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Differential Expression of the 67-kD Laminin Receptor and 31-kD Human Laminin-binding Protein in Human Ovarian Carcinomas

F.A. van den Brûle, A. Berchuck, R.C. Bast, Fu-Tong Liu, C. Gillet, M.E. Sobel and V. Castronovo

The expression of the 67-kD laminin receptor (67LR) and the 31-kD human laminin-binding protein (HLBP31), two proteins involved in cancer cell laminin interaction, was evaluated on 30 ovarian cancer specimens. Expression of the 67LR was increased (up to 2.5-fold, in 87% of the patients), while HLBP31 expression was downregulated in cancer cells compared with the normal tissue, as detected by northern blotting and immunohistochemistry. The immunohistochemical study demonstrated that the 67LR was significantly overexpressed ($P < 0.05$) in the group of patients whose cytoreductive surgery was suboptimal, and those with poor clinical outcome. No correlation was observed between HLBP31 expression and clinicopathological features. Increased expression of the 67LR appears to correlate with the invasive phenotype of ovarian cancer cells and suggests a role of the latter in ovarian cancer invasion.

Key words: ovarian cancer, laminin receptors
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INTRODUCTION

Ovarian cancer is the fourth most frequent cause of cancer death in women [1]. Mortality and morbidity arise mainly from silent intra-abdominal spread of the disease [2]. To develop new prognostic evaluation methods and effective therapies, the mechanisms that allow these tumours to invade have to be elucidated.

Penetration of the basement membranes (BM) by malignant cells is a crucial step in the invasion of aggressive carcinomas [3, 4]. It includes a succession of interactions including attachment, degradation and migration [3]. Several gene products have been identified as key elements in BM penetration including various cell surface laminin receptors. Among these, expression of the 67-kD high-affinity laminin receptor (67LR) is increased in a variety of highly metastatic cancer cells [5–7]. The 67LR appears to be responsible for both strong adhesion and chemotaxis of the tumour cell to laminin [8]. Recently, another laminin-binding protein, HLBP31, has been identified. It is homologous to the 31-kD IgE binding protein [9], the Mac-2 antigen [10] and to the 31-kD galactoside-binding lectin [11]. Decreased expression of this lectin compared to the normal tissue has been associated with the metastatic potential of cancer cells in various cancer types such as colon [12] and breast carcinoma (Castronovo *et al.*, manuscript in preparation).

Correspondence to F.A. van den Brûle at the Metastasis Research Laboratory, University of Liège, Tour de Pathologie +3, B23, Sart Tilman, B-4000 Liège, Belgium.
F.A. van den Brûle, C. Gillet, M.E. Sobel and V. Castronovo are at the Molecular Pathology Section, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20892; A. Berchuck and R.C. Bast are at the Duke University Medical Center, Durham, North Carolina 27710; and Fu-Tong Liu is at the Molecular and Experimental Medicine, Scripps Clinic and Research Foundation, La Jolla, California 92037, U.S.A.
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To study the molecular mechanisms involved in ovarian cancer invasion, we examined expression of the 67LR and HLBP31 in 30 samples of ovarian cancer at both the mRNA and protein level by northern and western blotting, and by immunoperoxidase staining techniques, and compared them with clinicopathological features of advanced ovarian tumours.

MATERIALS AND SAMPLES

Tissue samples

Thirty liquid nitrogen frozen epithelial serous ovarian carcinoma samples weighing from 2 to 5 g were obtained from the Department of Gynecological Oncology (Duke University Medical Center, Durham, North Carolina, U.S.A.) and used for RNA and protein extraction, and fixed for histological examination. RNA extraction was first realised, and the two latter techniques were performed when enough tissue was provided. Out of the 30 samples, 25 were from primary tumours and five from recurrences. These five samples were obtained after primary treatment, including chemotherapy. For each patient, clinical data and pathological reports, including histological type and grade, were available. Surgical staging was established according to the FIGO (Fédération Internationale de Gynécologie-Obstétrique) [13] grading system. Debulking status was defined by surgeons in comparable conditions using identical criteria during primary surgery: it was stated optimal when the surgeon was able to remove the tumour, except for nodules smaller than 1 cm, and suboptimal when the nodules were bigger than 1 cm. Follow-up status was based on a combination of clinical, radiological and second-look data. All patients in this study completed primary chemotherapy and the range of follow-up was between 6 months and 6 years.

RNA extraction and northern blot analysis

Total cellular RNA was isolated from the tissue powder of 23 ovarian carcinoma samples and from a wedge resection of a

normal ovary using the guanidine isothiocyanate extraction procedure and cesium chloride gradient centrifugation as described previously [14]. Since this method of detection uses homogenised tissues, and the normal specimen used contained a variety of normal ovarian cell types, not just the epithelial cells of origin of the carcinoma, it should be noted that the comparisons with this tissue are somewhat arbitrary. Unfortunately, for seven samples, the procedure yielded degraded RNA. Equal amounts (5 μg) of total RNA were separated by size through a 1.2% (weight/vol) agarose-formaldehyde gel and transferred to nitrocellulose filters as described previously [15]. 67LR and HLBP31 cDNA probes have already been described [9, 16]. Hybridisation signals on the blots were quantified by using the Scan Analysis software package for the Macintosh (BioSoft, Cambridge, U.K.), and normalised to densitometric readings of the 28S and 18S ribosomal RNA bands as seen on ethidium bromide-stained gels and blots. This was justified by the fact that the use of classical housekeeping gene probes such as GAPDH or β-actin detected large discrepancies in expression of these genes compared to RNA loading. All values are expressed in relative optical density units.

Immunoblots

For the 24 largest samples, total protein extracts were prepared in 1% sodium dodecylsulphate (SDS). Fifteen micrograms of protein extract, as determined by a bicinchoninic acid protein assay (Pierce Chemical Co, Rockford, Illinois, U.S.A.), were separated by size on 10% SDS–polyacrylamide gels, and transferred to a polyvinylidene difluoride membrane (Immobilon: Millipore Corp, Bedford, Massachussetts, U.S.A.), as described previously [15]. The 67LR was detected using the previously-described affinity-purified antibody 4099 [15]. HLBP31 was detected with anti-recombinant rat IgE binding protein (rIgEBP) antibody [9]. Signals obtained by an enhanced chemiluminescence (ECL) western blotting detection system (Amersham, U.K.) were quantified by densitometric analysis of the immunoblots as described above for the assessment of RNA levels.

Immunoperoxidase staining

Immunoperoxidase staining, a technique allowing discrimination between cell types in heterogenous tissues, was performed on paraffin sections of 27 formalin-fixed specimens. The avidin–biotin immunoperoxidase method was used as described previously [17]. The 67LR was detected by the previously-described affinity-purified antibody 4161 [18]. HLBP31 was detected using anti-rIgEBP antibody [9]. Three independent observers estimated the level of each laminin-binding protein expression by counting the cancer cells that were strongly stained by the corresponding antibody, expressed as a percentage of the total number of cells examined (at least 1000 cells per specimen). The tumour samples were compared to each other, and to a normal value, defined by the mean of the values for the normal serosal epithelium present in three samples (OC8, OC11 and OC13). These three normal references were selected amongst the samples to avoid any edge effect resulting in artificially high values for the normal epithelium.

Statistical analysis

The differences between the values of various groups of ovarian carcinoma specimen were determined by the non-parametric double-sided Wilcoxon–Mann–Whitney test, using the Statworks statistics package for the Macintosh (Cricket Software, Philadelphia, Pennsylvania, U.S.A.). This test is based on the number of times the values of the members of a group surpass those of the other group, and not on the comparison of the means and standard deviations of the groups.

RESULTS

Increased 67LR expression correlates with tumour aggressiveness

As expected, the 67LR cDNA probe hybridised to a transcript of approximately 1400 bases in all the carcinoma samples (Figure 1, a panels). When compared to the wedge resection RNA sample, 87% of the samples showed up to 2.5-fold increased expression of the 67LR on northern blots. Antibody 4099 detected a 67-kD band on immunoblots (Figure 1, b panels). The 67LR detected by immunoperoxidase was essentially associated with the neoplastic cells (Figure 1, c panels): as at the mRNA level, its expression in the cancer cells was increased in 81% of the cases compared to the normal serosal surface epithelium. In some samples, tumour heterogeneity was the source of apparent discordance between the results of the three techniques used, particularly in immunoblot, that analysed pools of proteins from normal and cancer cells, as well as from the extracellular matrix. The best correlation for the three techniques occurred in the most cellular tumours. Immunohistochemistry was the most reliable detection technique for cancer cells.

The 67LR protein level detected by immunohistochemistry was significantly increased in the samples from patients who had suboptimal debulking compared to specimens from patients with optimal debulking (1.3 ± 0.6 versus 0.9 ± 0.9, $P < 0.05$; Table 1). A positive and significant correlation was also found in the patients with poor clinical outcome (AWD, alive with disease; DOD, died of the disease) compared to those free of disease (NED, no evidence of disease) (1.4 ± 0.6 versus 0.7 ± 0.7, respectively, $P < 0.05$; Table 1). In most of the cases, patients with suboptimal debulking belong to the progressive disease group. Indeed, 79% of the women with optimal debulking remained NED during follow-up, and 77% of the women with suboptimal debulking had evident disease at least 6 months after diagnosis (AWD or DOD). Conversely, 83% of the patients who were disease-free had optimal cytoreductive surgery, and 71% of the patients with progressive disease had suboptimal cytoreductive surgery. Thus, the patients with increased 67LR, as detected by immunohistochemistry, fall into the category of women whose disease was difficult to surgically debulk, with subsequent, persistent disease. In our patient population in which most patients had advanced stage, moderate or poorly differentiated cancers, classical prognostic factors such as surgical staging or histological grading of the tumours were less predictive of outcome than has been reported in other studies [19]. The same statistical analysis performed on the primary lesions only (25 samples) showed the same statistically significant difference. Indeed, there was a significant increase of the 67LR in the group of patients with suboptimal cytoreduction compared to those with optimally debulked tumours [1.45 ± 0.46 (9 samples) versus 0.96 ± 0.96 (12 samples), mean ± S.D., $P < 0.05$], and in the group of patients with progression of the disease, compared with those with remission after treatment [1.45 ± 0.90 (11 samples) versus 0.85 ± 0.75 (10 samples), mean ± S.D., $P < 0.05$]. No correlation with other parameters such as differentiation grade or surgical stage was found.

Expression of HLBP31 in ovarian carcinoma samples

As shown in Figure 1 (a panels), the HLBP31 cDNA probe detected a message of approximately 1400 bases in the carcinoma

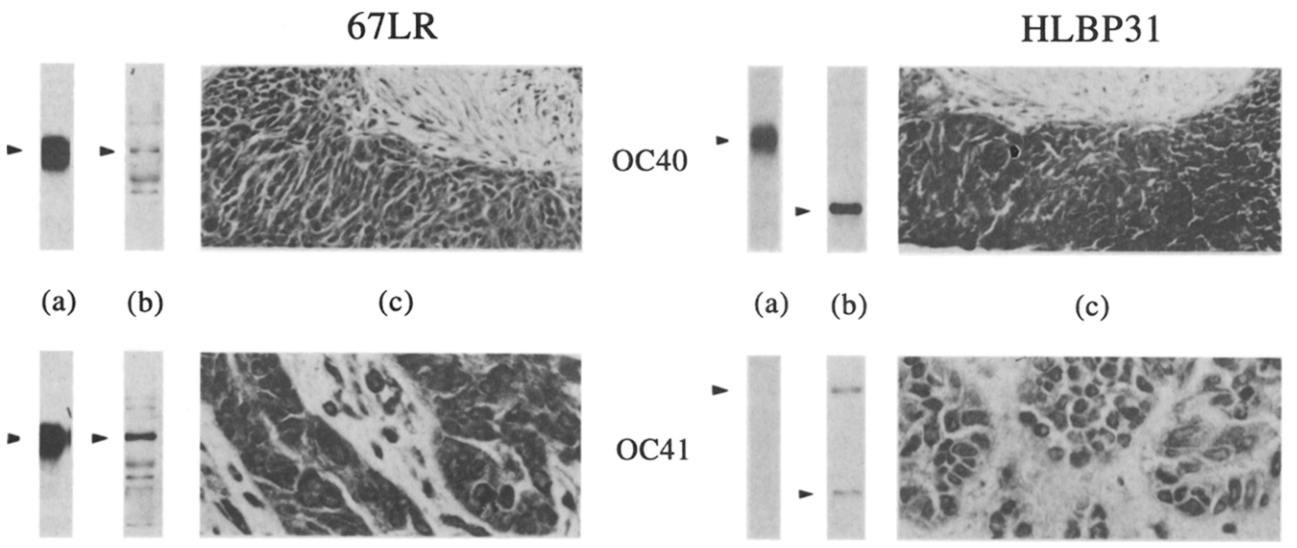

Figure 1. Typical examples of ovarian carcinoma specimens. OC40 and OC41 are two samples of primary serous carcinoma. Total cellular RNAs were extracted, run on 1.2% formaldehyde-agarose gels, transferred to nitrocellulose filters and hybridised to the 67LR or HLBP31 cDNA probes, showing a message of approximately 1400 bases for each transcript (a). Total tissue protein extracts were separated by electrophoresis on 10% reducing SDS–polyacrylamide gels and transferred to PDVF membranes. 67LR was detected using anti-67LR cDNA-derived peptide antibody 4099. HLBP31 was detected by anti-rIgEBP antibody (b). Immunoperoxidase staining was performed on paraffin sections of the specimens using the avidin–biotin immunoperoxidase method and anti-67LR cDNA-derived peptide 4161 for the 67LR or anti rIgEBP antibody for HLBP31 (magnification × 400) (c).

Table 1. Statistical analysis of the results according to various clinical characteristics.

	37LRP mRNA	HLBP31 mRNA	67LR IPX	HLBP31 IPX
Debulking				
Optimal	0.3 ± 0.3 (9)	0.3 ± 0.1 (9)	0.9 ± 0.9 (13)	1.7 ± 1.2 (13)
Suboptimal	0.3 ± 0.1 (11)	0.3 ± 0.2 (11)	1.3 ± 0.6 (12)*	1.7 ± 0.9 (13)
Follow-up				
NED	0.3 ± 0.1 (8)	0.3 ± 0.1 (8)	0.7 ± 0.7 (11)	1.5 ± 1.1 (12)
AWD + DOD	0.3 ± 0.1 (13)	0.2 ± 0.2 (13)	1.4 ± 0.6 (14)*	1.8 ± 1.0 (14)

Values are expressed as mean ± S.D. (number of patients). IPX, immunoperoxidase staining; NED, no evidence of disease; AWD, alive with disease; DOD, died of disease. *$P < 0.05$ from Wilcoxon–Mann–Whitney test.

samples. When compared to the RNA sample extracted from the normal wedge section, 87% of the samples showed decreased expression of HLBP31. Anti-IgEBP antibody revealed a 31-kD band on immunoblots (Figure 1, b panels).

Immunohistochemistry allowed us to specifically examine HLBP31 expression in the neoplastic cells of the specimens (Figure 1, c panels). HLBP31 expression in cancer cells was decreased in 67% of the cases compared to the normal epithelial cells. Thus, it seems that HLBP31 expression in ovarian cancer cells is downregulated compared to the normal tissue, and follows the same trend as in other neoplastic cell types, like colon [12] and breast cancer (Castronovo *et al.*, manuscript in preparation). No statistical correlation between HLBP31 expression and the clinicopathological data was found.

DISCUSSION

It is well known that most patients with epithelial ovarian cancer have metastatic implants throughout the peritoneal cavity at the time of diagnosis due to exfoliation of cells from the primary ovarian tumour. Relatively little is known, however, regarding the molecular events involved in ovarian cancer cell attachment to, and invasion of, the peritoneal surfaces. Currently, treatment of advanced ovarian cancer includes surgical debulking followed by platin-based multi-agent chemotherapy. Overall 5-year survival for patients with advanced stage disease is approximately 10–20%, with a median survival of 30 months [19]. In these patients, the strongest prognostic factor is the extent of residual disease following cytoreductive surgery. The favourable effect of optimal debulking on survival is thought to be due to the fact that the efficacy of chemotherapy is related to the number of cells present prior to initiation of treatment. On the other hand, it has been postulated that cancers that are amenable to optimal debulking are inherently less biologically aggressive. According to this theory, the poor outcome of suboptimally debulked cancers would be directly related to their higher invasiveness rather than to the presence of a greater number of cells prior to initiation of chemotherapy. The proponents of this latter theory believe that responsiveness to chemotherapy, rather than debulking, is the primary determinant of clinical outcome.

To clarify this controversy, studies are needed to elucidate the biomolecular events underlying ovarian tumour development, invasion and metastasis. In the current study, we examined the expression of two non-integrin laminin-binding proteins in

ovarian cancer. We used three different techniques to quantify expression of the two laminin binding proteins in the tumour samples, northern and western blotting, and immunohistochemistry. In some samples, tumour heterogeneity was the source of apparent discordance between the results of the three techniques used, particularly western immunoblot which analyses proteins homogenised from normal and cancer cells, as well as from the extracellular matrix. The best correlations between the results obtained by the three techniques were obtained with the most cellular tumours. Because of its cell type specificity, immunohistochemistry has been considered as the most reliable detection technique, allowing to discriminate, for instance, expression of laminin-binding proteins in cancer cells from various cell types such as endothelial cells and macrophages, expressing considerable amounts of 67LR and HLBP31, respectively.

We found that the expression of the 67LR and HLBP31 is inversely modulated in ovarian cancer, compared to a wedge resection of a normal ovary by northern blotting, and specifically to serosal epithelial cells by immunohistochemistry. These comparisons imply some technical difficulties such as, for northern blotting, contamination of the serosal cells by stromal and follicular cells in the normal wedge resection sample, and by infiltrating cells and endothelial cells in the tumour samples, and, for immunoperoxidase staining, wedge effect that gives artefactually high staining of the serosal cells. However, these data show the same result, and confirm those obtained with other tumour types, such as colon and breast carcinoma.

Moreover, the high expression of the 67LR in the cancer cells detected by immunohistochemistry was associated with suboptimal debulking and persistence of disease following completion of chemotherapy. These data suggest that increased 67LR expression may be related to the commitment of ovarian cancer cells towards the invasive phenotype. Similar to the findings of this study, 67LR expression has been found to be significantly increased in solid neoplasm of the breast, stomach and colon, compared to corresponding normal tissue [20], and this overexpression is directly correlated with metastatic potential. Increased 67LR expression in ovarian cancer could lead to more extensive invasion of the organs underlying the peritoneal surface, which would decrease the likelihood of achieving optimal debulking. The prognosis value of 67LR detection in ovary cancer remains to be determined by future large prospective studies.

Although HLBP31 expression in the cancer cells was not significantly correlated with clinical outcome, it was decreased compared to normal tissue in the vast majority of the patients in this study. These date provide further evidence that invasive cancer cells could display a specific pattern of laminin-binding proteins, e.g. increased 67LR and decreased HLBP31 expression. This inverse modulation is usually found in colon [12] and breast carcinoma (Castronovo *et al.*, manuscript in preparation).

In summary, these data suggest that the 67LR could be implicated in the invasiveness of ovarian cancers. Many other invasion and metastasis-related genes, such as other laminin-binding proteins, metalloproteinases and motility factors, remain to be investigated in order to better understand the specific biology of ovarian cancer. Hopefully, future studies will bring us close to the development of effective strategies for early diagnosis and treatment for this problematic disease.

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