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ORIGINAL ARTICLE

Single-molecule array assay reveals the prognostic impact of plasma LRIG1 in ovarian carcinoma

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ABSTRACT

Background: Ovarian carcinoma is the eighth most common cause of cancer death in women worldwide. The disease is predominantly diagnosed at a late stage. This contributes to high recurrence rates, eventually leading to the development of treatment-resistant disease. Leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1) is a transmembrane protein that functions as a tumor suppressor and regulator of growth factor signaling. LRIG1 levels have not been investigated in human plasma previously.

Materials and methods: A quantitative LRIG1-specific single molecule array assay was developed and validated. LRIG1 levels were quantified in plasma samples from 486 patients with suspicious ovarian masses.

Results: Among women with ovarian carcinoma, LRIG1 levels were significantly elevated compared to women with benign or borderline type tumors. High LRIG1 plasma levels were associated with worse overall survival and shorter disease-free survival both in the group of all malignant cases and among the stage 3 cases only. LRIG1 was an independent prognostic factor in patients with stage 3 ovarian carcinoma.

Conclusion: LRIG1 plasma levels were elevated in patients with ovarian carcinoma, and high levels were associated with poor prognosis, suggesting that LRIG1 might be an etiologic factor and a potentially useful biomarker in ovarian carcinoma.

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KEYWORDS
Ovarian carcinoma; plasma; LRIG1; Simoa; biomarker; prognosis

Introduction

Ovarian carcinoma accounted for 3.4% of all incident cancers in women worldwide in 2018, and it is the eighth most common cause of cancer death in women worldwide [1]. Ovarian carcinoma is predominantly diagnosed at a late stage, which contributes to high recurrence rates. Most ovarian carcinomas initially respond well to chemotherapy; however, among women with advanced-stage disease, treatment-resistant disease eventually develops almost invariably [1–3]. Although ovarian carcinoma screening based on the plasma levels of cancer antigen 125 (CA-125) may result in the earlier detection of the disease, this diagnostic 'stage shift' does not translate into an improved survival rate [4]. Additionally, in women diagnosed with a suspicious pelvic mass, it remains a clinical challenge to discriminate between malignant and benign disease. To this end, the plasma levels of CA-125 or human epididymal protein 4 (HE4), or their combination, together with menopausal status can be used in the risk of ovarian malignancy algorithm (ROMA) [5,6]. However, the predictive power of these biomarkers and ROMA are far from optimal. An additional clinical challenge in the management of ovarian carcinoma is the prediction of treatment response for individual patients. Primary and acquired chemoresistance result in disease relapse following chemotherapy, which contributes to the high mortality rates of ovarian cancer [7,8]. Therefore, there is an urgent need for new biomarkers that can detect ovarian cancer at an early stage, discriminate between malignant and benign disease, and predict therapeutic response and prognosis.

The human leucine-rich repeats and immunoglobulin-like domains (LRIG) gene family comprises three genes, *LRIG1*, *LRIG2,* and *LRIG3*, which encode three structurally similar transmembrane proteins [9]. LRIG1 functions as a tumor suppressor in different tissues and organs, including in the gastrointestinal tract and in the brain [10,11]. The exact mechanism behind the tumor suppressive functions of LRIG1 has yet to be determined. However, it has been shown that LRIG1 antagonizes the signaling of several oncogenic receptor tyrosine kinases [12] and enhances cellular sensitivity to bone morphogenetic protein (BMP) [13,14]. Intriguingly, LRIG1 can be shed from cells in a bioactive form, suggesting a possible paracrine function [15]. It has previously been shown that LRIG1 tumor mRNA expression is associated with increased survival in ovarian serous carcinoma [2]. However, to our knowledge, the clinical implications of LRIG1 protein expression have not been investigated in ovarian tumors

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previously, nor have the levels of LRIG1 in human plasma been assessed systematically before.

Based on current knowledge, we hypothesized that plasma LRIG1 might be a diagnostic, prognostic, or predictive biomarker in ovarian carcinoma. Therefore, we developed an LRIG1-specific single molecule array (Simoa) assay and used this assay to evaluate the LRIG1 plasma levels in 486 patients who were treated with surgical resection of a suspicious pelvic mass. The results showed that plasma LRIG1 levels were increased in patients with ovarian carcinoma and that high plasma LRIG1 levels were paradoxically associated with worse overall survival (OS).

Material and methods

Study cohort and sample processing

The healthy blood donor population comprised 13 anonymous volunteers from our department. Healthy donor blood sampling was performed in a completely anonymous and blinded manner. Both EDTA and citrate plasma samples were collected from each donor. None of the healthy donors had any known disease at the time of blood withdrawal. Neither sex, age, nor any other personal information was collected from the anonymous volunteers. The study population has been described previously and comprises 486 patients who had been admitted to Sahlgrenska Hospital for a suspicious pelvic mass [16]. Of these patients, 155 patients had benign diseases of the ovary, 73 had borderline-type tumors, 224 had malignant ovarian tumors, and 34 had ovary-localized metastases. The patient and tumor characteristics are summarized in Table 1. The sample collection and processing procedures have been described previously [16].

Table 1. Patient and tumor characteristics according to malignancy.

	Benign	Borderline	Malignant	Metastasis
Total	155	73	224	34
	31.9%	15.0%	46.1%	7.0%
Age[a]	61 (16–88)	51 (16–85)	63 (19–88)	61 (36–91)
Tumor stage				
Stage I		61	63	1
		83.6%	28.1%	2.9%
Stage II		1	14	
		1.4%	6.3%	
Stage III		11	124	1
		15.1%	55.4%	2.9%
Stage IV			23	
			10.3%	
Histology				
Serous	88	37		
	56.8%	50.7%		
Dermoid	5			
	3.2%			
Endometriosis	7			
	4.5%			
Simple	1			
	0.6%			
Stromal	2	1		1
	1.3%	1.4%		2.9%
Teratoma	1			
	0.6%			
Other	1	1	1	28
	0.6%	1.4%	0.4%	82.4%
LGSC			17	
			7.6%	
CCC			15	1
			6.7%	2.9%
Mucinous	50	33	20	2
	32.3%	45.2%	8.9%	5.9%
Endometrioid		1	24	1
		1.4%	10.7%	2.9%
HGSC			146	1
			65.2%	2.9%
Tumor type[b]				
Type 1			76	
			33.9%	
Type 2			147	
			65.6%	

[a]Mean age in years (range).
[b]Type 1 (LGSC, endometrioid, clear cell, and mucinous carcinomas) or type 2 (HGSC and undifferentiated carcinomas) [17].

LRIG1 Simoa assay

The LRIG1 Simoa assay was developed using the Homebrew Assay Development kit (Quanterix Corporation, Billerica, Massachusetts, USA). Simoa is a digital and automated enzyme-linked immunosorbent assay (ELISA) technology capable of measuring low concentrations of proteins in complex samples with high specificity [18]. Our in-house developed mouse monoclonal antibody 3F7, against the immunoglobulin-like domains of human LRIG1, was used as the capture antibody, and 0.2 mg/mL antibody was conjugated to paramagnetic beads through incubation with 0.3 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) at 4 °C. Our in-house developed mouse monoclonal antibody 2B7, against the leucine-rich repeats domain of human LRIG1, was used as the detector antibody. It was biotinylated using a 40:1 molar ratio of biotin to antibody. To optimize the assay, we used recombinant human soluble LRIG1 (sLRIG1) as the analyte and spiked plasma samples. Recombinant sLRIG1 comprised the amino acid sequence corresponding to the extracellular/luminal part of human LRIG1 (GenBank accession no. AF381545, amino acids 35–792) fused to a FLAG and 8xHis tag. The recombinant protein was produced in HEK293 cells and purified through immobilized metal affinity chromatography using a Ni-Sepharose Excel column (GE Healthcare, Uppsala, Sweden), yielding a final protein purity of >90% as assessed *via* Coomassie blue-stained polyacrylamide gels. In the final optimized Simoa protocol, the 2-step format was used, that is, 300,000 beads conjugated with the 3F7 capture antibody were incubated with the diluted sample together with 0.15 μg/mL biotinylated 2B7 detector antibody for 35 min followed by incubation with 150 pM β-galactosidase-conjugated streptavidin for 5 min. Resorufin β-galactopyranoside was used as the fluorogenic substrate. The samples were run on an HD-1 Simoa analyzer (Quanterix Corporation) according to the manufacturer's instructions. The dilutional linearity was determined using three different ethylenediaminetetraacetic acid (EDTA) plasma samples that were either spiked with 2 ng/mL sLRIG1 or not spiked and then serially diluted two-fold from 2× to 32×. The percentage of linearity was calculated according to the following equation: % *Linearity (from X dilution) = (dilution corrected concentration of sample at Y dilution)/(dilution corrected concentration of sample at X dilution)*. The percentage of spike recovery was calculated as % *Recovery = (concentration of*

spiked sample – concentration of unspiked sample)/(concentration of spike level). The specificity of the assay was determined by evaluating the ability of a 20-fold excess of unlabeled detector antibody to inhibit the signal in four different EDTA plasma samples according to the equation: % *Inhibition = (assay signal in sample with no spike – assay signal in sample spiked with excess of unlabeled detector antibody)/(assay signal in sample with no spike)*.

Statistical analyses

In the LRIG1 Simoa assay validation, statistical comparisons were performed using a paired Student's *t* test on GraphPad Prism, version 5.0, software (GraphPad Software, San Diego, California, USA). The other statistical analyses were carried out using IBM SPSS statistics, version 28. The Mann–Whitney U test or Kruskal–Wallis test was used for statistical evaluation of differences between one or more groups, respectively. Adjustment for multiple comparisons was performed with the Bonferroni method. For all statistical tests, the significance level was set to 0.05. For OS, cancer-specific survival, and disease-free survival (DFS), Kaplan–Meier curves were created and presented together with the log-rank *p* value and the 95% confidence interval (CI) of mean survival time. The data were dichotomized into LRIG1 plasma levels below or above the median value.

Ethics statement

All the methods were performed in accordance with relevant guidelines and regulations. The study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of the Sahlgrenska University Hospital (Dnr 201-15). All the patients/participants provided written informed consent to participate.

Results

Development of Simoa assay for the quantification of LRIG1 in biological fluids

To quantify LRIG1 in biological fluids, we developed an LRIG1 Simoa assay. To this end, we used our in-house developed monoclonal antibody 3F7 against the immunoglobulin-like domains of LRIG1 as the capture antibody and 2B7 against the leucine-rich repeats domain of LRIG1 as the detector antibody. The optimized protocol, as described in Materials and methods, showed good linearity (average % linearity at an eightfold sample dilution was 102% ±4.0% for nonspiked samples and 104% ±1.6% for spiked samples) and good spike recovery (87% ±8.7%). The specificity of the assay was high, as a 20-fold excess of unlabeled detector antibody inhibited 96.5% ±2.6% ($n = 4$) of the signal in EDTA plasma. The estimated limit of detection was 1.5 pg/mL, and the dynamic range was 40–2500 pg/mL.

Validation of the LRIG1 Simoa assay using human plasma

To investigate whether LRIG1 could be detected in human plasma using our new Simoa assay and whether the anticoagulant used influenced the performance of the assay, blood samples were collected from 13 apparently healthy donors using EDTA or citrate as the anticoagulant. The LRIG1 levels were measured with the LRIG1 Simoa assay, and the EDTA and citrate values were compared for each donor (Figure 1(A)). The mean LRIG1 level for the EDTA samples was 705 ± 224.5 pg/mL ($n = 13$) and for the citrate samples was 689 ± 193 pg/mL ($n = 12$), and there was no significant difference in the LRIG1 levels between the samples with the two different anticoagulants ($p = 0.54$, paired sample *t* test). Next, the stability of LRIG1 in EDTA blood was assessed.

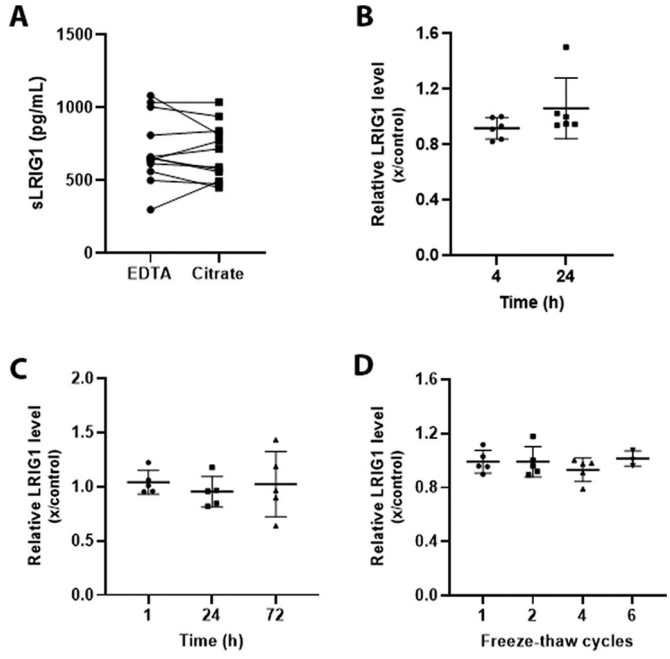

Figure 1. LRIG1 Simoa assay validation using human plasma. (A) Type of plasma anticoagulant did not affect the LRIG1 Simoa assay performance. Plasma was prepared with EDTA or citrate as the anticoagulant. The samples were analyzed with the LRIG1 Simoa assay, and the EDTA and citrate results were compared for each donor. There was no significant difference in the LRIG1 levels between the EDTA and citrate samples ($p = 0.54$, paired *t* test). (B) LRIG1 plasma levels were not affected by extended incubation of the blood. Blood samples were handled immediately after withdrawal (controls) or kept for 4 h at room temperature or 24 h at 4 °C prior to plasma preparation. The samples were then analyzed with the LRIG1 Simoa assay. Shown are the ratios between the values for the stored samples and their respective controls. The LRIG1 values for the blood samples that had been stored at room temperature or at 4 °C were not significantly different from the controls ($p = 0.077$ and $p = 0.74$, respectively, paired *t* tests). (C) LRIG1 plasma levels were not affected by extended incubation of plasma at room temperature. EDTA plasma samples were kept at room temperature for 0 h (controls), 1 h, 24 h, or 72 h prior to analysis with the LRIG1 Simoa assay. Shown are the ratios between the values for the stored samples and their respective controls. There was no significant difference between the samples that had been stored for 1, 24, or 72 h at room temperature and their respective controls ($p = 0.64$, $p = 0.26$, and $p = 0.66$, respectively, paired *t* tests). (D) LRIG1 plasma levels were not affected by several plasma freeze–thaw cycles. EDTA plasma samples were freeze–thawed only once (controls) or exposed to 1, 2, 4, or 6 extra freeze–thaw cycles. Shown are the ratios between the extra freeze-thawed plasma samples and their respective controls. There was no significant differences in LRIG1 levels between the samples that had been freeze-thawed 1, 2, 4, or 6 extra times and their respective controls ($p = 0.94$, $p = 0.75$, $p = 0.13$, and $p = 0.66$, respectively, paired *t*-tests).

Plasma was prepared either directly after withdrawal of the blood (control) or after storage of the blood at room temperature for 4 h or at 4 °C for 24 h. The LRIG1 ratios between the samples stored at room temperature for 4 h or at 4 °C for 24 h and the controls were close to 1 for all sample pairs (Figure 1(B)). Accordingly, there was no significant difference in LRIG1 levels associated with the blood storage conditions tested ($p = 0.077$ and $p = 0.74$, respectively, paired sample t test). The stability of LRIG1 in plasma was assessed by storing EDTA plasma samples at room temperature for 1, 24, or 72 h prior to the Simoa analysis (Figure 1(C)). The LRIG1 ratio between the plasma samples that had been stored at room temperature and the samples that were analyzed directly after withdrawal was close to 1 for all sample pairs, and there was no significant difference in the LRIG1 levels between the plasma samples that had been kept at room temperature for up to 72 h and the controls ($p = 0.64$, $p = 0.26$, and $p = 0.66$, respectively, paired t tests). To investigate the possible effects of repeated plasma freeze–thaw cycles, the plasma samples were freeze–thawed 1, 2, 4, or 6 additional times, prior to the analysis (Figure 1(D)). There was no significant effect on the LRIG1 levels following up to six extra freeze–thaw cycles of the plasma ($p = 0.94$, $p = 0.75$, $p = 0.13$, and $p = 0.66$, respectively, paired t tests). Taken together, these experiments showed that plasma LRIG1 analyzed by the LRIG1 Simoa assay was robust with regard to the anticoagulant used, time of blood or plasma being kept at room temperature or in the refrigerator, and the number of plasma freeze–thaw cycles.

LRIG1 plasma levels were increased in patients with ovarian carcinoma

To evaluate possible associations between plasma LRIG1 levels and clinical features of ovarian tumors, LRIG1 plasma levels were determined for 486 gynecological patients (Table 1). Two outlier patients with extreme LRIG1 plasma levels were excluded prior to statistical analysis. One was a patient diagnosed with high-grade serous ovarian carcinoma and showed an LRIG1 plasma level of 2.95 μg/mL. One patient was diagnosed with low-grade serous ovarian carcinoma and showed an LRIG1 plasma levels of 32.0 ng/mL. The reasons for these extreme LRIG1 values were not further investigated; future studies will reveal whether these kinds of outliers are real or if they represent technical artifacts. Among the remaining patients, the average LRIG1 concentrations in the plasma of the different malignancy groups were 577 ± 529 pg/mL in the benign group, 645 ± 1222 pg/mL in the borderline-type group, 817 ± 800 pg/mL in the malignant group, and 857 ± 926 pg/mL in the ovary-localized metastasis group (Figure 2(A)). The differences in LRIG1 plasma levels were significant between benign and malignant groups (Bonferroni adjusted $p = 3 \times 10^{-9}$), benign and metastasis groups (Bonferroni adjusted $p = 8 \times 10^{-5}$), borderline type and malignant groups (Bonferroni adjusted $p = 0.011$), and borderline type and metastasis groups (Bonferroni adjusted $p = 0.026$). In the benign group, LRIG1 plasma levels showed a weak correlation with patient age (Spearman's rho 0.224, $p = 0.005$). However, for patients with borderline-type tumors, ovarian carcinomas, and ovary-localized metastases there were no associations between LRIG1 levels and patient age.

LRIG1 plasma levels showed no association with ovarian carcinoma histological subtypes

The patients were classified and grouped according to the current WHO (2014) criteria for ovarian carcinoma histotypes. The average LRIG1 plasma levels were 613 ± 472 pg/mL for low-grade serous carcinoma (LGSC; $n = 17$), 1,058 ± 927 pg/mL for clear cell carcinoma ($n = 15$), 748 ± 952 pg/mL for mucinous carcinoma ($n = 20$), 731 ± 1,151 pg/mL for endometrioid carcinoma ($n = 24$), and 846 ± 739 pg/mL for high-grade serous carcinoma (HGSC; $n = 146$; Figure 2(B)). None of these differences were statistically significant ($p = 0.386$, Kruskal–Wallis).

LRIG1 plasma levels were increased in ovarian carcinoma stages 3–4 compared to stages 1–2

The ovarian carcinomas ($n = 224$) were classified and grouped according to tumor stage. Because of the low number of stage 2 cases ($n = 14$) and stage 4 cases ($n = 23$), these cases were grouped together with stage 1 ($n = 63$) and stage 3 ($n = 124$), respectively. The LRIG1 level was significantly higher in stages 3–4 than in stages 1–2 (Figure 2(C), $p = 0.003$, Mann–Whitney test).

LRIG1 and treatment response

The apparent response to chemotherapy is strongly dependent on the degree of surgery performed. Radical surgery is associated with a superior response to therapy. Therefore, to evaluate the effect of LRIG1 on the response to chemotherapy, we restricted the analysis to patients who did not receive radical surgery. In this group, the median LRIG1 plasma levels were 877 pg/mL ($n = 35$) for the complete responders and 1226 pg/mL ($n = 29$) for the noncomplete responders (Figure 2(D)). This difference was of borderline significance ($p = 0.051$, Mann–Whitney U test), suggesting that higher levels of plasma LRIG1 might be associated with a worse treatment response.

High LRIG1 plasma levels were associated with worse OS

The prognostic impact of LRIG1 plasma levels in the total malignant group ($n = 221$) and in the ovarian carcinoma stage 3 group ($n = 123$) was evaluated in association with OS, cancer-specific survival, and DFS. Kaplan–Meier survival analyses showed that plasma LRIG1 above the median was significantly associated with a worse OS both among all malignant cases (Figure 3(A), $p = 0.0001$, log-rank test) and among stage 3 cases only (Figure 3(B), $p = 0.001$, log-rank test), and with DFS among all malignant cases (Figure 3(C), $p = 0.031$, log-rank test) and among stage 3 cases only

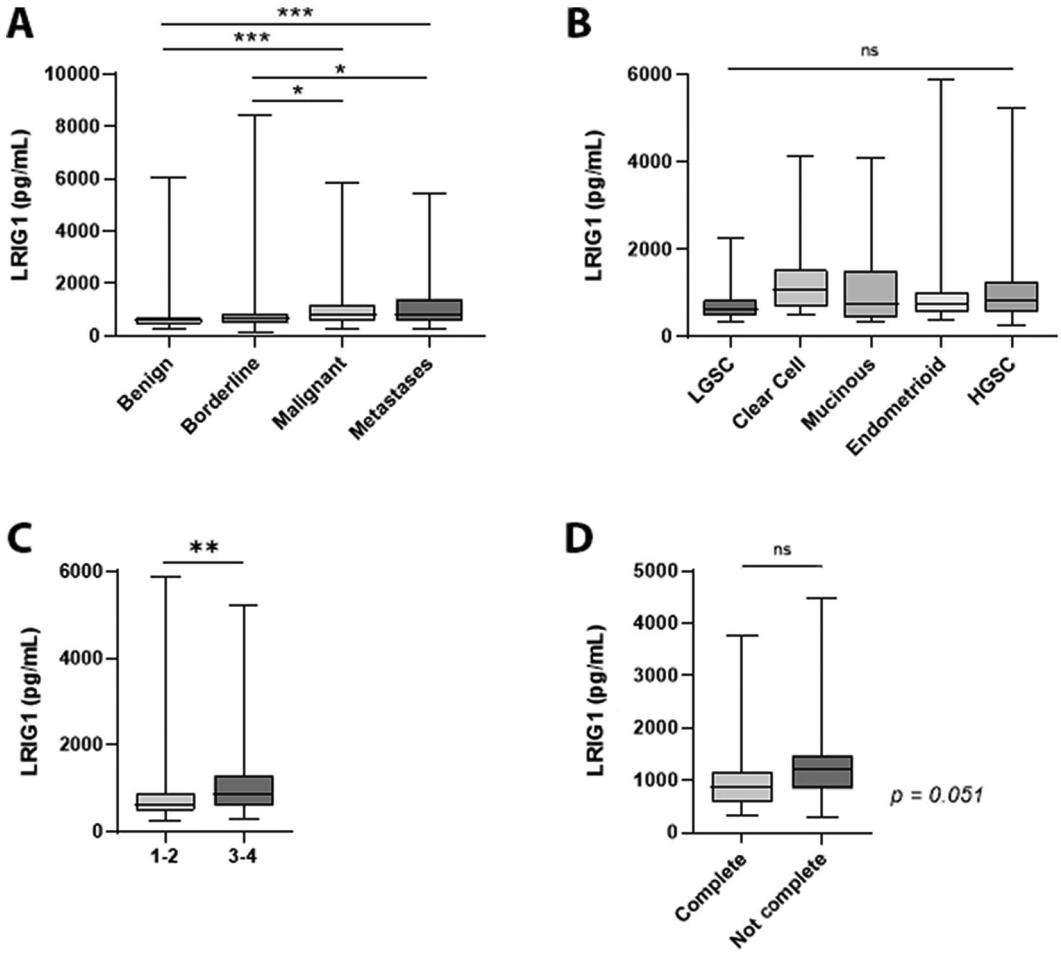

Figure 2. LRIG1 plasma levels in relation to clinical data. Shown are the LRIG1 plasma levels for the respective groups, displayed as boxplots. (A) LRIG1 plasma levels were increased in ovarian carcinoma and ovary-localized metastases. The average LRIG1 plasma concentrations for each group were as follows: benign cases ($n = 155$), 577 pg/mL, borderline cases ($n = 73$), 645 pg/mL, malignant cases ($n = 234$), 817 pg/mL, and ovary-localized metastases ($n = 34$), 857 pg/mL. (B) There was no difference in LRIG1 plasma levels between the major ovarian cancer histological subtypes. The average LRIG1 plasma levels for each histological subtype were as follows: HGSC ($n = 146$), 846 ± 739 pg/mL, endometrioid carcinoma ($n = 26$), 731 ± 1151 pg/mL, mucinous carcinoma ($n = 20$), 748 ± 952 pg/mL, clear cell carcinoma (CCC, $n = 15$), 1058 ± 927 pg/mL, and LGSC ($n = 16$), 613 ± 472 pg/mL (SD). (C) LRIG1 plasma levels were increased in stage 3–4 disease compared to stage 1–2 disease. Because of the low number of stage 2 cases ($n = 14$) and stage 4 cases ($n = 24$), these cases were grouped together with stage 1 ($n = 70$) and stage 3 ($n = 124$), respectively. (D) Complete treatment responders showed a trend toward lower LRIG1 plasma levels. The average LRIG1 level for the complete responders ($n = 35$) was 877 pg/mL and was 1226 pg/mL for the incomplete responders ($n = 29$). This difference was of borderline significance ($p = 0.051$, Mann–Whitney U test). The p values were determined with the Mann–Whitney U test or Kruskal–Wallis test, where applicable. Multiple comparisons were adjusted for using Bonferroni correction. Significant p values are denoted as $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$.

(Figure 3(D), $p = 0.04$, log-rank test). There was no significant association between LRIG1 above or below the median and cancer-specific survival ($p = 0.104$, log-rank test). Next, we performed a univariable Cox regression analysis to assess the impact of LRIG1 and other possible relevant factors on the survival outcome. This analysis showed that within the malignant group, high levels of LRIG1 ($p = 0.001$), CA-125 ($p = 0.002$), and HE4 ($p = 0.005$) along with age ($p = 0.004$) and nonradical surgery ($p < 0.0001$) were all individually associated with poor OS (Table 2). The hazard ratio for LRIG1 among all malignant cases was 2.8 (CI 95% 1.56–5.00). For stage 3 disease, LRIG1 ($p = 0.001$), age ($p = 0.031$), and nonradical surgery ($p = 7 \times 10^{-6}$) were significantly associated with poor OS, with a hazard ratio for LRIG1 of 4.1 (CI 95% 1.87–8.78). Notably, in type 1 and stage 3 disease, only LRIG1 among the analyzed factors was significantly associated with OS ($p = 0.01$), whereas in type 2 and stage 3 disease, both LRIG1 ($p = 0.02$) and nonradical surgery ($p \leq 0.001$) were associated with poor OS. To simultaneously assess the relationships between all the risk factors and OS, we performed multivariable Cox regression analyses, including all significant factors from the univariable analyses in the models (Table 2). For all malignant cases, HE4 ($p = 0.027$), age ($p = 0.006$), and nonradical surgery ($p \leq 0.001$) remained significant in the multivariable model, whereas among all stage 3 cases, all of LRIG1 ($p = 0.002$), age ($p = 0.046$), and nonradical surgery ($p < 0.001$) remained significant in the multivariable model. In type 2 and stage 3, only nonradical surgery remained significantly associated with worse OS ($p = 2.3 \times 10^{-5}$) in the multivariable model.

Discussion

We developed an LRIG1 Simoa assay to enable the quantification of LRIG1 in human biological fluids. The apparent sensitivity of the assay was 1.5 pg/mL, which was well below the observed LRIG1 levels in all the plasma samples analyzed. Furthermore, the assay showed excellent linearity, a wide

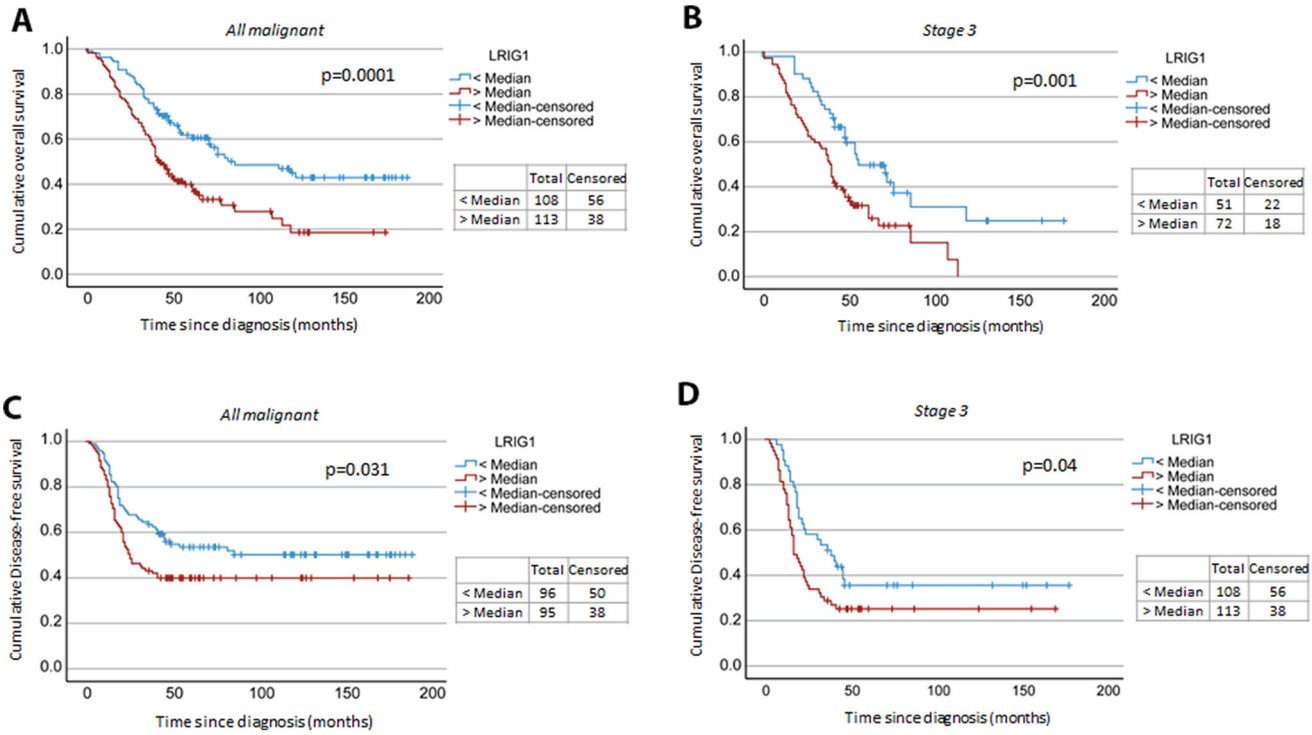

Figure 3. Kaplan–Meier curves showing overall and DFS according to LRIG1 plasma levels in ovarian cancer. (A–D) High LRIG1 plasma levels were associated with worse OS (A, B) and worse DFS (C, D) among all malignant cases (A, C) and among stage 3 cases only (B, D). The *p* values indicated in the figure were determined using the log-rank test. The number of patients per group is also indicated in the figure.

dynamic range, and high tolerance with regard to the preanalytical handling of the blood and plasma samples. The recorded average LRIG1 EDTA plasma level among the apparently healthy test individuals was 705 pg/mL, which was nine-fold higher than the previously reported mass spectrometry-based estimation of 78 pg/mL [19]. We did not investigate this discrepancy further. However, it is notable that the ELISA-based quantification of plasma LRIG2 [20] also yielded significantly higher levels than the corresponding mass spectrometry-based estimation (six-fold difference; 12 ng/mL vs. 2 ng/mL). Several reasons may account for the observed discrepancy between our Simoa assay results and the mass spectrometry estimations. First, the mass spectrometry estimations are based on spectral counting, which only yields an approximate estimation of the absolute protein abundance [21]. Second, immuno-based techniques such as Simoa rely on the actual expression of the epitopes recognized by the antibodies by the studied protein. At present, it cannot be ruled out that alternative forms of LRIG1, which might lack one or both epitopes recognized by the capture and detector antibodies, could exist in plasma or in the reference protein preparation. Therefore, the absolute LRIG1 levels in human plasma remain uncertain. Nevertheless, the robust performance of the LRIG1 Simoa assay demonstrated that it was well suited for the detection and relative quantification of LRIG1 in human plasma.

The observation that high LRIG1 plasma levels were associated with poor survival in ovarian carcinoma is intriguing and paradoxical. Here, plasma LRIG1 was associated with poor survival in all malignant cases, and in stage 3, it was an independent prognostic factor. In fact, in type 1 and stage 3 ovarian carcinoma, LRIG1 was the only significant factor predicting survival, hence outperforming CA-125, HE4, patient age, and radical surgery in prognostic power in this patient group. There was also a trend toward a worse treatment response among patients with high LRIG1 levels. These observations contrast with previous reports, where LRIG1 expression in general has been associated with good survival [22–29] and enhanced drug sensitivity in different types of cancer [30–34], but a double-edged sword function has also been proposed [35]. Here, the function of LRIG1 might be informative to consider. In this regard, LRIG1 was recently shown to function as a prominent promoter of BMP signaling [14]. If the primary function of LRIG1 is to promote BMP signaling, then one might expect that the effects of LRIG1 on tumor growth will mirror the role of BMP signaling in that particular tumor type. Intriguingly, whereas BMP signaling suppresses many other types of cancer, several recent reports suggest that BMP signaling is associated with worse survival in ovarian cancer and actually promotes ovarian carcinoma growth [36–39]. Based on these observations, we suggest that LRIG1 functions as a tumor promoter in ovarian carcinoma *via* its ability to promote BMP signaling. The role of LRIG1 and BMP signaling in the progression and treatment response of ovarian cancer will need further investigation.

Regarding the apparently conflicting results between the present study, which showed that low LRIG1 plasma protein levels were associated with *good* survival in ovarian carcinoma, and the study by Willis *et al.* [2], which demonstrated that low LRIG1 tumor mRNA levels are associated with *poor* survival in ovarian serous carcinoma, there are several possible explanations. Importantly, whereas Willis *et al.* analyzed tumor mRNA expression data, we analyzed plasma LRIG1 protein levels, and because the correlation between mRNA and protein levels is

Table 2. Univariate and multivariate Cox regression analyses of hazards ratios for different prognostic factors.

Univariable						
Patients included	Variable	Events	Total	HR	CI 95%	*p* value
Malignant	LRIG1 (Log 10)	127	221	2.79560	1.564–4.996	0.001
	CA125	107	195	1.00006	1.000032–1.000091	0.002
	HE4	53	87	1.00025	1.000104–1.000391	0.005
	Age	127	221	1.02200	1.007129–1.037096	0.004
	Nonradical surgery	124	217	3.66100	2.529280–5.298969	<0.0001*
Malignant/Stage 3	LRIG1 (Log 10)	83	123	4.05368	1.872502–8.775591	0.001
	CA125	72	109	1.00005	0.999985–1.000118	0.182
	HE4	32	36	1.00005	0.999850–1.000255	0.623
	Age	83	123	1.02000	1.001654–1.038915	0.031
	Nonradical surgery	82	122	2.84600	1.795933–4.509913	0.000
Malignant/Stage 3/HGSC	LRIG1 (Log 10)	69	98	2.79317	1.163308–6.706535	0.024
	CA125	59	87	1.00005	0.999980–1.000126	0.202
	HE4	25	26	0.99999	0.999763–1.000224	0.957
	Age	69	98	1.01976	0.997383–1.042635	0.082
	Nonradical surgery	68	97	3.45495	2.001619–5.963527	0.000
Malignant/Stage 3/Type 1	LRIG1 (Log 10)	14	25	8.34807	1.611–43.261	0.011
	CA125	13	9	1.00053	0.999–1.002	0.400
	HE4	7	10	1.00121	0.998–1.005	0.502
	Age	14	25	1.01810	0.980–1.057	0.348
	Nonradical surgery	14	25	1.89991	0.646–5.585	0.243

Multivariable							
Patients included	Variable	Events	Total	HR	CI 95%	*p* value	*p* value
Multivariable model 1							
Malignant	LRIG1 (Log 10)	42	72	1.43700	0.423–4.880541	0.561	0.000014
	CA125			1.00000	0.999484–1.000874	0.614	
	HE4			1.00000	1.000034–1.000561	0.027	
	Age			1.03600	1.010117–1.061548	0.006	
	Nonradical surgery			3.96800	1.989259–7.916870	0.000	
Multivariable model 2							
Malignant	LRIG1 (Log10)	104	191	1.83200	0.867921–3.869038	0.112	3.97E-11
	CA125			1.00000	1.000002–1.000071	0.037	
	Age			1.02000	1.003345–1.037384	0.019	
	Nonradical surgery			3.31700	2.175336–5.056549	0.000	
Multivariable model 3							
Malignant/Stage 3	LRIG1 (Log10)	82	122	3.98200	1.689–9.389	0.002	<0.001
	Age			1.02000	1.000–1.039	0.046	
	Nonradical surgery			2.73400	1.715–4.360	<0.001	
Multivariable model 4							
Malignant/Stage 3	LRIG1 (Log10)	82	122	3.99516	1.743794–9.153219	0.001	2.50E-07
	Nonradical surgery			2.72208	1.713931–4.323218	<0.0001*	
Multivariable model 5							
Malignant/Stage3/HGSC	LRIG1 (Log10)	68	97	2.38321	0.913004–6.220875	0.076	5.00E-06
	Nonradical surgery			3.26601	1.888482–5.648368	0.000	

often poor, the two studies might not be directly comparable. In fact, in the Cancer Genome Atlas (TCGA) ovarian cancer dataset [40], a frequent LRIG1 transcript variant (TCGA mRNA isoform_uc011bfu) lacks the 5′ end that encodes the leucine-rich repeats domain and might therefore encode a nonfunctional protein. Furthermore, it is not obvious whether high LRIG1 plasma levels reflect decreased or increased LRIG1 functionality in its tissue of origin, that is, whether high LRIG1 plasma levels reflect the proteolytic inactivation of cellular LRIG1 or if it mirrors the presence of high levels of active protein in the tissue of origin. Additionally, it remains possible that the measured plasma LRIG1 proteins originate from extratumoral sites, although the identity of this speculative LRIG1 source remains unknown. To resolve the apparent conflict between the plasma protein results presented herein and Willis and coworkers' tumor mRNA results, it will be important to analyze the levels of plasma LRIG1 protein and tumor LRIG1 mRNA in the same patients. To clarify whether plasma LRIG1 is produced by tumor cells or by other sources, xenograft experiments might be informative.

Conclusions

In summary, we presented a sensitive and robust Simoa assay for the quantification of LRIG1 in human body fluids and showed that plasma LRIG1 was an ovarian cancer biomarker associated with poor patient OS. In fact, in type 1 and stage 3 ovarian carcinoma, plasma LRIG1 was superior to all the other prognostic factors tested. In future studies, we find it important to further explore the functional role of LRIG1 in ovarian carcinoma progression and further investigate the possible clinical usefulness of LRIG1 plasma tests.

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Author contributions

ALLM and HH conceived the study and performed the experiments. AL performed the statistical analyses. ALLM, AL, KS, DL, and HH analyzed the data. ALLM and HH wrote the first draft. All authors contributed to the writing and review of the final draft.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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