L1 (CAM) (CD171) in ovarian serous neoplasms

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Summary

Purpose of the investigation: The evaluation of L1 (CAM) as a tumor progression marker and as a prognostic factor in serous ovarian tumors. *Methods:* L1 (CAM) protein expression was assessed by immunohistochemistry and Western blot in serous ovarian tumors [cystadenomas (n = 20), borderline tumors (n = 14) and carcinomas (n = 47)], and was correlated with stage,grade, progression-free survival time (PFS) and overall survival. *Results:* L1 (CAM) immunoreactivity correlated significantly with stage and grade. It increased from benign tumors to early carcinomas and to advanced stage carcinomas progressively and significantly. In Stage III G3 carcinoma patients, low L1 (CAM) expressing tumors exhibited better response to chemotherapy and were associated with statistically significantly longer PFS (p = 0.002). *Conclusion:* L1 (CAM) expression represents a novel diagnostic marker in serous ovarian neoplasms that shows characteristics of tumor progression. L1 expression was associated with chemotherapy response.

Key words: L1 (CAM); CD171; Ovarian cancer; Biomarker; Prognostic factor; Tumor progression.

Introduction

The L1 (CAM) molecule was first cloned and identified to be a 200-kDa transmembrane glycoprotein from mice [1]. L1 (CAM) belongs to the immunoglobulin (Ig) superfamily of ''recognition'' molecules and mediates cell-cell adhesion by using a Ca 2+ independent homophilic binding mechanism [2, 3]. These homophilic interactions are considered to promote neurite outgrowth and other morphogenic events [4, 5]. In addition, L1 (CAM) participates in heterophilic interactions with other adhesion molecules such as NCAM, TAG-1/axonin-1, integrins, CD24, laminin and proteoglycans [6-13]. Initially L1 (CAM), (subsequently designated CD 171), was extensively studied in the nervous system [14-16]. However, L1 (CAM) is expressed also by hematopoietic cells and certain epithelial cells as well as by a variety of human tumor cell lines [17-19].

Recently L1 (CAM) emerged as a promising new biomarker for the diagnosis and prognosis of human ovarian and endometrial carcinomas [20-22]. Not only was L1 (CAM) expression correlated with disease progression, even in early stage carcinomas, but serum/ tissue levels were associated with recurrent disease and short survival as well [20]. L1 (CAM) has been advocated by the same team of investigators as a marker that ''provides an alternative classification of gynecologic tumors according to their aggressiveness rather than their histology'' [21].

The reliability of L1 (CAM) as a new marker and its possible use as a prognostic marker would be benefited by additional and independent studies designed to confirm its relation with stage, grade and other histological morphologic findings (invasive fronts, tumor aggregates, etc.) that have been used as traditional indicators of "tumor aggression'' and survival.

Materials and Methods

Out of 95 ovarian cancers that were operated on in the hospital between 2001 and 2005, all Stage I and poorly differentiated Stage III serous neoplasms that received six cycles of postoperative TC (175-180 mg/m^2 paclitaxel and carboplatin) after calculating the area under the concentration curve (= 5) with available specimens and follow-up were selected for further analysis.

The samples included were further selected from patients that had serous neoplasms and were matched to other risk factors (age, family history). Genetic marker analysis was not available. Control ovaries were obtained from oophorectomy specimens of women operated on for benign disease (fibroid uterus) and were matched for age with the study group. All above-mentioned samples were obtained from each patient.

One hundred and one pathological samples from paraffin-embedded tissue were included. The histology of the surgical specimens were 47 serous invasive ovarian tumors, 14 serous borderline tumors, 20 serous benign cystadenomas and 20 normal ovaries were used as the control. Tumor stage, histology and grade of these cases are summarized in Table 1.

Institutional Review Board (IRB) approval was given. Clinicopathologic information was obtained from medical records.

Cancer patients were classified after staging laparotomy (the most common initial surgical procedure consisted of abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy and lymph node sampling) was performed. Nine were classified as Stage I disease (FIGO I) (4 with good differentiation - G1 and 5 with moderate differentiation - G2) and 38 as Stage III (poorly differentiated - G3) ovarian cancers.

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All slides were jointly reviewed by two pathologists with special interest in gynecological pathology. The direct effects of chemotherapy were assessed using the World Health Organization criteria.

At the completion of primary chemotherapy, response was assessed on the abdominopelvic CT plus serum CA 125, physical examination and a statement in the medical record about being clinically disease-free. For survival analysis the Stage III patients were dichotomized by a L1 immunohistochemistry score of 3 (the calculated mean for Stage III L1 expression was 2.88). The two groups (high vs low expression) that were compared did not differ in age, family history or level of cytoreduction. The mean follow up was three years. At the time of the analysis 11/38 had died of their disease. Different regimens were given as second- and third-line therapy and the different groups (after further classifying them according to second-line chemotherapy) had small numbers to be analyzed separately. Therefore although both the progression-free interval and the overall survival were calculated, we report the progression-free interval as the main outcome of the study.

Immunohistochemistry Western blotting and evaluation of data

At least one tissue sample per case was studied. L1 was immunolocalized on 4-μm tissue sections using one monoclonal antibody, clone UJ127 to L1CAM (Abcam laboratories, Cambridge Science Park, England) that recognizes a heavily glycosylated protein (between 200-220 kD) produced by a gene located in Xq28. The immunostaining technique included blockage of endogenous peroxidase activity with 3% H_2O_2 and antigen retrieval with 1 μM EDTA pH 8 (Labvision UK) in a microwave oven at 700 W for 10 min. The sections were incubated at 4°C with the primary antibody at a dilution of 1:200. Detection was performed using a commercially available kit (Envision, Dako) according to the manufacturer's instruction. The specificity of immunostaining was checked by substituting the primary antibody with non-related isotypic (IgG1) mouse immunoglobulin at a comparable dilution. Immunoreactivity of the small nerves was used as an internal positive control for L1 (CAM).

Two pathologists evaluated L1 immunoreactivity in tumor cells independently and blindly, without knowledge of patient identity or clinical outcome. Immunoreactivity was assessed based on the intensity of immunostaining (immunointensity II) and the percentage of immunopositive tumor cells (immunopositivity IP). A two-score system for II and IP was used. Extent of immunoreactivity was estimated as the semiquantification for percentage of immunopositive cells that was scored on a scale of 1 (1-10%), 2 (11-50%), 3 (51-80%) and 4 (> 80%). Intensity of immunoreactivity was graded subjectively on a scale of: 0, negative; 1, weak; 2, moderate; 3, strong with 3 corresponding to a signal of high intensity equal to that of enclosed small nerve branches. To facilitate comparative and statistical analysis of the data, a combined immunoreactivity score coded as coded L1X was calculated by multiplying the values for extent and intensity of immunostaining (Table 1).

Western blotting

The specificity of our results was also evaluated on portions of all studied samples by Western blot analysis. The tissues were lysed with NET-Triton lysis buffer (0.01 M Tris-Cl, 0.1 NaCl, 1 mM EDTA pH 7.4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate and a cocktail of protease inhibitors) for the protein extraction. Aliquots of lysates containing 20 μg of total protein for L1 detection were run on 8-12% NuPAGE tris-acetate gel (Invitrogen, Carlsbad, CA, USA) under denaturing and reducing conditions. Proteins were transferred to PVDF membranes (BioRad, USA). Nonspecific binding of antibody to the membrane was blocked by one-hour incubation with 5% (w/v) non-fat dry milk/0.01 (v/v) Tween 20 in PBS.

Immunoblot analysis for detection of the ectodomain part of L1 was performed using mouse monoclonal (UJ127.11) L1CAM antibody (Abcam, UK) and anti-mouse monoclonal antibody (1:500 dilution, Zymed Laboratories, USA). Human F-actin monoclonal antibody (Serotec, UK) was used as a protein marker for the quantification of the protein bands. Membranes were then immersed in ECL detection solution (Santa Cruz, CA, USA) and exposed to XAR-5 film (Kodak, USA) for autoradiography. Protein bands were quantified using an Epson GT-8000 laser scanner. The ratio of L1 protein band intensity relative to F-actin band intensity was calculated for each sample.

Table 1. — *L1 immunoreactivity score (L1X) means in normal, benign, borderline, Stage I, Stage III, G1, G2 and G3 ovarian lesions*.

Tumors		L1X
Histology		
Carcinoma serous	(n = 38)	3.38
Borderline serous	(n = 14)	1.33
Benign serous	(n = 20)	0.31
Normal ovaries	(n = 20)	0
Grade		
Low grade carcinomas G1	(n = 4)	0.5
High grade carcinomas G2 and G3	(n = 5) (n = 38)	3.64
Stage		
Stage I carcinomas	(n = 9)	1.67
Stage 3 carcinomas	(n = 38)	2.88

Statistical analysis

The Mann-Whitney U and the the Kruskal-Wallis non-parametric tests were used for L1 (CAM) expression. Progression-free survival and overall survival were estimated by the method of Kaplan and Meier. The log-rank test was used to compare differences between survival curves. Univariate and multivariate analyses were performed using Cox proportional hazards regression. The covariates (age, family history or level of cytoreduction of the univariate analyses) were used for the multivariate analysis (all patients were Stage 3/G3).

Results

L1 immunoreactivity was not found in the normal ovarian stroma or in the normal ovarian surface epithelium. Focally, few endothelial cells showed immunoreactivity. Moreover, occasional tubal epithelial cells showed weak staining while hilar nerves showed intense immunoreactivity as expected (not shown).

Both the extent and intensity of the immunoreactivity showed variation that could not be considered patternless. The lowest immunoreactivity scores were noted in normal ovaries and benign tumors and the highest in advanced stage tumors (Figure 1a). The results of the semiquantitative evaluation of immunoreactivity are summarized in Table 1, according to the stage and grade. When the Kruskal-Wallis test was applied in benign, bor-

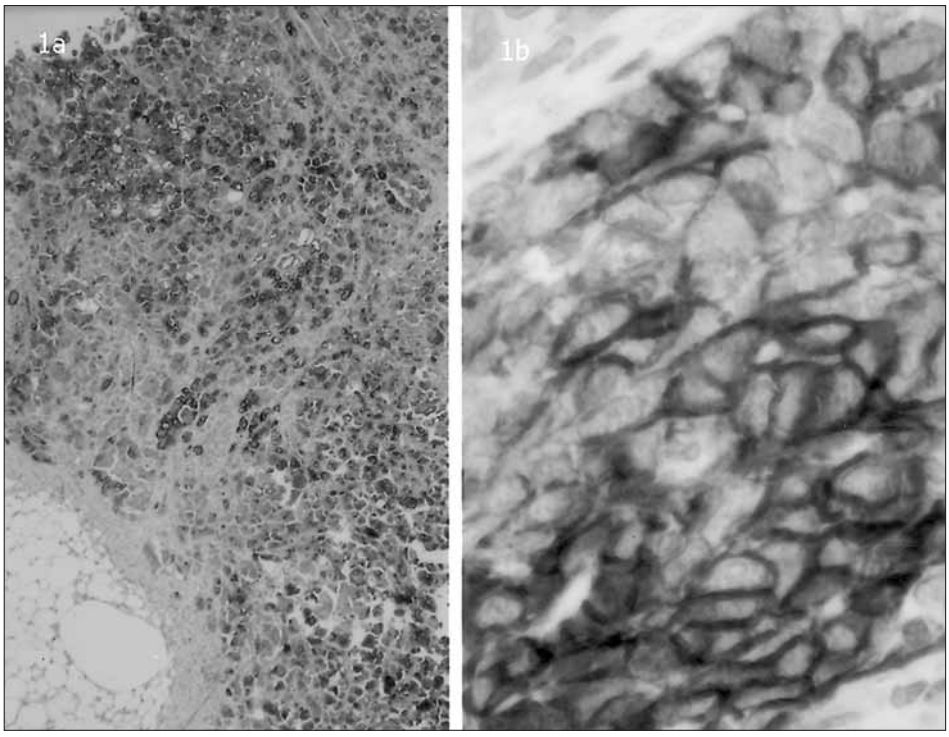

Figure 1a. — Extensive and intense L1 immunostaining of infiltrating ovarian serous carcinoma (original magnification 40 x).
Figure 1b. — Intense L1 immunoreactivity with predominant localization at the tumor cell membrane (original magnification 500 x).

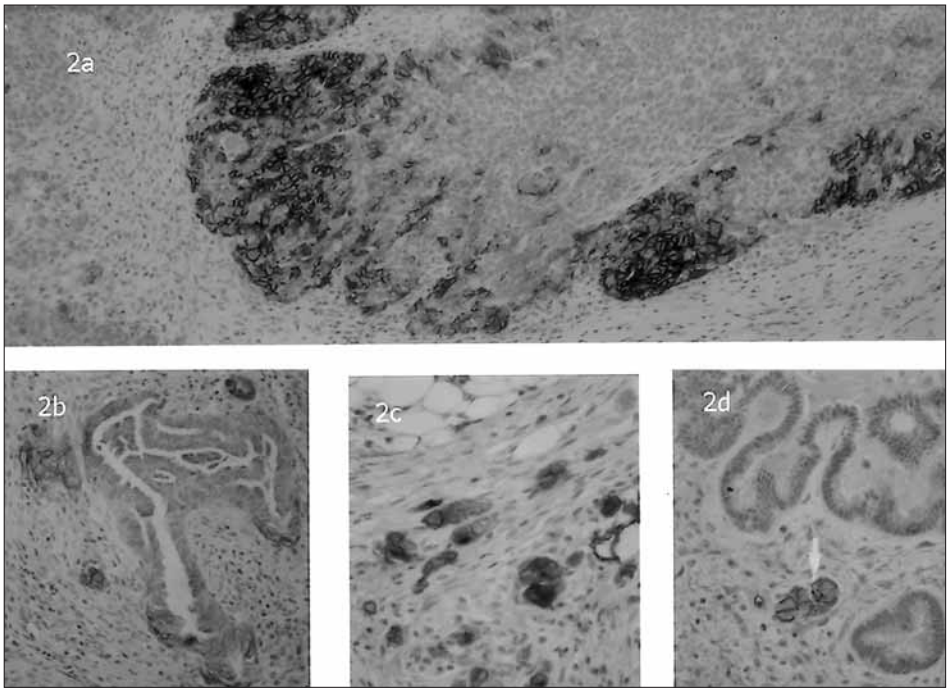

Figure 2a. — L1 immunostaining is enhanced at the invasive fronts of solid tumor aggregates (original magnification 100 x).
Figure 2b. — L1 immunostaining is increased at the polar regions of carcinomatous glands (original magnification 200 x).
Figure 2c. — Increased L1 immunostaining was most prominent in cells invading singly or in tiny aggregates (original magnification 500 x).
Figure 2d. — Microscopic focus of early stromal invasion in a serous tumor that otherwise showed the morphology of a borderline serous tumor. Notable is the markedly enhanced L1 immunoreactivity of the few invading tumor cells (arrow) (original magnification 250 x).
Microscopic focus of early stromal invasion in a serous tumor that otherwise showed the morphology of a borderline serous tumor. Notable is the enhanced L1 immunoreactivity of invading tumor cells (original magnification 250 x).

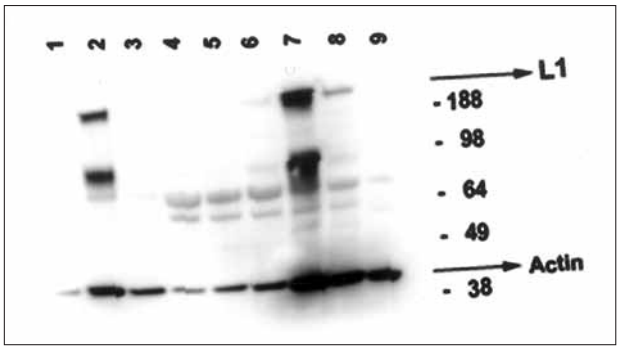

Figure 3. — Western blot analysis of L1 protein in cell extracts of ovarian carcinomas. 1: normal ovaries, 2: serous-type carcinoma, 3: borderline serous, 4/5: mucinous type borderline carcinoma, 6: benign serous, 7: ovarian carcinoma, 8: endometrioid carcinoma.

derline, Stage 1 and Stage 3 the result was highly significant ($p < 0.001$).

Carcinomatous cells showed predominantly membranous localization of the immunoreactivity (Figure 1b). Indeed increased immunostaining was noted at the invasive fronts of solid tumor aggregates (Figure 2a), at the invading branches of carcinomatous glands (Figure 2b) and it was most prominent in cells invading singly or in tiny aggregates (Figure 2c).

In addition, microinvasive foci in two borderline tumors showed preferential L1 (CAM) immunostaining (Figure 2d), despite the absence of statistically significant increase of immunoreactivity in borderline compared to benign tumors ($p = 0.35$).

Advanced (Stage 3) tumors demonstrated significantly higher L1 (CAM) immunostaining scores than Stage I ($p = 0.04$). Tumors of higher grade showed significantly higher L1 (CAM) immunoreactivity scores ($p = 0.03$) (Table 1).

In the retrospective analysis of L1 (CAM) expression by Western blotting analysis on 33 serous carcinomas, 14 serous borderline tumors and ten benign serous tumors we identified L1 (CAM) in most examined ovarian carcinomas of advanced stage (in 20 of 30 specimens of women with Stage III serous-type carcinoma) (Figure 3). We did not detect L1 (CAM) in 9/10 benign serous specimens as well as in the healthy control ovaries. Moreover, L1 (CAM) protein levels varied among the different groups with higher levels detected in G3 undifferentiated ovarian carcinomas (ratio L1 (CAM)/actin: 100%) followed by G2 serous-type carcinoma (ratio L1(CAM)/actin: 80%) and borderline serous (ratio L1(CAM)/actin: 30%).

All (n = 10) low L1 (CAM) expressing tumors exhibited complete response rate to TC while in high L1 (CAM) expressing tumors (n = 28), 18 had a complete clinical response, eight had a partial clinical response, and two had progressive disease ($p < 0.01$).

Patients with FIGO Stage III, G3 with high (n = 28) versus low (n = 10) L1 (CAM) expression had a statistically significant ($p = 0.001$) shorter median progression-free survival (PFS) of 17.50 (median) (mean 18.73, standard deviation 10.27) versus 28.50 (median) months (mean 29.37, standard deviation 5.06), respectively. The PFS survival curve which was statistically significant (log-rank = 0.006) is shown in Figure 4. The Cox regression model for progress-free survival when L1 expression was increased showed a hazard ratio = 3.8279 (95% CI 0.91-15.92, $p = 0.045$).The multivariate analysis verified L1 (CAM) expression as an independent prognostic factor for PFS. Despite the statistically significant PFS in Stage III patients, the overall survival of the patients was not statistically different ($p = 0.213$) with a L1 (CAM) increased expression median of 28.50 months and a L1 (CAM) decreased expression median of 29 months for the two groups, respectively. Due to the short follow-up (3 years) all low stage and borderline patients were alive and disease free.

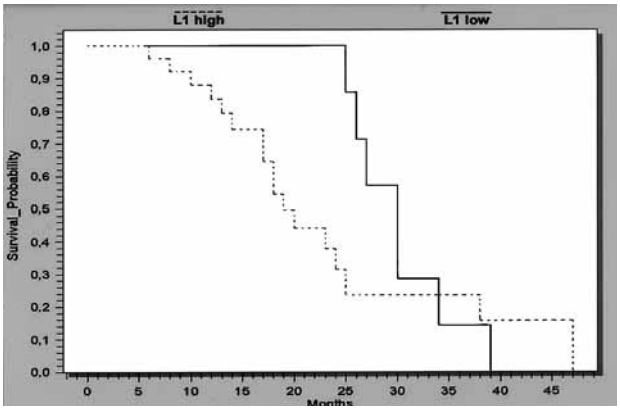

Figure 4. — Progress-free survival curve.

Discussion

L1 (CAM) has been advocated as a novel prognostic marker in ovarian neoplasms. Our study offers additional support to this notion, directly and indirectly. In patients with serous ovarian carcinomas, Stage III and grade 3, L1 (CAM) low expression was associated with a better response to the chemotherapy (TC = carboplatin paclitaxel) and prolonged progress-free survival (PFS). All the other risk factors (age, stage, grade, level of cytoreduction, and family history) were not statistically different between the two L1 (CAM) expression groups (low vs high) and multiple regression analysis showed it is an independent prognostic factor. This is in accordance with a recent report that suggested a link between L1 (CAM) expression and chemoresistance of ovarian carcinomas [23].

In addition, we noted that L1(CAM) expression (as determined by IHC and Western blotting) correlated significantly with established prognostic parameters such as the stage and grade of serous ovarian carcinomas. L1 (CAM) expression increased significantly and progressively from benign to aggressive ovarian neoplasms, in a manner analogous to that of a genuine tumor progression marker.

Furthermore, certain immunohistological findings suggested that L1 (CAM) could be pivotal in tumor cell invasion. We noted increased L1 (CAM) immunoreactivity at the invasive fronts of aggressive carcinomas as well as at the invasive component of microinvasive serous tumors. L1 (CAM), being an adhesion molecule, could promote cancer invasion through interactions with other critical adhesion molecules and extracellular matrix proteins.

L1 (CAM) could find some usage in the preoperative evaluation of a given ovarian carcinoma. High L1 (CAM) expression in a small biopsy sample could predict an aggressive behavior or an advanced stage and could assist in designing a more suitable management protocol.

Recent work has shown that antibodies to L1 (CAM) have therapeutical potential and can reduce cell proliferation in vitro [24, 25], and in vivo growth in a xenograft mouse model for human ovarian carcinoma [26]. Thus, L1 (CAM) might be a novel target for antibody-based therapy as second-line therapy against aggressive human ovarian tumors.

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