p, as a noninvasive, broad**
 184 **usage, multicancer biomarker.**

185 **Top:** Schematic representation of an L1-expressing cancer cell within a vascularized tumor.
 186 An active L1 locus is transcribed (blue box, in black chromosome) to synthesize bicistronic
 187 L1 mRNAs (wavy lines). Translation occurs in the cytoplasm to produce ORF1p (blue circle)
 188 and ORF2p (purple oval). An L1 ribonucleoprotein particle (RNP) is formed by the
 189 association between the L1 mRNA, ORF1p and ORF2p. By entering in the nucleus, the L1
 190 RNP can insert a new L1 copy into another locus (black box in grey chromosome) through
 191 DNA cleavage and a process called Target-Primed Reverse Transcription (TPRT) (1).

192 Taylor, Wu *et al.* describe the presence of ORF1p in the bloodstream (blue dots in red
193 vessels) of patients diagnosed with a subset of epithelial cancers (7). The mechanism by
194 which ORF1p is released from cancer cells and reaches the bloodstream is currently
195 unknown (dashed arrows with question mark).

196 **Bottom:** Ultrasensitive detection of circulating ORF1p from blood samples. The procedure is
197 based on a simple blood test. Taylor, Wu *et al.* measured plasmatic levels of ORF1p by
198 digital immunoassays relying on a bead-based “sandwich” immunocomplex, which combines
199 capture and detection antibodies or nanobodies with high affinity for ORF1p (7). Two
200 strategies were employed: Single Molecule Arrays (Simoa), and Molecular On-bead Signal
201 Amplification for Individual Counting (MOSAIC). Simoa is performed in a plate format with
202 each well containing a single bead through an enzymatic detection, whereas MOSAIC relies
203 on the evaluation of positive and negative beads by flow-cytometry through a secondary
204 amplification process and fluorescence. Ultimately, the presence of circulating ORF1p can
205 be used for the diagnosis and monitoring of cancer patients in a noninvasive manner, and
206 can apply to multiple cancer types, such as colorectal or ovarian cancers.

207