LASP-1 interacts with ErbB2 in ovarian cancer cells.

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Running title: LASP-1 & ErbB2 interaction in ovarian cancer cells
Abstract

LASP-1 was identified as a protein following mass spectrometric analysis of phosphoproteins consequent to signaling by ErbB2 in SKOV-3 cells. It has been previously identified as an oncogene and is located on chromosomal arm 17q 0.76Mb centromeric to ErbB2. It is expressed in serous ovarian cancer cell lines as a 40kDa protein. In SKOV-3 cells, it was phosphorylated and was inhibited by Lapatinib and CP7274714. LASP-1 co-immunoprecipitated with ErbB2 in SKOV-3 cells, suggesting a direct interaction. This interaction and phosphorylation were independent of the kinase activity of ErbB2. Moreover, the binding of LASP-1 to ErbB2 was independent of the tyrosine phosphorylation of LASP-1. LASP-1 was neither expressed on the surface epithelium of the normal ovary nor in the fallopian tube. It was expressed in 28% of ovarian tumours (n=101) that did not significantly correlate with other clinical factors. In tumours from patients with invasive ductal carcinoma of the breast who had ErbB2 amplification (3+), LASP-1 was expressed in 3/20 (p <0.001). Analysis of the expression of an independent dataset of ovarian and breast tumors from TCGA showed the significant co-occurrence of ErbB2 and LASP-1 (p<0.01). These results suggest that LASP-1 and ErbB2 interaction could be important in the pathogenesis of ovarian cancer.
1. Introduction

Epithelial ovarian cancer is one of the leading causes of death due to gynaecological cancer. Though chemotherapy, radiotherapy and surgery are the common treatment modalities, new methods of targeted cancer treatment have been devised that exploit the differences in molecular attributes observed between cancer and normal cells. As a result, several receptor tyrosine kinases (RTK) such as vascular endothelial growth factor (VEGF) and ErbB2 receptors that play a crucial role in human carcinomas have emerged as clinically useful drug targets in anti-neoplastic therapy [1]. Subsequently, many new small molecule inhibitors and monoclonal antibodies have been developed to inhibit receptor kinase activity, producing an anti-tumor effect [2, 3]. Most of the molecular changes associated with the cancer cells are found to be due to alterations at the protein level, leading to its differential expression and/ or post-translational modifications, mainly phosphorylation. Hence, the role of these kinases can be uncovered by comprehensive protein analysis of tissue or body fluids. ErbB2 is a potent oncogene in many cancers and there are licensed drugs that inhibit its function. However, a comprehensive analysis of its signaling has not been performed. In ovarian cancer, clinical trials have shown that Trastuzumab [4] and Lapatinib [5] have very low response rates, suggesting poor efficacy. We undertook a comprehensive qualitative analysis of phosphoproteins following signaling by ErbB2.

In order to identify new substrates subsequent to phosphorylation, we analyzed phosphoproteins by mass spectrometry in SKOV-3 cells in the presence or absence of tyrosine kinase inhibitors [6]. The phosphoproteins were enriched initially by using Ferric affinity chromatography (Pierce phosphoprotein enrichment column). The samples were from SKOV-3 cells grown without serum, stimulated by EGF and in the presence of two tyrosine kinase inhibitors, Lapatinib [7] and CP724714 [8]. The phosphoproteins were
analyzed by nano-LC ESI-MS/MS by LTQ Orbitrap Discovery (Proteome exchange (PXD018046)). We identified LASP-1 as a protein in the analysis. As it was an oncogene previously reported in breast cancer [9], we chose to examine it in detail.

LASP-1 belongs to a family of LIM and SH3 domain containing proteins and its functions are probably distinct from LASP-2. It was originally isolated from a cDNA library of nodal metastases from breast cancer. It is located on the chromosome arm 17q11-21.3, 0.76 Mb centromeric to ErbB2 that is amplified in breast and ovarian cancer. The cDNA is 410 bp (261aa) and it codes for a 40 kDa protein. It has a LIM domain at the N-terminal region which is an arrangement of eight cysteine and histidine residues that are known to mediate protein-protein interactions as a modular binding surface. The LIM domain is followed by two nebulin like repeat regions which enable it to bind to actin. There is a direct interaction between the repeat region and actin via palladin at the stress fibres. This region is also involved in interactions with the Kelch related protein (Krp1), CD44 and ezrin to cellular contacts with the extra cellular matrix and promotes cell migration. The C terminal domain contains a SH3 motif that interacts with other proteins like palladin and is important in maintaining structural integrity [10]. There are two sites for phosphorylation. Serine 146 is phosphorylated by cyclic AMP and GMP protein kinases and is important in cellular localization and migration [11]. In contrast, tyrosine 171 is phosphorylated by c-Abl kinase and is associated with loss of LASP-1 from focal cell adhesions [12]. In serum starved cells that are quiescent, LASP-1 is localized to the peripheral edge of the cell. Upon growth factor stimulation, LASP-1 localizes to focal adhesions and thereafter to actin rich membrane ruffles on the surface of the cell [12].

There are preliminary reports on the expression of LASP-1 in ovarian tumours [13]. In breast tumours, LASP-1 showed increased expression as evaluated by immunohistochemistry and this correlated with tumour size and nodal positivity [14].
Interestingly, LASP-1 is expressed in bovine normal ductal epithelium and is increased during lactation. Further, LASP-1 is involved in a reciprocal translocation with MLL gene in acute leukemia [15]. Overall, the preliminary evidence suggests a role for LASP-1 in the pathogenesis of cancer.

In this report, we address whether LASP-1 is downstream of the ErbB2 signaling pathway and its expression in ovarian and breast cancer.

2. Materials and Methods

2.1. Cell line, inhibitors and antibodies

SKOV-3 cell line was obtained from Dr. Hani Gabra and was authenticated by STR profiling [16]. OVCAR-3, OVCAR-5, CAOV-3, CAOV-2, OAW42, HEK293T and PEO4 cells were obtained from ATCC. All the cell lines were grown in DMEM+10% FBS at 37°C and 5% CO2. FT33 cells were obtained from Dr. Ron Drapkin and grown in the recommended conditions [17]. All the cell lines were verified for mycoplasma by the PCR method [18].

Lapatinib was obtained from Chemie-Tek (USA). CP724714 was purchased from Selleck(USA). 10mM (Lapatinib) and 15mM (CP724714) stocks were prepared in DMSO.

EGF was purchased from Sigma and used at a concentration of 100ng/ml. LASP-1 rabbit polyclonal (sc-292342), LASP-1 mouse monoclonal (sc-37459) and HA rabbit polyclonal antibodies were purchased from Santacruz biotechnology (USA). GAPDH (2118S) and ErbB2 (2165S) antibodies were purchased from Cell signaling technology (USA).

2.2. Plasmids

PCDNA3.1 LASP-1 WT and Y171F were gifts from Dr. Raman Dayanidhi University of Uledo USA [19, 20]. The cDNA encoding LASP-1 was cloned in PCDNA3.1 using EcoRI and BamHI. The ATG is from the HA tag [21]. PCDNA3 ErbB2 Kinase dead (KD) (ErbB2/K753A) and Wildtype (WT) were a gift from Dr. Miller, Stony Brook USA [22].
2.3 Patient data

Clinical data for this study was extracted from case records of patients diagnosed with epithelial ovarian cancer (EOC) between 2005-2008 who had undergone surgery and treatment at Cancer Institute (WIA) Chennai, India. All the patients (n=293) were treated either with primary surgery or after 3-4 cycles of neoadjuvant chemotherapy with carboplatin and paclitaxel. The intent of surgery was to achieve complete macroscopic cytoreduction. After completion of all treatments, patients were on follow-up and evaluated with physical examination CA125 estimation and CT scans as required. The patients were staged according to the International Federation of Gynaecology and Obstetrics classification. Only 101 patients had representative tumour blocks. All the slides were reviewed for the confirmation of pathology. The grading of tumours was performed according to the WHO 2003 classification [23]. In addition, we identified 20 patients with invasive intraductal carcinoma of the breast whose tumours had amplification of ErbB2 by FISH and 3+ and 2+ expression by IHC. This study was conducted after obtaining approval from the Institutional Ethics committee (Cancer Institute WIA).

FISH was performed by Lalpath, an accredited laboratory according to international guidelines. IHC for ErbB2 was performed as described below using a rabbit polyclonal ab(A0485) (Dako, Denmark) to ErbB2.

2.4 Immunoblotting and Immunoprecipitation

Cells at 80% confluency were initially washed with PBS buffer and then were lysed using ice cold lysis buffer (500mM NaCl,1mM Tris-Hcl pH 8.0, 1mm EDTA, 1% Triton X-100, fresh Protease inhibitor, 2mM Sodium Fluoride, 1mM fresh PMSF and 2mM Sodium Orthovanadate). The lysates were centrifuged at 10,000 rpm for 20 mins to remove the debris. The supernatant was stored at -80ºC. For immunoblot, the lysates were quantified by BCA
assay and 40µg of total protein was resolved on SDS-PAGE and transferred to PVDF 0.45µm (Amersham, UK).

Blocking was done in 5% skimmed milk for 1 hr at RT. The blot after the washes was incubated with primary antibody overnight at 4°C, washed with PBST and then incubated with secondary antibodies for 1hr at RT. The blots were then visualized by ECLfemto (Pierce, USA) on X-ray film.

For immunoprecipitation, the protein A/G agarose beads (sc-2003) were initially washed with lysis buffer and then incubated the lysate with antibody at a final concentration of 1.5µg/mL at 4°C overnight. It was followed by washes with lysis buffer and 1M NaCl. The beads were boiled at 95°C for 5 minutes and then centrifuged at 10,000rpm for 10 minutes. The supernatant was then immunoblotted as mentioned above.

2.5 Immunohistochemistry (IHC)

Blocks from 101 patients were retrieved. 3-micron sections of formalin fixed paraffin embedded tissue were cut onto glass slides and dried at 60°C overnight. The sections were deparaffinized with three changes of xylene and rehydrated in a descending series of ethanol. After washes with water, the LASP-1 antigen was retrieved by heat induced epitope retrieval at 95°C in a water bath for 30 mins at 2 intervals of 15 mins each. The slides were then cooled after the retrieval to room temperature. After washes with Phospho buffered saline (PBS) (2x5mins each), the endogenous peroxidase was blocked with hydrogen peroxide. The slides were washed with PBS and then blocked with 2% skimmed milk for 15 mins. The slides were next incubated with primary LASP-1 rabbit polyclonal antibody (sc-292342) and ErbB2 (2165S) at 1:25 and 1:400 dilutions overnight at 4°C. These slides were then washed with PBS (3x5mins each) and later incubated with anti-rabbit secondary antibody conjugated with biotin (link) at 1:20 dilution for 1 hr. After the washes with PBS(1x5mins), they were incubated with
avidin conjugated with peroxidase (label) for 45 mins. After the washes with PBS (3x5mins each), the slides were then incubated with Diaminobenzidine (DAB) for 45 mins. They were next washed with water followed by counterstaining with haematoxylin. The slides were finally mounted with a DPX mountant [24].

For double immunohistochemistry, the initial steps of dewaxing and antigen retrieval were same as mentioned above. After retrieval, it was followed by washes with Tris buffered saline (TBS) for 5x3 times. The endogenous peroxidase was then blocked by 3% Hydrogen peroxide, followed by blocking with 2% skimmed milk for 15 mins. The slides were then incubated with LASP-1 mouse monoclonal (sc-37459) overnight at 4°C. The slides were washed with TBS and next incubated with anti-mouse secondary conjugated with biotin for 45 mins. After the washes with TBS (1x5 mins), they were then incubated with avidin conjugated with peroxidase for 30 mins. After the washes with TBS (2x5 mins each), the slides were next incubated with Diaminobenzidine (DAB) for 5 mins. After the initial DAB staining, the slides were again blocked with 2% skimmed milk for 30 mins. The slides were next incubated with ErbB2 rabbit polyclonal antibody (1:400) overnight at 4°C. The slides were then washed with TBS (3x5 mins each) and later incubated with anti-rabbit secondary conjugated with biotin for 45 mins. After the washes with TBS (1x5 mins), they were again incubated with avidin conjugated with alkaline phosphatase for 30 mins. After the washes with TBS (3x5 mins each), the slides were next incubated with Fast red for 5 mins. The slides were then washed with water, counterstained with haematoxylin and finally mounted with DPX [25].

2.6 Quantitative analysis of LASP-1

The expression of LASP-1 was evaluated according to the intensity and extent of staining. The intensity of staining was compared against a normal tissue and scored as faint (1), moderate (2) and strong positive (3). The extent of staining also depended on the number of cells stained in a section. 0 – 30% was scored as 1, 30-60% as 2 and more than 60% was scored as 3. A
combined score was used in the final analysis. The combined score of 9 was considered high, 6 & 4 considered moderate and the scores of <4 were considered low. The sections were evaluated by a pathologist (KM), who was not aware of the clinical data.

2.7. Plasmid, transformation and isolation

The plasmids were eluted from the filter paper by 100µl of TE buffer, incubated at 37°C for 10 mins and then centrifuged at 10,000 rpm for 30 mins. This was followed by the transformation of the plasmid into competent DH5 alpha cells as per standard protocols [26].

A single colony was inoculated into 500ml of transformed E.coli and was processed for plasmid isolation using Alkaline lysis method according to standard protocols [27].

2.8. Transfection of plasmids

0.5 x 10^6 cells of HEK293T were plated in a 6 well plate overnight. The following day, the medium of the cells was changed 2 hrs prior to transfection. In a sterile tube, 3µg of plasmid DNA was added to 200µl of media. Then 9µg of Polyethanolaamine (PEI cat no: 23966, Polysciences, USA) reagent was added to this complex in the ratio of 3:1. It was followed by incubation at RT for 15 mins. The complex was then added to the cells dropwise and incubated for 48 hrs prior to lysate preparation.

2.9. Statistical and Bioinformatic analysis

Overall survival (OS) and event free survival (EFS) were the outcome measures. An event was defined as local or distant recurrence or doubling of CA125 on more than 2 occasions. OS was defined as the period from diagnosis until the time of death from any cause. EFS was defined as the period from diagnosis until the occurrence of an event as defined above.

The Cox proportional hazards model was used to calculate the prognostic factors and to assess any impact on EFS and OS [28]. Univariate and multivariate analysis were performed to evaluate these factors. The survival curves were plotted using the Kaplan and Meier method.
All the statistical analysis was performed using SPSS version 17. The correlation of expression of LASP-1 with survival TCGA ovarian data was performed (c-Bioportal) [30, 31].

3 Results

3.1 Expression of LASP-1 in ovarian cancer cell lines

A protein of 40kDa was identified on the Western blot by the rabbit polyclonal anti-LASP-1 (sc-292342) upon transfection of full-length cDNA of LASP-1 in HEK 293 cells (Fig.1A). To establish the size of LASP-1 and specificity of antibodies, we performed the following experiment. In SKOV-3 cells, immunoprecipitation was performed with a rabbit polyclonal antibody and in the Western blot a mouse monoclonal antibody against LASP-1 (sc-37459) was used. A 40kDa band was detected which confirmed the specificity of antibodies and the size (Fig.1B). Although the cDNA for LASP-1 codes for a 28kDa protein, the size of LASP-1 varied between 40-44kDa. This is probably due to post-translational modifications.

Upon evaluation of cellular lysates from a panel of ovarian cancer cell lines, LASP-1 was expressed in SKOV-3, CAOV-2, CAOV-3, OVCAR-3, OVCAR-5 and OAW42 but absent in FT33. The expression of LASP-1 was reduced in A2780 and PEO4 cells (Fig.1C). Maximal expression was observed in OVCAR-5 and SKOV-3 cell lines as compared to others.

3.2 Phosphorylation of LASP-1 and interaction

LASP-1 was identified as a protein during our phosphoproteomic study of signaling by ErbB2 [6]. This raised the possibility that LASP-1 could be a substrate and potentially phosphorylated by ErbB2. Lapatinib and CP724714 were the inhibitors chosen as they are relatively specific to ErbB2 [32]. Lapatinib is a licensed drug and is slightly more specific to ErbB2 with an IC50 of 9.2nM [7]. CP724714 is highly selective to ErbB2 as compared to EGFR with an IC50 of 10nM for kinase activity [8].
To confirm this, we performed an experiment where LASP-1 was immunoprecipitated from SKOV-3 cells. Following SDS PAGE and transfer, the blot was probed with an anti-phosphotyrosine antibody (4G10). Although there was no increase in the phosphorylation of LASP-1 following EGF stimulation, it was inhibited completely by Lapatinib and partially by CP724714 (Fig.2A). This suggests that signals could be dependent on ErbB2.

To examine whether LASP-1 and ErbB2 interact, we performed experiments in SKOV-3 cells that expressed both proteins abundantly. Even under stringent conditions (lysis buffer and 1M NaCl), we were able to show the reciprocal interaction between LASP-1 and ErbB2 (Fig.2B).

We next examined whether the interaction between ErbB2 and LASP-1 was dependent on the kinase activity. To confirm this, LASP-1 was overexpressed together with wild type and kinase dead ErbB2 in HEK293T cells and the lysates were then immunoprecipitated for LASP-1 with the rabbit polyclonal antibody. Following SDS-PAGE and transfer, the blot was probed with ErbB2 antibody. The ErbB2 band was present in both the wild type and kinase dead transfected lanes but was absent in the untransfected lane (Fig.2C). This shows that the interaction between ErbB2 and LASP-1 is independent of the kinase activity of ErbB2.

We also examined whether the phosphorylation of LASP-1 was dependent on the kinase activity of ErbB2. LASP-1 was overexpressed together with wild type and kinase dead ErbB2 in HEK293T cells and the lysates were then immunoprecipitated for LASP-1 with the rabbit polyclonal antibody. Following SDS-PAGE and transfer, the blot was probed with an anti-phosphotyrosine antibody (4G10). The phosphorylation of LASP-1 increased in transfected cells as compared to untransfected but was unaltered even in the presence of Kinase dead (KD) version of ErbB2 (Fig.2D). This shows that the phosphorylation of LASP-1 is independent of the kinase activity of ErbB2.

We also examined whether the interaction of ErbB2 with LASP-1 was dependent on the phosphorylation of LASP-1. To confirm this, ErbB2 was overexpressed together with the wild
type LASP-1 and its mutant(Y171F) in HEK293T cells and the lysates were then immunoprecipitated for ErbB2 with the rabbit polyclonal antibody. Following SDS-PAGE and transfer, the blot was probed with LASP-1 antibody. The LASP-1 band was present in both the wild type and mutant lanes but was reduced in the untransfected lane (Fig.2E). This confirmed that the interaction of ErbB2 with LASP-1 was independent of the tyrosine 171 phosphorylation of LASP-1.

3.3 Clinical data

One hundred and one patients with epithelial ovarian cancer (EOC) diagnosed between 2005-2008 with archival material were analysed. Clinicopathological data are presented for 101 patients with a median age of 49 years (Table 1). Most of the patients had high-grade serous carcinoma (77/101) and had stage III (63/101) disease. Of the 101 patients, 46 patients had undergone primary surgery whereas 55 patients had received neoadjuvant chemotherapy.

3.4 Expression of LASP-1 in primary tumours

Expression of LASP-1 was evaluated by IHC using a specific rabbit polyclonal antibody to LASP-1(sc-292342). The control tissue of a patient with hepatocellular carcinoma showed cytoplasmic expression (Fig.3A). However, there was no expression on the surface epithelium or the stroma of normal ovaries or fallopian tube (Fig.3B &C).

On examination of 101 malignant tumours there was a variable degree of cytoplasmic expression in 28 tumours (28%). It was expressed at high levels in (n=1), moderately (n=16) and weakly (n=11) (Supp. Table 1). 22/28 tumours showing cytoplasmic expression of LASP-1 were high grade serous while the rest of the positive tumours were non-serous. The representative tumour images with high, moderate and negative staining are shown (Fig.3 D, E, F).
3.5 Association of clinicopathological factors and the expression of LASP-1 and clinical outcome

Univariate and multivariate analysis were performed to evaluate the prognostic factors in 101 patients. In univariate analysis, stage (p<0.01), residual disease (p<0.01), histology (p=0.01) and extent of surgery (p<0.01) significantly associated with outcome (Table 2). The expression of LASP-1 did not show any impact on EFS or OS. In multivariate analysis, only stage (p<0.01) and extent of surgery (p<0.01) significantly associated with outcome (Table 3).

There was no significant difference in survival between patients whose tumours expressed LASP-1 versus not expressed (Fig.4). In addition, we examined the expression of LASP-1 at the mRNA level in the TCGA dataset (n=594) in HGSOC and it was 6%. The expression of LASP-1 also did not correlate with outcome (Supp Fig.1).

3.6 Correlation between LASP-1 and ErbB2 in patients with ovarian and breast cancer.

We queried the TCGA dataset for both ovarian and breast cancer whether LASP-1 and ErbB2 were expressed simultaneously. The query for RNA was (LASP-1: EXP>=1 ERBB2: EXP>=1). Interestingly, we found out that at the RNA level, the expression of both LASP-1 and ErbB2 tend to co-occur in these tumours that was significant (Supp Fig.2 A &B). To evaluate this experimentally, we selected 20 patients with invasive ductal carcinoma who had amplification (FISH) and overexpression (3+ and 2+ by IHC) of ErbB2. LASP-1 was expressed only in 3/20 breast tumours with overexpression of ErbB2. To evaluate this association between LASP-1 and ErbB2 in breast cancer, we did double IHC on one breast tumour that had amplification of ErbB2 and expressed LASP-1. This showed that both the proteins were expressed simultaneously (Fig.5A). We also selected 20 ovarian tumours that expressed LASP-1. In the case of ovarian tumours, ErbB2 was not expressed in any of the...
tumours. This relationship between ErbB2 and LASP-1 in both ovarian and breast cancer was highly significant using the chi square test (p<0.001) (Fig.5C).

4 Discussion and Conclusion

In this report, we present the preliminary evidence on the role of LASP-1 in the pathogenesis of ovarian cancer. We identified LASP-1 as one of the specific proteins during qualitative phosphoproteomic analysis of ErbB2 in SKOV-3 cells [6]. Essentially, the experiment was the stimulation of cells in the presence or absence of inhibitors of ErbB2 and mass spectrometry of enriched phosphoproteins from cellular lysates.

LASP-1 and 2 are members of the nebulin family of proteins. In normal cells, these proteins are assembled in a variety of cytoskeletal structures and have a role in the function of actin proteins. LASP-1 and 2 are localised to focal adhesions where they interact with several cytoskeletal proteins [10]. These observations support the results on different malignant cell lines where over-expression of LASP-1 leads to migration, invasion and proliferation in vitro. Conversely, inhibition of LASP-1 by siRNA in malignant cell lines lead to abrogation of these functions. Further, overexpression of LASP-1 promotes tumourigenicity in nude mice [10]. Thus, by all accounts it behaves like an oncogene.

In the original report, it was shown to be amplified in 8% of human breast tumours, often together with ErbB2 [9]. Since then, there are increasing reports evaluating its expression in diverse malignancies such as hepatocellular carcinoma [33], cholangiocarcinoma [34], non-small cell lung [35], renal [36], gastric [37], bladder [38] and oral carcinomas [39]. The expression of LASP-1 was assessed in tumours from patients with breast cancer (n=177) and it was observed that nuclear and cytoplasmic expression of LASP-1 correlates with tumour size and nodal positivity [14]. Expression of LASP-1 as a prognostic factor was first studied in hepatocellular carcinoma (n=144) [33]. LASP-1 staining was observed in both cytoplasm
and nucleus. It was highly expressed in the cytoplasm for 50% of tumours, while nuclear staining was observed in 32 (27.2%) of patients. However, only cytoplasmic staining of LASP-1 correlated with survival, and it associated with HBS Ag and serum alpha protein that are known prognostic factors for hepatocellular carcinoma. In renal cancer (n=216), tumours were evaluated for the expression of LASP-1 and 124 tumours had an increased expression of LASP-1 [36]. This overexpression correlated adversely with survival. Expression of LASP-1 has also been studied in 50 tumours from oral cancer and its expression correlated only with tumour size and a significant difference was found in LASP-1 expression levels between T1/T2 groups and T3/T4 groups indicating that levels are higher in advanced compared with early stages. Finally, the expression of LASP-1 as a prognostic factor was studied in gastric cancer [37]. Its expression was significant and associated with tumour size, invasive depth, TNM staging, and lymph node metastasis and p53 expression. Multivariate analysis showed that the expression of LASP-1 was an independent prognostic factor. It is still uncertain whether the expression of LASP-1 in the cytoplasm or nucleus or both indicate an adverse prognosis.

The expression of LASP-1 has been examined previously in primary ovarian tumours (n=26) and the effect of its knockdown has been evaluated in SKOV-3 cells. It was expressed in 14/26 tumours and was shown to be involved in migration and proliferation [13]. A study had shown that there was a strong gain of the region 17q12 in ovarian cancer and LASP-1 to be a potential candidate gene [40]. However, there have been no studies examining its expression in a large cohort of primary ovarian tumours and evaluating it against other conventional prognostic factors. In this report, we have shown that it is expressed in 28% of tumours. Even in those patients who had primary surgery, the expression of LASP-1 was not a significant factor correlating with outcome. Similarly, in patients with high grade serous adenocarcinoma, expression was not a significant factor correlating with outcome. This is
corroborated by mRNA data from TCGA analysis (n=594) that had examined only high-grade serous adenocarcinoma [30, 31]. The overexpression was not a prognostic factor as only 101 tumours were being evaluated. At the RNA level, evaluation of the expression in a large number of tumours (n=594) in the TCGA [30, 31] database also showed no correlation with outcome. Interestingly, as both ErbB2 and LASP-1 are localised to the same chromosome 17q and only 0.76Mb apart, we evaluated the expression of both using the TCGA data. There was significant co-expression of both genes in serous adenocarcinoma of the ovary. There was a suggestion that tumours that expressed ErbB2 did not express LASP-1 and vice versa. This was experimentally addressed by examining a small cohort of invasive ductal carcinomas of the breast that had amplified ErbB2 and overexpression (3+) at the protein level. There was a significant correlation in that only 3/20 of these tumours expressed LASP-1. Both LASP-1 and ErbB2 co-localized in these cases, that was shown by double immunohistochemistry. This suggests that LASP-1 could be in the signaling pathway of ErbB2.

In the subsequent experiments, although there was no significant increase in phosphorylation of LASP-1 upon stimulation of cells with EGF, we were able to confirm inhibition by using specific inhibitors. It was also shown that both proteins interact and was independent of the kinase activity of ErbB2. It was also shown that unphosphorylated LASP-1 (Y171F) can bind to ErbB2. It has been shown previously that unphosphorylated LASP-1 binds to AGO [20]. The phosphorylation of LASP-1 was not dependent on the kinase activity of ErbB2. These results can be interpreted to suggest that the two tyrosine kinase inhibitors inhibited the phosphorylation of LASP-1 through an undetermined mechanism. Further, the physiological effect of the interaction between ErbB2 and LASP-1 has to be explored.

There are reports showing phosphorylation of LASP-1 at S146 and Y171 in different cell lines but not in SKOV-3 cells. It was shown to be phosphorylated at S146 by protein kinase A
and protein kinase G [11]. It was phosphorylated at Y171 in leukemic cells from patients with CML and in K562 cells [41]. The LIM domain is shown to interact with the members of CXCR family. The binding of LASP-1 to CXCR1-3 is independent of phosphorylation of LASP-1, but the binding of CXCR-4 to LASP-1 is dependent on the phosphorylation of LASP-1 at S146 [10]. It was recently shown that the LIM domain can also interact with UHRF1 [42]. It has been reported that the 2 nebulin repeats have a direct interaction with F actin [21]. The SH3 domain is known to interact with many proteins like vimentin [43], Zyxin [44], ZO2 [45] and Palladin. Recently it has been reported that the SH3 domain can bind to DNMT1/G9a [42]. Recent evidence has shown that LASP-1 co-immunoprecipitated with PTEN and there was an inverse correlation between these 2 proteins suggesting that LASP-1 could activate the PI3K-AKT pathway by the degradation of PTEN [10].

Recently, proteins like Ephrin B1 [46] and IQGAP1 [47] are known to co-immunoprecipitate with ErbB2.

The above results suggest that either the expression of ErbB2 and in its absence that of LASP-1 may drive the tumorigenesis through the same signaling pathway in ovarian cancer at the protein level. This observation is difficult to prove conclusively as both are potent oncogenes. A way forward would be to knock down either one in cells where both are expressed endogenously and examine the effect by in vitro assays on proliferation and clonogenicity. Further assessment of whether LASP-1 has a functional role in malignant transformation can be performed. Further, the interaction between ErbB2 and LASP-1 can be verified by using proximity ligation assay wherein PLA probes are used and the proximity would be seen as spots under a fluorescent microscope [48]. The above data in ovarian cancer set the background for more detailed experiments.
Acknowledgment, Declaration of interest: We thank Dr. Dayanidhi Raman for providing us the LASP-1 and its mutant (Y171F) constructs. We declare that there are no conflicts of interest.

Data availability statement: All the data generated or analysed during this study are included in this published article and its supplementary files. All the raw data is available with the corresponding author on request.

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Figure Legends

Figure 1: Evaluation of expression of LASP-1 by rabbit polyclonal antibody.

A) Western blot of untransfected and transfected lysates of HEK293T cells for expression of LASP-1. B) LASP-1 was immunoprecipitated with a rabbit polyclonal antibody from SKOV-3 cells and blotted with a mouse monoclonal antibody (Lane-1). As a control, lane-2 shows a lysate from SKOV-3 cells. C) Western blot of ovarian cell line lysates for LASP-1. GAPDH was used as loading control. These are representative images of 2 independent experiments.

Figure 2: Effect of Lapatinib and CP724714 on the phosphorylation of LASP-1 and the interaction of endogenous ErbB2 with LASP-1.

A) SKOV-3 cells were cultured in the presence of Lapatinib (10 µM) & CP724714 (15µM) for 24 hrs in serum free media. EGF (100ng/ml) was then added to the media for 60 mins prior to lysis. Immunoprecipitated samples were then assessed by Western blot for the phosphorylation of LASP-1 by an anti-phosphotyrosine 4G10 Ab. The omission of primary antibody served as control for immunoprecipitation. The same blot was stripped and reprobed for LASP-1. B) Western blot of the immunoprecipitated samples from SKOV-3 lysate by rabbit polyclonal antibodies for ErbB2 and LASP-1 respectively. C) Samples from untransfected and transfected lysates of HEK293T cells immunoprecipitated with rabbit polyclonal against LASP-1 and blotted with antibody against ErbB2. The omission of primary antibody served as control for immunoprecipitation. The total LASP-1 is shown as loading control. The transfection in these lysates was shown by the expression of ErbB2. GAPDH was used as a loading control for the protein. D) Samples from untransfected and transfected lysates of HEK293T immunoprecipitated with rabbit polyclonal against LASP-1 and blotted with anti-phosphotyrosine 4G10 and antibody against ErbB2. The omission of
primary antibody served as control for immunoprecipitation. The same blot was stripped and reprobed for LASP-1. These are representative images of 2 independent experiments.

E) Samples from untransfected and transfected lysates of HEK293T cells immunoprecipitated with rabbit polyclonal against ErbB2 and blotted with antibody against LASP-1. The omission of primary antibody served as control for immunoprecipitation. The total ErbB2 is shown as loading control. The transfection in these lysates was shown by the expression of LASP-1. GAPDH was used as a loading control for the protein.

**Figure 3: Expression of LASP-1 on the surface epithelium of Normal Ovary, in Fallopian tube and Ovarian tumours.**

Immunohistochemical expression of LASP-1 was performed on normal ovaries (n=3) fallopian tube (n=3) and on ovarian tumours (n=101). Representative photograph of (A) Positive control (hepatocellular carcinoma) (B) Normal Ovarian surface Epithelium (C) Fallopian tube and ovarian tumours showing (D) High (E) Moderate and (F) Negative expression of LASP-1 at 40X magnification using Nikon E200 microscope

**Figure 4: Correlation of expression of LASP-1 with outcome.**

Kaplan Meier curve showing the correlation of expression of LASP-1 in 101 ovarian cancer tumours with (A) Overall survival and (B) Event free survival. The dotted line represents patients whose tumours did not show expression of LASP-1 (n=73) and the straight line represents patients whose tumours showed expression of LASP-1(n=28). The comparison was not statistically significant.
Figure 5: Expression of ErbB2 and LASP-1 on a breast tumour and their correlation with patients with breast and ovarian cancer.

Expression of LASP-1 performed on a breast tumour that had amplification of ErbB2 by double immunohistochemistry. The expression of LASP-1 is shown as brown and that of ErbB2 is shown in pink. The representative photographs showing A) dual staining of ErbB2 and LASP-1 on an ErbB2+ and LASP-1+ tumour. B) Dual stain of ErbB2 and LASP-1 on an ErbB2+ and LASP-1- tumour using Nikon E200 microscope. C) Paraffin sections of breast tumours (n=20) who had amplification of ErbB2 and overexpression by IHC (3 & 2+) were selected. Ovarian tumours (n=20) which showed expression of LASP-1 were selected. These tumours were evaluated for the expression of LASP-1 and ErbB2 respectively by specific antibodies. 1) Expression of ErbB2 and LASP-1 in ErbB2 + breast tumours (n=20) 2) Expression of LASP-1 and ErbB2 in LASP-1 + ovarian tumours (n=20). The relationship between the expression of ErbB2 and LASP-1 in ErbB2 + breast tumours and LASP-1+ ovarian tumours was statistically significant (chi-square test) (p<0.001).
Table 1: Clinicopathological characteristics of patients

| Characteristics          | All patients |
|-------------------------|--------------|
| Total no                | 101          |
| Age                     |              |
| <40                     | 73           |
| >40                     | 28           |
| FIGO stage              |              |
| Stage I                 | 17           |
| Stage II                | 08           |
| Stage III               | 63           |
| Stage IV                | 11           |
| NA                      | 02           |
| Histological type       |              |
| Serous                  | 77           |
| Non serous              | 24           |
| Grade                   |              |
| Grade I                 | 4            |
| Grade II                | 28           |
| Grade III               | 68           |
| NA                      | 1            |
| Treatment               |              |
| Primary surgery         | 47           |
| Chemotherapy            | 53           |
| NA                      | 1            |
| Surgery                 |              |
| Primary                 | 46           |
| IDS                     | 41           |
| Biopsy                  | 14           |
| Residual bulk           |              |
| Optimal                 | 66           |
| Suboptimal              | 17           |
| Inoperable              | 18           |
| Recurrence              |              |
| Yes                     | 42           |
| No                      | 59           |
| Survival status         |              |
| Alive                   | 41           |
| Dead                    | 60           |
## Table 2: Univariate analysis

| Variable          | Number | Overall survival | Event free survival |   |
|-------------------|--------|------------------|---------------------|---|
|                   |        | HR               | 95% CI              | P value |
|                   |        | HR               | 95% CI              | P value |
| **Age**           |        | Baseline         | Baseline            |   |
| <48               | 51     | 1.56             | 0.93-2.6            | 0.08 |
| >48               | 50     | 1.74             | 0.94-3.24           | 0.07 |
| **Stage**         |        | Baseline         | Baseline            |   |
| I                 | 19     | 2.8              | 0.7-11.2            | 0.145 |
| II                | 8      | 3.9              | 1.4-10.9            | 0.009* |
| III               | 63     | 11.5             | 3.5-37.09           | <0.0001* |
| IV                | 11     | 1.56             | 0.93-2.6            | 0.08 |
| **Histology**     |        | Baseline         | Baseline            |   |
| Serous            | 78     | 0.38             | 0.18-0.81           | 0.012* |
| Non serous        | 23     | 0.343            | 0.13-0.87           | 0.025* |
| **Grade**         |        | Baseline         | Baseline            |   |
| I                 | 5      | 1.4              | 0.18-11.2           | 0.72 |
| II                | 28     | 4.03             | 0.55-29.2           | 0.16 |
| III               | 68     | 4.03             | 0.55-29.2           | 0.16 |
| **Residual disease** |   | Baseline         | Baseline            |   |
| <1cm              | 66     | 2.7              | 1.4-5.29            | 0.002* |
| >1cm              | 17     | 3.1              | 1.5-6.5             | 0.002* |
| Inoperable        | 18     | 3.1              | 1.5-6.5             | 0.002* |
| Surgery           |        | Baseline         | Baseline            |   |
| Primary           | 46     | 1.49             | 0.8-2.7             | 0.18 |
| IDS               | 41     | 0.2              | 0.1                 | <0.0001* |
| Inoperable        | 14     | 0.33             | 0.41                | 0.001* |
| LASP-1            |        | Baseline         | Baseline            |   |
|Absent             | 73(73%)| 0.22             | 0.57-1.8            | 0.94 |
|Present            | 28(28%)| 0.69             | 0.24-1.2            | 0.143 |

HR: Hazards ratio, CI: Confidence interval and * denotes p<0.05
Table 3: Multivariate analysis

| Variable        | Number | Overall survival | Event free survival |
|-----------------|--------|------------------|---------------------|
|                 |        | HR               | 95% CI              | P value | HR               | 95% CI              | P value |
| Stage           |        |                  |                     |         |                  |                     |         |
| I               | 19     | Baseline         | 3.6                 | 1.1-12  | 0.034*           | 3.5                 | 0.88-14.5 | 0.07   |
| II              | 8      |                  | 2.6                 | 1-6.6   | 0.043*           | 2.7                 | 0.96-7.8  | 0.059  |
| III             | 63     |                  | 8.1                 | 2.7-24  | <0.0001*         | 5.1                 | 1.3-19.4  | 0.016* |
| IV              | 11     |                  | 1.75                | 0.64-2.1 | 0.6      | 0.59                | 0.26-1.36  | 0.22   |
| LASP-1          |        |                  |                     |         |                  |                     |         |
| Absent          | 73 (73%) | Baseline       | 1.4                 | 0.7-2.7 | 0.29               | 1.36                | 0.63-2.9  | 0.42   |
| Present         | 28 (28%) |                  | 2.6                 | 1.39-5.2 | 0.003*         | 1.53                | 0.6-3.8   | 0.36   |
| Residual disease|        |                  |                     |         |                  |                     |         |
| <1cm            | 66     | Baseline         | 1.4                 | 0.7-2.7 | 0.29               | 1.36                | 0.63-2.9  | 0.42   |
| >1cm            | 17     |                  | 2.6                 | 1.39-5.2 | 0.003*         | 1.53                | 0.6-3.8   | 0.36   |
| Inoperable      | 18     |                  | 0.82                | 0.43-1.5 | 0.539          | 0.52                | 0.22-1.2  | 0.14   |
| LASP-1          |        |                  |                     |         |                  |                     |         |
| Absent          | 73 (73%) | Baseline       | 1.37                | 0.74-2.5 | 0.3      | 0.45                | 0.19-1.0  | 0.068  |
| Present         | 28 (28%) |                  | 2.6                 | 1.39-5.2 | 0.003*         | 1.53                | 0.6-3.8   | 0.36   |

HR: Hazards ratio, CI: Confidence interval and * denotes p<0.05
Figure 1
Figure 3
Figure 4

A) Overall Survival (OS)

- LASP-1+ve (n=28) vs LASP-1-ve (n=73)

B) Event free survival (EFS)

- LASP-1 +ve (n=28) vs LASP-1 -ve (n=73)

Cumulative survival vs Duration (Years)

- Overall Survival (OS): p=0.9
- Event free survival (EFS): p=0.13
Figure 5

A) H and E

B) LASP-1 & ERBB2

C) No of positive patients breast tumours ovarian tumours

1) LASP-1 +ve ErbB2+ve
   n=17

2) LASP-1 -ve ErbB2+ve
   n=3

ErbB2 + ve LASP-1 + ve

n=20

Figure 5