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Original contribution

Expression of L1 retrotransposon open reading frame protein 1 in gynecologic cancers ☆

Zhouchunyang Xia MSc [a], Dawn R. Cochrane PhD [b], Basile Tessier-Cloutier MD [a], Samuel Leung MSc [c], Anthony N. Karnezis MD, PhD [d], Angela S. Cheng BSc [c], David A. Farnell MD [a], Jamie Magrill BSc [b], Dietmar Schmidt MD [e], Stefan Kommoss MD [f], Felix K.F. Kommoss MD [g], Friederich Kommoss MD [h], Jessica N. McAlpine MD [i], C. Blake Gilks MD [a], Martin Koebel MD [j], Joseph T. Rabban MD, MPH [k], David G. Huntsman MD [a,b,*]

[a] *Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC V5Z 1L3, Canada*
[b] *Department of Molecular Oncology, BC Cancer Agency, Vancouver, BC V5Z 4E6, Canada*
[c] *Genetic Pathology Evaluation Centre, Vancouver General Hospital, Vancouver, BC V6H 3Z6, Canada*
[d] *Department of Pathology and Laboratory Medicine, University of California, Davis, CA 95817, USA*
[e] *Insitute of Pathology and Cytology, Viersen, 69120, Germany*
[f] *Department of Obstetrics and Gynecology, University of Tübingen, 72076, Germany*
[g] *Institute of Pathology, Heidelberg University Hospital, 69120, Germany*
[h] *Institute of Pathology, Medizin Campus Bodensee, Friedrichshafen, 88048, Germany*
[i] *Department of Obstetrics and Gynaecology, University of British Columbia, Vancouver, BC V5Z 1L3, Canada*
[j] *Department of Pathology and Laboratory Medicine, University of Calgary, Calgary, AB T2N 2T9, Canada*
[k] *Department of Pathology and Laboratory Medicine, University of California, San Francisco, CA 94158, USA*

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Summary LINE-1 (L1) retrotransposons are mobile genetic elements capable of "copy-and-pasting" their own sequences into random genomic loci, and one of the proteins it uses to achieve mobility is LINE-1 open reading frame 1 protein (L1ORF1p). L1ORF1p expression is found across many epithelial cancers, including small cohorts of ovarian and endometrial cancers, and is highly expressed in cancers with mutant p53 expressions. Here we aimed to gain insights into L1ORF1p expression levels within specific histotypes of ovarian cancers: high-grade serous (n = 585), low-grade serous (n = 26), clear cell (n = 132), endometrioid (n = 148), and mucinous (n = 32) ovarian cancers, as well as endometrial cancers (n = 607) using tissue microarray (TMA's). We demonstrated that L1ORF1p expression is associated with advanced stage and serous histotype in gynecological cancers. Like previous studies, we found a higher proportion of L1ORF1p expression in cases with aberrant p53 expression. We evaluated the expression of L1ORF1p in serous tubal intraepithelial carcinomas (STICs) (n = 6) and p53 signature lesions

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* Corresponding author at: BC Cancer Research Centre, 675 West 10th Avenue, Vancouver, BC V5Z 1L3.
E-mail address: dhuntsma@bccancer.bc.ca (D. G. Huntsman).

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(n = 2) in fallopian tubes. Three STIC cases displayed aberrant p53 overexpression with corresponding L1ORF1p expression in the same tissues, but such correlation was not seen in the two p53 signature lesions, suggesting that L1 protein may be expressed after dysplastic transformation. The remaining three STIC cases have *TP53* nonsense mutations with absent p53 expression but a strong and clear L1ORF1p expression within the STIC lesions. While L1ORF1p may not be prognostic in gynecological cancers, it may be useful clinically as a diagnostic IHC marker for p53 null STIC lesions and this warrants further investigation.

1. Introduction

Epithelial ovarian carcinoma is the most lethal gynecological cancer and the fifth leading cause of cancer related deaths in women in the United States and in Canada [1]. There are five major histotypes of epithelial ovarian carcinoma which differ in their molecular and clinical characteristics: high-grade serous ovarian carcinoma (HGSC; 68%-70%), clear cell ovarian carcinoma (CCOC; 9%-12%), endometrioid ovarian carcinoma (ENOC; 8%-11%), low-grade serous ovarian carcinoma (LGSC; 3%-4%), and mucinous ovarian carcinoma (MC; <3%) [2,3]. Endometrial carcinoma, although responsible for less mortality than ovarian carcinoma, is the most frequently diagnosed gynecologic cancer in the western world [4]. Endometrial carcinoma is also categorized by histomorphology into histotypes; the three most common being endometrioid endometrial carcinoma (EEC; 85%), serous endometrial carcinoma (SEC; 3%-10%), and clear cell endometrial carcinoma (CCEC; 2%-3%) [4]. Furthermore, Endometrial carcinoma can be molecularly classified into four groups using the ProMiSe classifier: mismatch repair deficient (MMR-D), mutant polymerase epsilon (POLE), mutant p53 (p53abn) and wild type p53 (p53wt); which can better prognostically classify EC tumors than histopathology [5-7].

Retrotransposons are a type of mobile genetic element that comprises approximately 42% of the human genome [8]. While there are several different classes of retrotransposons, the only autonomous retrotransposons (capable of propagating on their own) are LINE-1 (L1) retrotransposons. L1 elements account for 17% of the human genome [9-12]. However, the majority of L1 elements have lost the capability to retrotranspose due to mutations, truncations and/or sequence inversion. Approximately 70–100 L1 retrotransposons remain intact and potentially functional [9]. L1 elements are generally suppressed in normal somatic cells via promoter methylation and additional mechanisms, but often in cancer development, these mechanisms fail and L1 s are activated [13-15]. Somatic L1 retrotranspositions occur in approximately 50% of human cancers, and epithelial cancers exhibit high numbers of L1 insertions [9,16-18]. The majority of these L1 retrotranspositions are considered passenger events in cancer, correlating with genomic instability while having the potential to contribute to genomic instability through their methods of genomic integration [12,19].

L1ORF1p is one of the essential proteins L1 elements encodes for retrotransposition [12,20]. The immunohistochemical expression of L1ORF1p has been found across different cancers as a marker of L1 activities [12,17,21]. One study found L1ORF1p to be immunoreactive in 93.5% of HGSC cases [17]. An association between *TP53* mutation and increased L1ORF1p expression was also noted in this study, where 86% of the HGSC cases were also *TP53* deficient, and a higher expression of L1ORF1p was found in *TP53* deficient lung carcinoma, pancreatic carcinoma and secondary glioblastoma compared to *TP53* wildtype case [17]. A study with L1ORF1p immunofluorescence in ovarian cancers demonstrated a positive association of higher L1ORF1p expression with the patient age and the metastatic potential of the tumor [22].

Here we aimed to see if a similar relationship between L1ORF1p and mutant p53 expression occurs in our gynecological cancer cohorts. In addition, we surveyed the expression of L1ORF1p in a small cohort of HGSC precursor lesions with p53 mutations to assess the activation of L1 elements in HGSC development.

2. Materials and methods

2.1. Immunohistochemistry

We assessed the expression of L1ORF1p in retrospective cohorts of ovarian and endometrial cancers using tissue microarrays (TMAs) previously constructed with duplicate 0.6-mm cores from formalin-fixed paraffin-embedded (FFPE) tissues. Three ovarian TMAs (n = 1307) and two endometrial TMAs (n = 848) were used (details in Supplementary Table 1). 4um slides of each TMA were cut onto Superfrost plus slides (Fisher Scientific). Processing of the slides was done using the automated Ventana Benchmark and Discovery systems (Ventana Medical Systems, Tucson, AZ). The recommended IHC staining protocol for the Ventana Discovery XT was used, with minor deviations including primary antibody incubation of 2 hours, counterstain

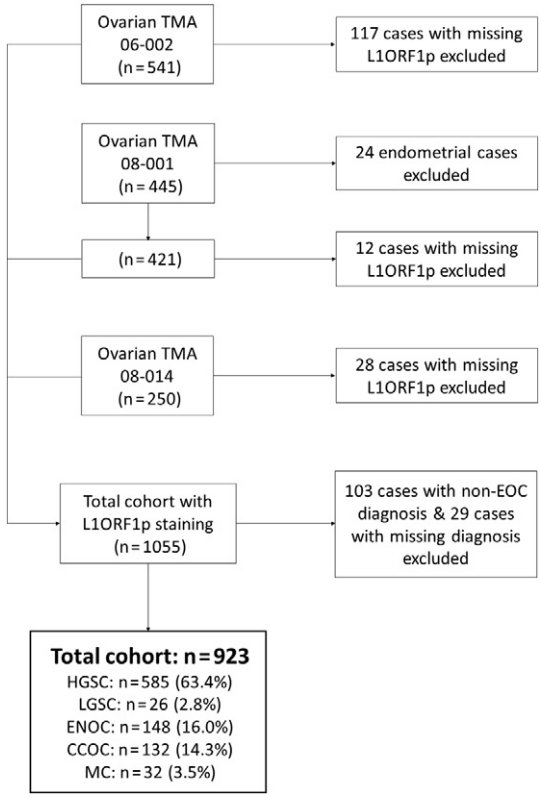

Fig. 1 Flowchart for the case selection process of the ovarian TMAs. A total of 923 cases were included, with 5 of the common epithelial ovarian carcinoma subtypes, HGSC, LGSC, ENOC, CCOC, and MC.

incubation of 12 minutes and post counterstain incubation of 4 minutes. Slides were stained with antibody against L1ORF1p (L1RE1, mouse monoclonal 4H1, #MABC1152; Millipore Sigma) diluted to 1:100 using Discovery antibody diluent from Ventana (#760–108). Diffuse cytoplasmic staining was observed, as has been described previously [17]. Normal fallopian tube was used as the negative control, and tumors known to have active *TTC28*-L1 were used as positive controls. Overall project processes were approved by the BC Cancer Agency Research Ethics Board at the University of British Columbia (REB #H09–02153 & #H18–01457).

To assess the expression of L1ORF1p in HGSC precursor lesions, we stained fallopian tube (FT) tissues with serous tubal intraepithelial carcinoma (STIC) lesions (identified and selected by pathologist B.G., M.K., J.R.), using the same IHC protocol. We also stained serial sections of each tissue block with p53 (P53, mouse monoclonal DO-7, #M7001; Dako) diluted to 1:400 using Discovery antibody diluent, for comparison between L1ORF1p and p53 expression. In addition, IHC for p53 was performed on normal fallopian tube fimbria from 53 patients and was assessed for p53 signature lesions by a pathologist (A.K.). We selected two normal FT with p53 signature lesions, and two normal FT without p53 signature lesions within this cohort for L1ORF1p staining.

All TMAs and whole tissue sections were scored by an anatomical pathologist (B.T-C.). L1ORP1p staining localized to the cytoplasm. For L1ORF1p, a score of 0 indicates negative staining, 1 indicates weak, 2 indicates moderate, and a 3 indicates strong staining. Staining patterns were noted as diffuse if the staining intensity is constant and uniform across all tumor cells in the core, and variable if spots of cells with strong stains appear against a background of weaker stains. For cases where both tissue cores contained tumor, the predominant staining score pattern was retained for the analysis. For p53, as the IHC expression is often related to mutational status of the gene [23], we denoted the complete loss of p53 (p53 nonsense mutation/null) as <= 1% positivity, a normal

Table 1 Distribution of clinical characteristics by L1ORF1p IHC status

	Total	Negative	Weak	Moderate	Strong
Age at surgery					
Mean (SD)	59.5 (12.8)	55.5 (12.7)	57.3 (13.3)	60.4 (12.5)	63.7 (11.7)
Median	59.0	54.1	56.3	59.8	63.6
IQR	50.2-69.1	47.1-64.4	48.8-67.2	51.2-70.6	54.9-72.5
Range	19.2-90.9	23.8-88.0	19.2-89.0	19.2-90.1	34.5-90.9
Missing	110	41	24	36	9
FIGO stage					
I	233	96 (41.2%)	41 (17.6%)	79 (33.9%)	17 (7.3%)
II	235	70 (29.8%)	42 (17.9%)	83 (35.3%)	40 (17.0%)
III	448	53 (11.8%)	73 (16.3%)	204 (45.5%)	118 (26.3%)
IV	58	4 (6.9%)	6 (10.3%)	27 (46.6%)	21 (36.2%)
Missing	81	34 (42.0%)	19 (23.5%)	21 (25.9%)	7 (8.6%)
Histology					
High-grade serous	585	53 (9.1%)	89 (15.2%)	269 (46.0%)	174 (29.7%)
Low-grade serous	26	17 (65.4%)	8 (30.8%)	1 (3.8%)	0 (0.0%)
Endometrioid	148	65 (43.9%)	41 (27.7%)	37 (25.0%)	5 (3.4%)
Clear cell	132	54 (40.9%)	16 (12.1%)	53 (40.2%)	9 (6.8%)
Mucinous	32	17 (53.1%)	4 (12.5%)	11 (34.4%)	0 (0.0%)

p53 expression (p53 wildtype) as between 1%–80% positivity, and an overexpression (p53 missense mutation) as >80% positivity.

2.2. Statistical analysis

For univariable associations, χ^2 test was used for categorical biomarker and Kruskall-Wallis/Wilcoxon rank sum test was used for continuous biomarker data. Kaplan–Meier plots are used to assess survival, and the optimal staining intensity cut points for separating groups within each cohort were based on correlation with disease specific survivals. Statistical significance was set at $\alpha = .05$. The programming language R (software version 3.5.2) was used for all above statistical analysis and visualization of data.

3. Results

3.1. Cohort description and clinical correlations with L1ORF1p expression in epithelial ovarian cancers

Cases with missing L1ORF1p due to incomplete cores are omitted, and a total of 923 patients with a confirmed diagnosis of HGSC, LGSC, ENOC, CCOC, and MC were included in the analysis (Fig. 1). Distribution of stage and grade by L1ORF1p expression are listed in Table 1. Briefly, 63.4% of the whole cohort were HGSC, 2.8% were LGSC, 16.0% were ENOC, 14.3% were CCOC, and 3.5% were MC. The majority of the patients were diagnosed at stage III (48.1%), while stage II (24.7%) and stage I (21.6%) patients were similarly distributed, and the least number of patients were diagnosed at stage IV (5.5%). The distribution of

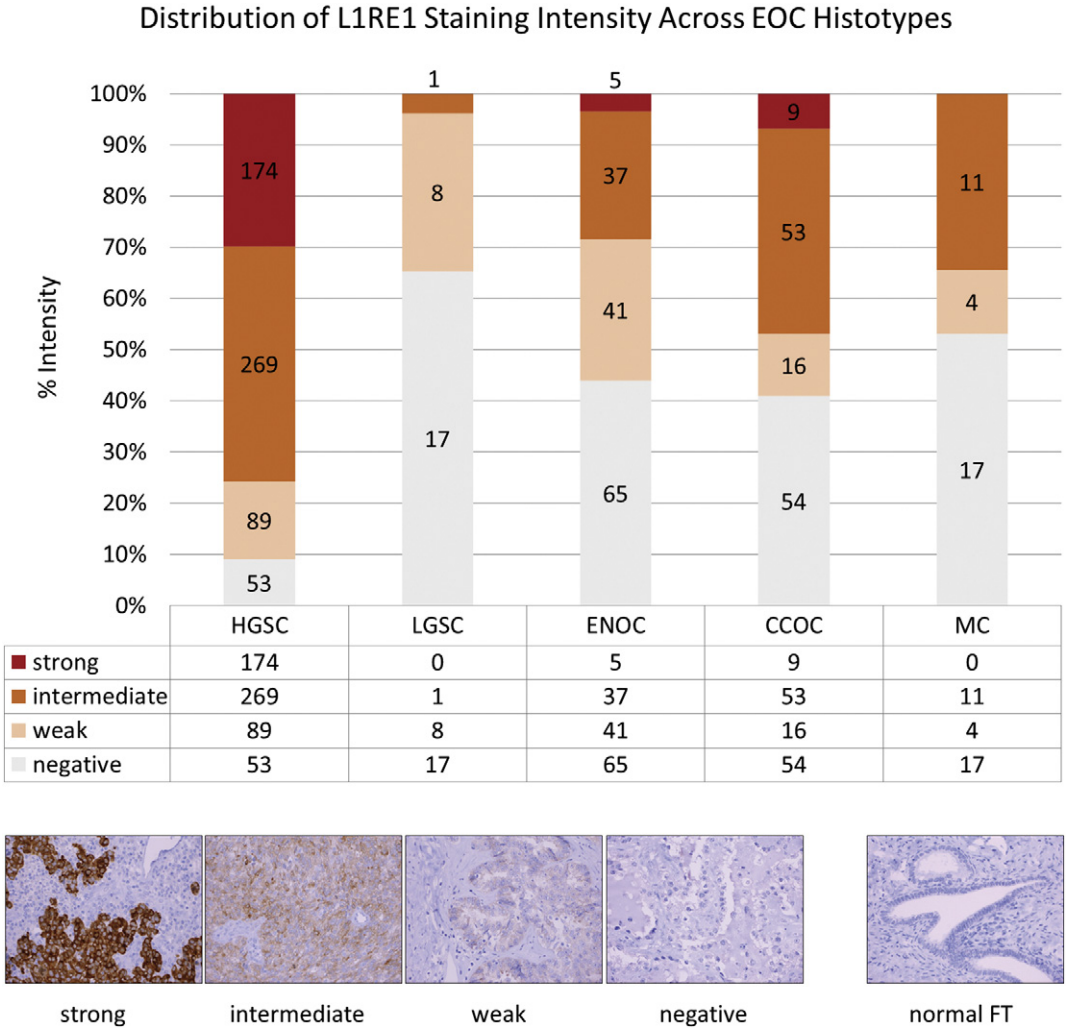

Fig. 2 Distribution of L1ORF1p expression across the different epithelial ovarian carcinoma histotypes. The numbers of cases within each score are shown on the graph, as well as in the table. The corresponding staining intensity for each score category is shown below the graph. L1ORF1p expression is significantly higher in HGSC compared to the other histotypes ($P < .0001$). Complete absence, overexpression and cytoplasmic phenotype all indicate mutated p53.

Table 2 Association of L1ORF1p expression (negative versus any positive) with histotypes. HGSC correlated with more L1 positivity. χ^2 test, $\alpha = .05$

Variable	Levels	Total	Negative	Any positive	*P*
Histotype	High-grade serous	585	53 (9.1%)	532 (90.9%)	<.0001
	Other	338	153 (45.3%)	185 (54.7%)	

Table 3 Association between L1ORF1p and p53 IHC status across the whole cohort

Variable	Levels	Total	Negative	Any positive	*P*
p53	Complete absence	63	1 (1.59%)	62 (98.4%)	<.0001
	Overexpression	136	8 (5.88%)	128 (94.1%)	
	Cytoplasmic	6	0 (0.00%)	6 (100.0%)	
	Wildtype	76	22 (28.9%)	54 (71.1%)	

L1ORF1p expression across the 5 epithelial ovarian cancer histotypes is shown in Table 1 and Fig. 2, and the associations of stage and grade with L1ORF1p expression levels are shown in Table 1. Within each of the four histotypes analyzed, HGSC has the highest proportion of positive L1ORF1p expression (90.9%), followed by CCOC (59.1%), ENOC (56.1%), MC (46.9%) and LGSC (34.6%). Staining intensity levels of weak, intermediate and strong was observed in HGSC, CCOC, and ENOC, with HGSC having the greatest proportion of strong staining (29.7%, 6.8%, and 3.4% respectively); while MC and LGSC were the two histotypes with a complete absence of strong staining intensity. Within all cases that displayed L1ORF1p positivity, 96.0% displayed a diffuse expression pattern, with tumor cells uniformly stained across the core and 4.0% displayed a variable pattern; all cases displayed cytoplasmic localization of L1ORF1p (data not shown). L1ORF1p expression was significantly higher in patients diagnosed with stages III/IV ($P < .0001$), grade 3 ($P < .0001$), and of the HGSC subtype ($P < .0001$) (Table 2).

3.2. Evaluation of L1ORF1p expression and survival in epithelial ovarian carcinoma

To determine if LINE-1 expression can be used as a prognostic marker in epithelial ovarian carcinomas, we assessed the overall survival (OS), disease specific survival (DSS), and progression-free survival (PFS) of our cohort in correlation with L1ORF1p expression. The optimal cut-off points for significant correlation between L1ORF1p staining pattern and survival was determined to be "negative" versus "any positive staining" for the whole cohort and then used for subtype specific analysis. In the whole cohort survival analysis, L1ORF1p expression significantly correlated with worse OS ($P < .001$, HR 1.75), DSS ($P < .001$, HR 2.06), and PFS ($P < .001$, HR1.94) (Supplementary Fig. 1a). However, L1ORF1p expression did not correlate with survival when each histotype was analyzed separately, except for in ENOC, where DSS was significantly worse at the 5-year cut-off ($P = .0478$, HR 2.976) (Supplementary Fig. 1b).

3.3. Association between L1ORF1p expression and p53 expression

In a pan-cancer survey of L1ORF1p expression, L1ORF1p was found to be associated with p53 expression [17]. To assess whether the same trend occurs in our cohort, we compared L1ORF1p expression with p53 (scored in 2016 by pathologist F.K., n = 268). Expression of L1ORF1p was significantly associated with mutated p53 ($P < .0001$) across the whole cohort (Table 3), however, there was no significant association was found within individual histotypes (Table 4).

Given that L1ORF1p expression correlates with mutant p53 expression, and L1 insertions have been seen in the precursor lesions of other cancers, we hypothesized that L1ORF1p can be detected in the HGSC precursor lesions of STIC, which also express mutant p53. We performed IHC staining of six STIC lesions in the fallopian tube, three of which have missense *TP53* mutations displaying p53 overexpression on IHC (Fig. 3A), and three of which have nonsense *TP53* mutations displaying p53 null expression on IHC (Fig. 3B). L1ORF1p expression was observed in neoplastic areas and corresponded to areas of p53

Table 4 No association was found between L1ORF1p and p53 IHC status in HGSC

Variable	Levels	Total	Negative	Any positive	*P*
p53	Mutated	183	7 (3.83%)	176 (96.2%)	1.000
	Wild-type	18	1 (5.56%)	17 (94.4%)	

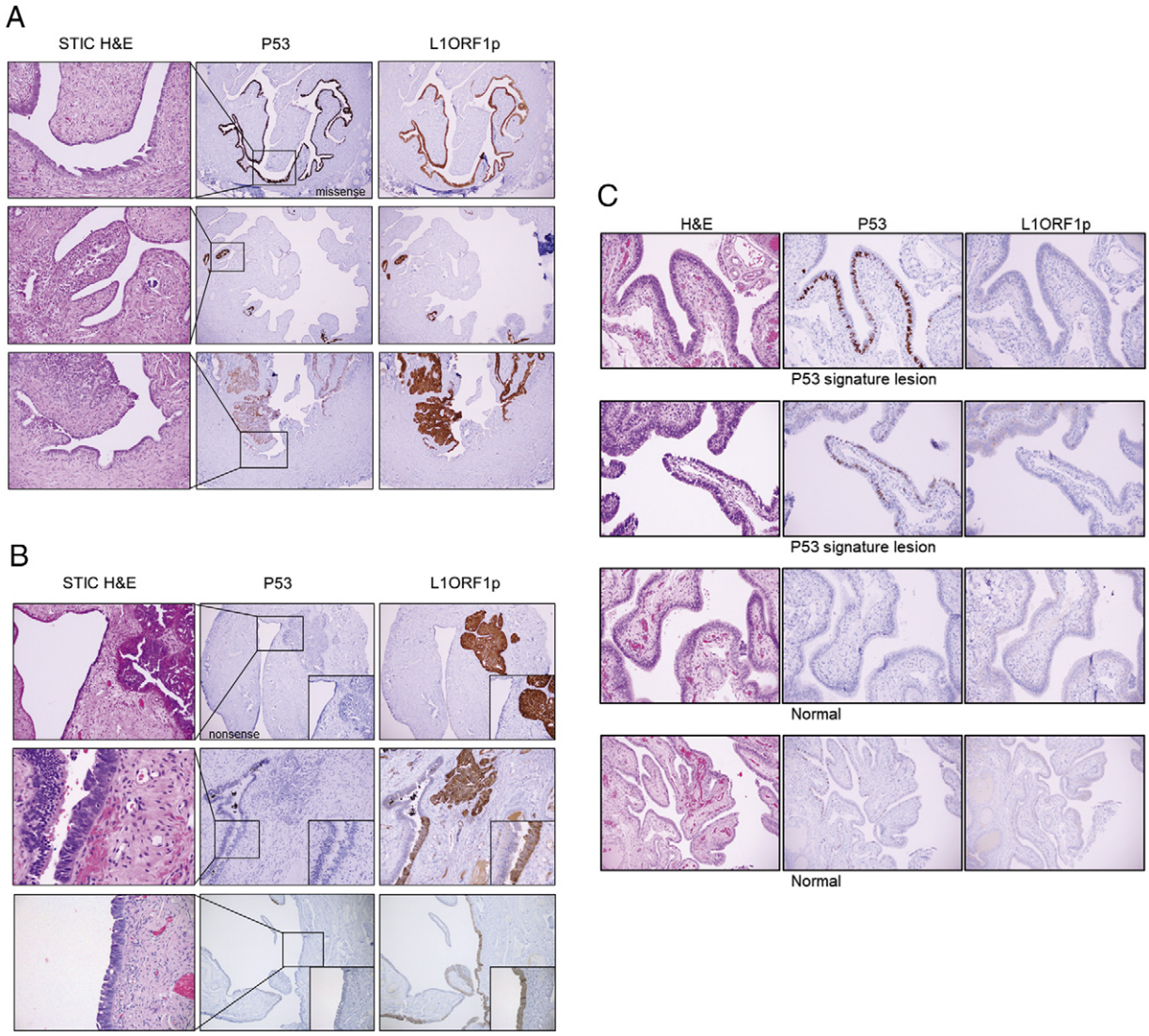

Fig. 3 L1ORF1p staining in high-grade serous ovarian cancer precursors. A, Distinct STIC morphology is observed in the H&E slides. L1ORF1p expression correlated with p53 overexpression. B, Distinct STIC morphology is observed in the H&E slides. This suggests the possibility of L1ORF1p as a surrogate marker that can identify p53 null expression. C, Two FT contain p53 signature lesions. Very faint L1ORF1p stains are observed in the epithelial cells throughout the section, with no preference for areas of p53 lesions, which likely indicate negative L1ORF1p activity.

overexpression, as well as STIC lesions with p53 null expression (Fig. 3A and B).

The accumulation of mutant p53 proteins in non-neoplastic cells (p53 signature lesions), as measured by IHC overexpression, can be found in otherwise normal fallopian tube epithelium that appears otherwise histologically normal. The p53 signature lesion is proposed to precede the formation of STIC lesions, in which the cells display histologic abnormalities (loss of nuclear polarity or cellular stratification, nuclear enlargement, nuclear pleomorphism and an atypical chromatin pattern). We assessed whether L1ORF1p was also expressed in these pre-neoplastic tissues. We found no evidence of L1ORF1p expression in the p53 signature lesions tested (Fig. 3C).

3.4. Evaluation of L1ORF1p expression in endometrial cancers, and association with MMR and p53 mutation status

We evaluated the expression of L1ORF1p across two cohorts of endometrial cancer, a Vancouver (Van) cohort (n = 153) and a German (Ger) cohort (n = 454). Four histotypes of endometrial cancers were included in the cohort, endometrioid endometrial (ENEC), serous endometrial (SEC), clear cell endometrial (CCEC), and mixed type endometrial cancers. The distribution of histotypes in the Van cohort is as follows: ENEC was 80.4% (123/153), SEC 10.5% (16/153), mixed type 3.9% (6/153) and others 5.2% (8/153), and in the Ger cohort: ENEC was 87.9% (399/454), SEC 7.5%

(34/545), mixed type 3.3% (15/545), and CCEC 1.3% (6/545). A high proportion of negative and weak L1ORF1p positivity was observed in the endometrioid histotypes within both cohorts, and a high proportion of intermediate and strong L1ORF1p positivity was observed in the serous histotype (Fig. 4A).

The optimal cut-off points for separating staining intensity was determined to be negative/weak staining versus intermediate/strong staining, based on L1ORF1p correlation with disease specific survival and was used for all association analysis. In the whole cohort analysis for the Vancouver cohort, negative/weak staining was associated with improved PFS (P = .00114, HR = 2.614), but no significant association was found for OS and DSS, nor was association found in histotype-specific analysis. In the German cohort, negative/weak staining was associated with improved OS (P = .00845, HR = 1.741) DSS (P = .0185, HR = 1.940), and PFS (P = .0125, HR = 1.987) for the whole cohort, but also no association was found within histotypes. (data not shown).

We next evaluated the expression of L1ORF1p across the four molecular subgroups of the ProMisE classifier [5]. The MMR-D and p53wt groups had a significant proportion of negative/weak staining compared to the p53abn group (P < .0001) (Fig. 4B). Within the p53wt group, negative/weak L1ORF1p expression appears to be significantly correlated with improved PFS (P < .001, HR = 7.404) in the Vancouver cohort, however the correlation was weaker in the German cohort that has a larger sample size (P = .0672, HR = 2.572) (Supplementary

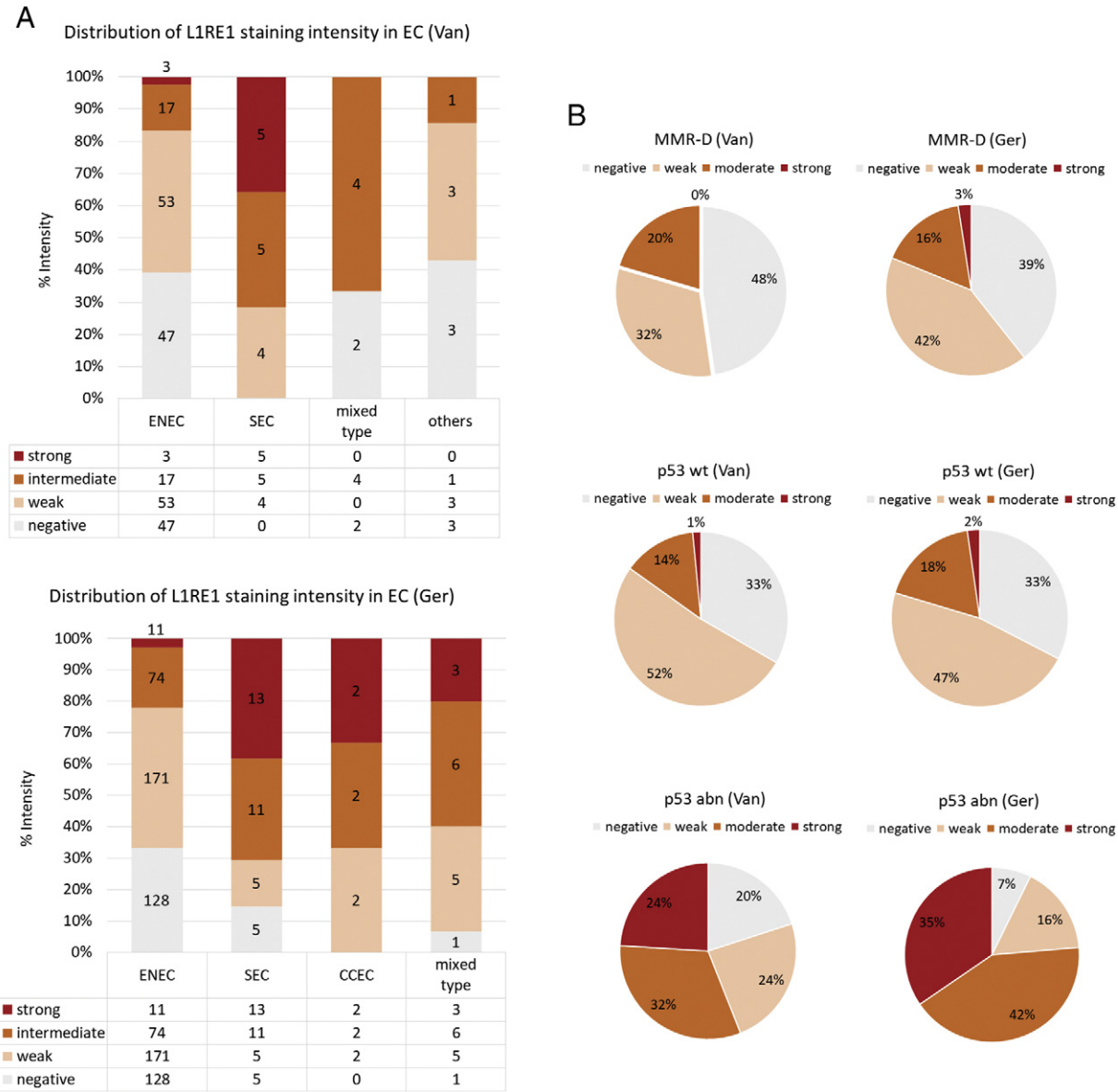

Fig. 4 Distribution of L1ORF1p expression across the two endometrial cohorts and amongst the molecular classifications. A, The distribution of ENEC and SEC was similar in both cohorts, where ENEC tends to have a high proportion of negative/weak expression, and SEC have a high proportion of intermediate/strong expression. B, Negative/weak L1ORF1p expression correlated with MMR-D and p53 wt within both cohorts.

Fig. 2). We did not find any significant correlation with survival in the MMR-D and p53abn groups.

4. Discussion

In this study we investigated L1ORF1p expression in two major types of gynecological cancers, epithelial ovarian carcinoma and endometrial carcinoma. We have demonstrated that L1ORF1p expression is associated with high-grade and more advanced stage ovarian and endometrial carcinomas, and it is expressed most frequently in the high-grade serous histotypes of both cancers. Previous studies have shown similar trends where L1ORF1p expression was higher in more genomic unstable and aggressive cancers (summarized in Ardeljan et al [24]), presumably due to the loss of p53 functions [25].

L1ORF1p is a high affinity RNA binding protein that is essential to the subsequent delivery of the L1 RNA into the nucleus for reverse transcription and genomic insertion. Studies in breast cancer demonstrated that the nuclear localization of L1ORF1p protein significantly correlated with poorer survival [26,27], however, we only observed cytoplastic localization within our cohorts. This observation may be due to the nature of the antibody and/or assay used, such that nuclear localization leads to protein interactions that decrease antibody binding affinity and detection. Indeed, we expected to observe cytoplasmic localization as nuclear localization is only rarely observed for L1ORF1p across different cancers [17,21,28].

We performed survival analysis and observed that L1ORF1p expression correlated with worse OS, PFS, and DSS survival in the overall epithelial ovarian carcinoma cohorts, although these correlations were no longer significant when accounting for the individual histotypes. Given the high proportion of L1ORF1p positivity in HGSC (90.9%) and the high proportion of HGSC in the cohort (60.4%), the observed association with worse prognosis in the whole cohort is likely a reflection of the worse survival in HGSC compared to the other histotypes, rather than the association with L1ORF1p expression. While there was a weak correlation between negative L1ORF1p expression and DSS in endometrioid ovarian carcinoma at the 5-year cut-off, it was no longer significant past 5 years. HenceL1ORF1p expression is not a prognostic marker in epithelial ovarian carcinomas.

The association between L1ORF1p expression and mutant p53 overexpression across many epithelial cancers is well established [17,29]. In our study, while p53 mutant overexpression was similarly associated with higher L1OR1p expression in the overall ovarian cancer cohort, this association was not found when subtype-specific analysis was performed. This is likely due to the low number of cases without p53 mutation in HGSC and the low number of cases with p53 mutations in all other histotypes, which made subtype-specific comparisons difficult. Nonetheless, studies have shown that p53 restrains L1 retrotransposition, which likely explains our observed correlation between L1ORF1p expression and the loss of p53 [25].

Serous tubal intraepithelial carcinoma (STIC) is an established precursor lesion to the ovarian carcinoma histotypes HGSC, and displays aberrant p53 expressions [30-32]. Given the function of the p53 protein in restricting L1 activation [25], and the presence of L1ORF1p expression in premalignant tissues in other epithelial cancers [21], we performed IHC on six STIC lesions, three of which displayed p53 overexpression and three with p53 null expression, to confirm the presence of L1 activation. As expected, we observed high L1ORF1P expressions in all six STIC cases, which suggests an early involvement of L1 activities during HGSC development. We also examined the expression of L1ORF1p in two histologically normal fallopian tubes with p53 signature lesions, and we were not able to detect L1ORF1p expression corresponding with the p53 lesions, suggesting that L1 elements may not be activated in the pre-neoplastic lesions of HGSC. Indeed, our results are similar to a recent study which surveyed 32 STIC cases and 12 p53 signature lesions and showed that 69% of STIC cases displayed strong L1ORF1p expressions, while none of the p53 signature lesions displayed L1ORF1p expression [29]. Our findings contribute to this body of evidence, which suggests that L1 elements are active post p53 mutations in the early development of HGSC, and that p53 is likely a required but not a sufficient event for L1 activation during such neoplastic transformation. While our study is limited in sample size, we observed a strong L1ORF1p expression in all STIC cases with p53 null expression, such expression could be a potential diagnostic marker for histologically questionable p53 null STIC lesions.

In our analysis of L1ORF1p expressions in endometrial cancers, we assessed its expressions within the subgroups of the TCGA based ProMiSe classifier [5]. We saw that endometrioid endometrial carcinomas had similar L1ORF1p expression patterns to endometrioid ovarian carcinomas as expected, since both cancers are similar in histology and genomic features. The serous histotypes of endometrial carcinoma displayed stronger L1ORF1p expression, similar to high-grade serous ovarian carcinomas. As expected, L1ORF1p had weaker expression in p53wt subgroup, and stronger expression in the p53abn group. Interestingly, moderate and strong L1ORF1p expressions within the p53wt subgroup in the Vancouver cohort conferred significantly worse survival, while only a trend towards worse survival was seen in the German cohort. This discrepancy between the two cohorts could be due to the difference in cohort sizes, such that the effect appears to be more statistically significant due to the small number of cases in the Vancouver cohort. Nonetheless, this could mean that L1ORF1p expression may be identifying a small group within the p53wt category with higher risk, or it may be identifying cases with underlying p53 mutations despite having a p53wt IHC stain. In either case, L1ORF1p may have the potential to be an additional marker for classifications in constructing future tools and warrants further investigations. Studies in colorectal cancers have

demonstrated that increased L1 hypomethylation was correlated with more MSI-stable cases [33], and we observed a similar trend in our endometrial cancer cohort, where MMR-D (which indicates microsatellite instability) cases had weaker L1ORF1p expression.

In conclusion, we have shown that L1ORF1p expression tends to be much higher in ovarian and endometrial carcinomas with genomic instability, and it has potential utility as an IHC marker in both research and clinical settings.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2019.06.001.

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