LINE-1 retrotransposon-mediated DNA transductions in endometriosis associated ovarian cancers

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HIGHLIGHTS

- An active LINE-1 retrotransposon from *TTC28* gene is present in 32% of EAOCs.
- *TTC28* LINE-1 events are near ubiquitous across multiple EAOC tumor samplings.
- *TTC28* LINE-1 is likely activated early during EAOC development.
- *TTC28* LINE-1 may be used to track EAOC development.

ARTICLE INFO

ABSTRACT

Objective. Endometrioid (ENOC) and clear cell ovarian carcinoma (CCOC) share a common precursor lesion, endometriosis, hence the designation endometriosis associated ovarian cancers (EAOC). Long interspersed nuclear element 1 (LINE-1 or L1), is a family of mobile genetic elements activated in many cancers capable of moving neighboring DNA through 3′ transductions. Here we investigated the involvement of specific L1-mediated transductions in EAOCs.

Methods. Through whole genome sequencing, we identified active L1-mediated transductions originating within the *TTC28* gene in 34% (10/29) of ENOC and 31% (11/35) of CCOC cases. We used PCR and capillary sequencing to assess the presence of specific *TTC28*-L1 transductions in formalin-fixed paraffin-embedded (FFPE) blocks from six different anatomical sites (five tumors and one normal control) for four ENOC and three CCOC cases, and compared the results to the presence of single nucleotide variations (SNVs)/frame shift (fs) mutations detected using multiplex PCR and next generation sequencing.

Results. *TTC28*-L1 mediated transductions were identified in at least three tumor samplings in all cases, and were present in all five tumor samplings in 5/7 (71%) cases. In these cases, *KRAS*, *PIK3CA*, *CTNNB1*, *ARID1A*, and *PTEN* mutations were found across all tumor sites while other selected SNV/fs mutations of unknown significance were present at varying allelic frequencies.

Conclusion. The *TTC28*-L1 transductions along with classical driver mutations were near ubiquitous across the tumors, suggesting that L1 activation likely occurred early in the development of EAOCs. *TTC28*-L1 transductions could potentially be used to determine clonal relationships and to track ovarian cancer progression.

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1. Introduction

Ovarian cancer is the most lethal gynecological cancer, and it is the 5th leading cause of cancer related deaths in women in the United States [1]. Endometrioid ovarian carcinoma (ENOC) and clear cell ovarian carcinoma (CCOC) each account for approximately 10% of all ovarian

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cancer cases [2]. Both cancers are low grade subtypes that are often diagnosed at stages I and II. When diagnosed at stages III/IV, ENOC has a more favorable prognosis compared to CCOC, as CCOC tends to have a much lower response rate to chemotherapy [2,3]. A recent study of the genomic profile of ovarian cancers stratified ENOC into three subgroups: ultramutator (high mutation load), micro-satellite instable (MSI), and micro-satellite stable (MSS), and CCOC cases into two subgroups: APOBEC (localized hypermutation) and mutation signatures similar to one previously associated with age, though not age-related in CCOC [4].

Despite having different pathology and genomic profiles, both ENOC and CCOC are associated with endometriosis, hence the designation endometriosis associated ovarian cancer (EAOC). Endometriosis is characterized by ectopic growth of endometrial epithelium and stroma. Women with endometriosis have a 2- to 3-fold increased risk of developing ENOC and CCOC [5]. Common genes with mutations implicated to drive tumorigenesis include *ARID1A* (30%), *PTEN* (20%), and *CTNN1B* (38–50%) in ENOC, and *ARID1A* (46–57%) and *PIK3CA* (33–46%) in CCOC. Cancer driving mutations in *ARID1A*, *PTEN*, and *PIK3CA* have also been found in endometriotic lesions both adjacent and distant to the tumors, indicating a clonal relationship between these diseases [6–9]. In addition, canonical cancer mutations have been found in deep infiltrating endometriosis that is not associated with cancer [7]. Given that driver mutations in EAOCs are only found in a subset of patients, and certain changes can occur in endometriosis without cancer, additional types of biomarkers are needed to distinguish women with endometriosis at risk of developing cancer.

In our whole genome sequencing of 29 ENOC and 35 CCOC cases, we observed frequent rearrangement events originating from an active LINE-1 (L1) retrotransposon located in the *TTC28* gene on chromosome 22q12. Long interspersed nuclear elements 1 (LINE-1 or L1), are repetitive mobile DNA elements that accounts for 17% of the human genome [10–13]. L1s encode an endonuclease and a reverse transcriptase that allows them to "copy-and-paste" their own sequences into random genomic locations via RNA intermediates [10,12]. They are also capable of taking unique downstream DNA fragments with them during this genetic propagation in a process called 3′ transduction [10]. While most L1s are not functional due to structural rearrangements, there are approximately 80–100 full-length L1s that are potentially active [10]. L1 activation is important in the germ cells during embryogenesis [14], but epigenetic silencing typically occurs in adult [12]. In the cancer genomes, however, L1s become activated as part of a general degradation of genomic and epigenetic integrity [12].

The effects of L1 retrotransposon activation in cancer development and progression has been studied in various cancers, including head and neck, prostate, ovarian, colorectal, gastric, esophageal, pancreatic, and breast cancer [10,11,13,15–21]. While most studies surveyed the landscape of somatic L1 retrotranspositions by detecting the presence of novel inserted L1 sequences in tumors, Tubio and colleagues studied the effect of 3′ transductions events mediated by a set of active L1s in cancers [10]. This specific *TTC28*-L1-mediated transduction from chromosome 22q12 is one of the most frequently active L1s found in colon, head and neck, and lung cancer samples and is the only active L1 element in 93% of breast cancer samples [10]. Specific L1-mediated transductions have been less extensively studied in EAOCs [17,21].

Here we used PCR and capillary sequencing to validate the presence of *TTC28*-L1-mediated transductions in different tumor samplings of ENOC and CCOC, and we compared the results to targeted resequencing of selected single nucleotide variations (SNVs)/frame shift (fs) mutations in the same tissues to infer biological timing of L1 transductions. Our data showed that these transduction events appeared with canonical driver mutations for majority of the cases, and suggests that they likely occurred early in tumorigenesis. We hope to use *TTC28*-L1 mediated DNA transductions to gain insights into the tumor development of EAOCs.

2. Methods

2.1. Whole genome sequencing (WGS)

The cohort and methods have been described previously by Wang et al. [4]. Briefly, tumor (frozen tissue) and matched normal (buffy coat) DNA libraries were constructed for 29 ENOC and 35 CCOC cases, and sequenced using Illumina HiSeq 2500 V4 chemistry. Analyses were performed using bioinformatic methods described in Wang et al. supplemental materials [4].

2.2. Case selection

Four ENOC and three CCOC cases with the highest numbers of *TTC28*-L1-mediated transductions detected by WGS were selected from our previous study [4]. All materials were provided by the OVCARE gynecological tissue bank. H&E slides for all available formalin-fixed paraffin-embedded (FFPE) blocks were reviewed by expert pathologists (T.N. and B.T-C.). Five FFPE tumor tissue blocks with the highest cellularity (>90%) and one representative FFPE normal tissue block were selected for each of the seven cases. Overall project processes were approved by the BC Cancer Agency or the University of British Columbia Research Ethics Board (REB #H08-01411 and #H09-02153).

2.3. DNA extraction from FFPE blocks

FFPE tissue blocks were cut 10um thick and applied on charged glass slides, where relevant areas, identified by a pathologist (T.N.), were macrodissected. DNA was extracted from FFPE tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen) as per manufacturers' protocols. All DNA was quantified using the broad range DNA assay Qubit fluorometer (Life Technologies).

2.4. PCR validation of TTC28-L1-mediated transduction events

To validate all L1 transduction events, primers were designed using Primer3 to generate 170-250 bp amplicons, which spanned the transduction junction. Primers were validated on WGA (whole genome amplified) DNA extracted from frozen tumor tissues (positive control) and buffy coats (negative control) for each of the cases. Primers were tested at an annealing temperature of 60 °C, using PCR SuperMix High Fidelity mastermix (ThermoFisher). Successful PCR was indicated by a single band at 200-300 bp using gel electrophoresis. For failed PCRs (no band, a smear, or multiple bands), a gradient PCR from 55 °C to 64 °C was performed. If the PCRs were still unsuccessful, Platinum® Taq DNA Polymerase High Fidelity (ThermoFisher) were used in substitution. Successful PCRs were validated via sequencing using ABI 3130XL automated capillary sequencing as per manufacture protocol. Validated primers were then used on the FFPE-extracted DNA samples with 50 ng input.

2.5. Microfluidic PCR validation of selected SNV/Frameshift mutations

From the whole genome sequencing results, six likely pathogenic missense and/or frameshift mutations were selected for each case for validations on the FFPE tissue blocks. Primer sets were designed using Primer 3 to amplify the specific gene regions. Forward primers were tagged with CS1 (5′-ACACTGACGACATGGTTCTACA-3′) and reverse primers with CS2 (5′-TACGGTAGCAGAGACTTGGTCT-3′) sequencing tags. PCR products (150–200 bp) were amplified using the Fluidigm 48 × 48 Access Arrays, as per manufacturers' protocol, with input of 50 ng FFPE derived DNA. DNA barcodes (10 bp) with Illumina cluster-generating adapters were added to the libraries post-Fluidigm harvest, and cleaned-up using Agencourt AMpure XP beads (Beckman Coulter). Barcoded PCR products were then quantified using the high sensitivity
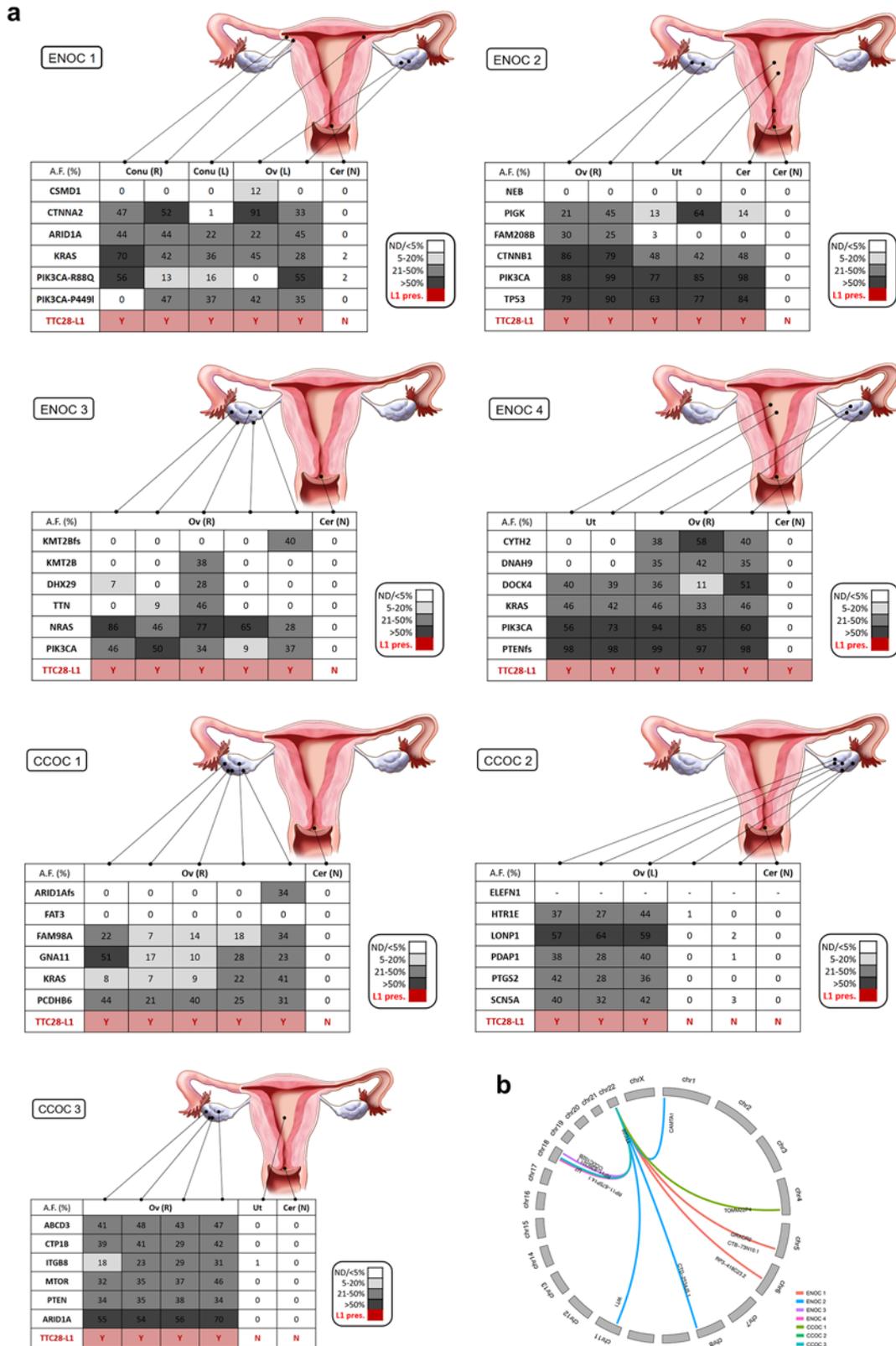
DNA assay Qubit fluorometer (Life Technologies) and pooled to one total library by normalizing to equal amounts of PCR product. In total, 48 samples were pooled and denatured according to Illumina standard protocols, and sequenced using a MiSeq 300 cycle V2 kit on the Illumina MiSeq for ultra-deep validations. Uni-directional barcode sequencing was performed. Analysis was performed using the bam and VCF files generated through Illumina MiSeq reporter.

3. Results

3.1. TTC28-L1 mediated transductions are frequent events in clear cell and endometrioid ovarian cancer

Whole genome shotgun sequencing of 29 ENOC and 35 CCOC cases to a median coverage of 51× and 37× for the tumor and matched normal DNA respectively was performed in a previous study [4]. Here, we studied this same data set generated via a structural variant caller (deStruct [22]), and found that the only recurrent rearrangement events originated from the *TTC28* gene at the chromosome 22q12 locus (Fig. 1). Previously thought to be a fragile site for translocation events, we now know that this is a L1-retrotransposon mediated 3′ transduction event, as indicated by the clustering of breakpoints within 1 kb downstream of an active L1. This retrotransposon-mediated transduction event was observed in 34% (10/29) of ENOC, and 31% (11/35) of CCOC cases. None of the transductions targeted exons. There was no association between *TTC28*-L1 mediated transduction and common mutations or landscape features (data not shown). Because L1 elements are highly repetitive throughout the genome, there is difficulty aligning the sequences. Consequently, there could be additional L1 retrotransposition events that were undetected.

3.2. TTC28-L1 mediated transductions are early events in ENOC and CCOC oncogenesis

We validated these *TTC28*-L1 transductions on high quality gnomic DNA extracted from patient frozen tumors and buffy coats via PCR, with primers designed to flank the sequences around the insertion breakpoint, such that only samples with the transductions amplified. Amplified PCR products are sequence on the Sanger to confirm the presence of breakpoints with DNA sequences from both sides. While the *TTC28*-L1 transductions that failed at the PCR or Sanger validation stages were excluded, each case had at least one validated transduction event (for a list of validated *TTC28*-L1 transductions, see Supplemental Table S1). As expected, validated *TTC28*-L1 transduction events were found only in the tumor and not the buffy coat samples.

To infer the cellular timing of these *TTC28*-L1-mediated transductions, we took archival FFPE tumor samplings from five different sites for each of the seven patients. We hypothesized that the same transduction events detected at multiple tumor sites should indicate early activation. For each case, we selected six somatic SNVs/fs mutations detected via WGS and with highest frequencies and predicted impact, and performed targeted re-sequencing in the tumor samplings to further characterize the timing of the *TTC28*-L1 mediated transductions relative to WGS detected mutations (for a detailed list of SNVs/fs, see Supplemental Table S2).

We detected *TTC28*-L1 insertions in all FFPE tumor sites in the majority of the cases (5/7 cases, ENOC 1–4 and CCOC1) (Fig. 2). In these cases, SNVs and fs mutations were detected to varying allelic frequencies for each tumor block, reflecting both inter- and intra-tumor heterogeneity (Fig. 2 and Supplemental Table S2). For each case, SNVs/fs mutations that were found in every tumor block sampled tend to be canonical cancer driver mutations, such as mutations in *ARID1A, PIK3CA, KRAS, PTEN,*

Fig. 1. Circos Plot showing the WGS results of retrotranspositions originating from *TTC28* on chromosome 22q12 (hg19 coordinates 29,059,272–29,065,303). Blue lines indicate ENOC cases and red lines indicate CCOC cases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. a). *TTC28*-L1 retrotransposition presence and allelic frequencies for five selected SNV/fs mutations at six anatomical sites for each of the seven ENOC and CCOC cases. Three shades of grey portray differences in allelic frequencies: dark grey indicates high frequency (>50%) at the point mutation; lighter grey indicates a frequency between 20% and 50%; lightest grey indicates low frequency (5%–20%). Red box indicates the presence of *TTC28*-L1 retrotransposition, and white box indicates no variance or retrotransposition detected. Numbers indicate the allelic frequencies in percentage. A.F., allelic frequencies; Ov, ovary; Cer, cervix; Ut, uterus; R, right; L, left; N, normal; ND, not detected. b). Circos plot displays retrotransposition events originating from *TTC28* (Chr.22q12) for the seven selected cases. All retrotransposition targets were in non-coding spaces. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and CTNN1B (Fig. 2 and Supplemental Table S2). These results suggest that *TTC28*-L1 was likely activated early during tumorigenesis, and was mediating DNA transductions along with SNVs/fs mutations in the earlier tumor clones. In one of the five cases (ENOC 4) the *TTC28*-L1 transduction was also detected in the histologically normal cervical tissue block used as a normal FFPE control, while the insertion event was not found in the same patient's buffy coat DNA. A second sampling from this tissue block yielded the same result, which may indicate the presence of histologically normal cells clonally related to the tumor, and suggest that L1 activation could occur even earlier.

In a single CCOC case (CCOC 2), we observed what appears to be two distinct clonal populations within the same ovarian tumor, where three of the five tumor blocks surveyed had highly similar SNVs/fs allelic frequencies in addition to presence of the *TTC28*-L1 transduction event, while the other two tumor blocks contained neither SNVs/fs nor the *TTC28*-L1 transduction events.

Four cases in our cohort (ENOC 1, ENOC 2, ENOC4, and CCOC3) had synchronous tumors in the ovary and the uterus. In the three endometrioid ovarian carcinoma cases (ENOC 1, ENOC2, and ENOC 4), we observed the *TTC28*-L1 transduction event as well as several shared SNV mutations in both the ovarian and uterine tumors. The observation of the same *TTC28*-L1 transductions in tumors at anatomically different locations further suggests that L1 activation occurred early in these diseases. In the clear cell ovarian carcinoma case (CCOC 3), SNVs/fs mutations and the *TTC28*-L1 event were found in all the samplings of the ovarian tumor but were not seen in uterine tumor.

4. Discussion

Herein we described the presence of DNA transductions mediated by a specific and active retrotransposon, *TTC28*-L1, in the endometriosis associated ovarian carcinoma subtypes: ENOC and CCOC. Our data showed that *TTC28*-L1 mediated transductions was detectable across all tumor sites in the majority of the cases. The presence of *TTC28*-L1 transduction was sometimes but not always accompanied by SNV/ frameshift mutations at various allelic frequencies. It is highly unlikely that the same retrotransposition event arose independently at every tumor site, which indicates that *TTC28*-L1 transductions were not subclonal events, but more likely events that occurred early in tumorigenesis. In addition, the presence of *TTC28*-L1 transductions correlated with high allelic frequencies of driver mutations implicated in the development of these cancers, including mutations in *ARID1A, CTNNB1, PIK3CA,* and *PTEN* [8], supporting our hypothesis that *TTC28*-L1 retrotransposons are activated early in EAOC development.

Targeted resequencing was previously used by our group to describe a clonal relationship between the endometrial and ovarian cancers in three of the four synchronous endometrioid and ovarian tumors, ENOC 2, ENOC 4 and CCOC 3 [23]. We also found somatic mutations and *TTC28*-L1 events shared between the uterine and ovarian tumors in ENOC 2 and ENOC 4, however, we did not detect any of our selected mutations nor the *TTC28*-L1 event in the uterine tumor from CCOC 3. CCOC 3 was unusual in several ways: different pathologies observed in the ovary (clear cell) and uterus (endometrioid), and there was only one single shared somatic event (a different event from the ones we repeated) as reported by Anglesio et al. [23]. It is possible this somatic event had occurred before either the seeding of endometriosis or the activation of the *TTC28*-L1 retrotransposon.

It is interesting to note that 3′ transductions accounts for approximately one quarter of all somatic L1 retrotranspositions, and the majority of L1 retrotranspositions result in solo L1 insertions, either as a full L1 or truncated at the 5′ end [10]. This meant that there are likely more L1 insertions undetected in our cohort. Past studies on somatic L1 retrotranspositions in various cancer types reported much more solo L1 insertions than 3′ transduction events; however, the lack of L1 3′ transductions detected could be due to bias in L1 sequencing and analysis methods [16,18–20]. Nonetheless, we have found *TTC28*-L1

transduction events at a high frequency in our cohort, which could be a unique feature of EAOCs.

While somatic L1 insertions are common in certain tumor types, there is little evidence that they are oncogenic. Only two studies showed that somatic L1 retrotranspositions (solo L1 insertions) directly initiated oncogenesis in colorectal cancer via a somatic L1 insertion in an exon of the tumor suppressor gene *APC* [24,25]. The majority of somatic L1 insertions target heterochromatic regions with no obvious functional impact [10,12]. We also observe that our L1 transductions targeted noncoding regions within out cohort.

Most research in the field focuses on using L1 activations as a marker of tumor development, by assessing the global hypomethylation of L1 loci and the expression of L1 mRNA and proteins throughout stages of cancer development and metastasis. The general conclusion is that somatic L1 retrotranspositions are passenger events that occur after a permissive environment has been established during cancer development, such as dysregulated epigenetic control [10,12,17]. Evidence of L1 activation as an early event include a recent study that found stepwise decrease of methylation across different L1 loci have been observed between normal endometrium, contiguous endometriosis (endometriosis adjacent to tumor), and ENOCs/CCOCs [17]. In gastrointestinal (GI) cancers, shared somatic L1 retrotranspositions (solo insertions) have been found between precancerous lesions and colon tumors [20], as well as between Barrett's esophagus (precursor lesion of esophageal cancer) and concomitant esophageal tumors [19], suggesting early activation of L1 during GI tumor development. This is likely the scenario in our cohorts, where the *TTC28*-L1 was activated early during ENOC/CCOC tumorigenesis, but after a retrotransposition-permissive environment was established.

The exact relationship between *TTC28*-L1 activation and malignant transformation in these two ovarian cancer subtypes is challenging to elucidate. Further studies will be needed to assay endometriosis tissues that are adjacent to tumors, distant from tumors, as well as endometriosis without cancer involvement. Such studies could determine whether *TTC28*-L1 transduction is a useful marker for cancer risk of endometriosis.

EAOCs suffer from a lack of effective early detection tools. *TTC28*-L1 and other L1s can potentially be used as biomarkers to identify early malignant transformations, especially in the subset of CCOC/ENOC cases where common coding mutations are not present. In addition, a better understanding of L1 activation and transduction patterns over the course of ENOC and CCOC progression may help elucidate the underlying mechanisms of ovarian carcinogenesis and progression.

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