The IgLON Family in Epithelial Ovarian Cancer: Expression Profiles and Clinicopathologic Correlates

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Abstract

Purpose: The IgLON family of cell adhesion molecules, comprising OPCML, HNT, LSAMP, and NEGR1, has recently been linked to cancer, through two of its members being proposed as tumor suppressors. We examined the expression profile of the family in human sporadic epithelial ovarian cancer and the normal ovary.

Experimental Design: We determined the expression level of each IgLON in a panel comprising 57 tumor and 11 normal ovarian samples by quantitative real-time reverse transcription-PCR. The results were statistically tested for associations with clinicopathologic variables.

Results: *OPCML*, *LSAMP* and *NEGR1* exhibited reduced expression in the tumor samples relative to the normal samples, whereas *HNT* expression was elevated. Statistically significant changes were specific to histologic type. The expression levels of individual IgLONs were correlated, the most significant finding being a positive correlation between *LSAMP* and *NEGR1*. *LSAMP* expression was also negatively correlated with overall survival and was found to be a negative predictor of outcome.

Conclusions: The expression of the IgLON family is altered in sporadic epithelial ovarian tumors in comparison to the normal ovary. In our small but representative cohort of patients, we have found significant correlations and associations in expression and clinicopathology that suggest a wider role of the family in ovarian cancer.

Ovarian cancer is the leading cause of gynecologic death in both the U.S. and the U.K., in fact, it ranks fourth as a cause of death from any cancer in women (Cancer Research UK statistics; ref. 1).[3] The incidence of the disease increases with age, peaking in around the seventh to eighth decade (2). Approximately 90% of ovarian cancers are epithelial ovarian cancers (EOC), with 5% to 10% being familial in origin (3, 4). Although, if diagnosed early, it has a very good prognosis, with a 92% 5-year survival rate, most cases (75%) are diagnosed at a late stage of disseminated disease when the prognosis is much less favorable, with only a 25% 5-year survival rate (5). Treatment of ovarian cancer is usually based on surgical cytoreduction and platinum-based chemotherapy (6). Although primarily a chemoresponsive disease, chemoresistance develops in most cases, resulting in the high aforementioned mortality rate.

The IgLONs are an immunoglobulin subfamily of glycosylphosphatidylinositol-anchored cell adhesion molecules comprising opioid binding protein/cell adhesion molecule–like (OPCML/OBCAM; ref. 7), limbic system–associated membrane protein (LSAMP/LAMP; ref. 8), neurotrimin (HNT; ref. 9), and neuronal growth regulator 1 (NEGR1/Kilon; ref. 10). Our knowledge of the functions of these proteins is limited and mainly derives from studies of chick and rat brain, the tissue where they are primarily expressed. Their patterns of expression in the brain are overlapping but also distinct, with *OPCML* being mainly expressed in the gray matter (11), *HNT* in the sensorimotor cortex and the cerebellum (9), *LSAMP* in cortical and subcortical neurons of the limbic system (8), and *NEGR1* in the cerebrum and brain stem (10). In these studies, it is suggested that the IgLONs could have an important role in cell adhesion and cell-cell recognition, mediated by both homo- and heterophilic interactions in *cis* and *trans* that are likely to be stabilized by clustering of the proteins on the cell surface (12). It has been recently suggested that IgLONs function mainly as heterodimers termed Diglons (13).

OPCML has been shown to exhibit functional characteristics of a tumor suppressor gene in an ovarian cancer cell line *in vitro* and also *in vivo* when xenografted into nude mice (14). Abrogated expression was found in 83% of sporadic EOC samples examined. The mechanism underlying *OPCML* silencing was shown to be CpG island hypermethylation. This first report of an IgLON involvement in cancer was followed by a further one, where the *LSAMP* promoter was shown to be

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[3] http://cancerresearchuk.org/aboutcancer/statistics/mortality.

methylated in 26% of a panel of sporadic clear cell renal cell carcinomas (15). Moreover, expression of *LSAMP* in clear cell renal cell carcinoma lines inhibited cell proliferation.

We have investigated the expression of the four members of the IgLON family in a panel of 11 normal human ovaries and 57 tumor samples from patients with EOC. Our primary objective has been to observe each gene separately, as well as to attempt to draw conclusions from the interrelationships of data that emerge. In this extensive study, we report significant associations between the expression levels of the IgLONs and various clinicopathologic variables.

Materials and Methods

Patient samples. Primary human ovarian tumor material and nonmalignant tissues were obtained from patients having undergone gynecologic surgery in the Lothian University Hospitals National Health Service Trust. Institutional ethical approval was granted for this work by the Lothian University National Health Service Trust, Medicine/Clinical Oncology Research Ethics Subcommittee. Tissue samples were excised and stored in liquid nitrogen. Nonmalignant tissue samples were derived from patients that underwent bilateral oophorectomies for suspected malignancy, but were found to have benign histologies; samples were collected from apparently normal contralateral ovaries.

In this study, a panel comprising 57 sporadic ovarian tumors and 11 normal ovaries was used. The features of the panel are shown in Table 1. Overall, our unselected cohort is very representative of the presentation of 1,400 women from the Scottish community over 20 years to a single center in a completely unselected fashion from primary care (16). Our present cohort is exactly as expected for stage distribution in a Scottish population; although it is skewed slightly towards a more adverse grade distribution. The overrepresentation of grade 3 tumors may account for a slightly shorter median survival of 2.25 years compared with 2.50 years.

Table 1. The panel of normal ovaries and EOC samples

Description	*n*
All samples	68
Normal	11
Tumors	57
Serous	29
Clear Cell	12
Mucinous	4
Endometrioid	8
Mixed	1
Unclassified	3
Median age at diagnosis (years)	64
FIGO stage	
I	8
II	4
III	37
IV	8
Grade	
1	2
2	11
3	41
Median survival (y)	2.25

Abbreviation: FIGO, Federation Internationale des Gynaecologistes et Obstetristes.

RNA preparation. Total RNA was prepared from the ovarian tissues and DNase I-treated using the Absolutely RNA reverse transcription-PCR Miniprep Kit (Stratagene, La Jolla, CA), as per the manufacturer's instructions.

Quantitative real-time reverse transcription-PCR. Quantitative reverse transcription-PCR was done using Rotorgene 2000 and 3000 real-time thermal cyclers (Corbett Research, Australia). Fifteen-microliter reactions were set up using 40 ng of DNase I-treated total RNA, 0.3 μmol/L each of forward and reverse primer and the Quantitect SYBR Green one-step reverse transcription-PCR kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's guidelines. Primers were designed using Primer 3 v0.2. Primer sequences are available upon request. All reactions were carried out in triplicate for the standard curve samples and in quadruplicate for the experimental samples and the negative control (no template). Fluorescence was detected using the FAM channel (source 470 mm, detector 510 nm). Analysis and quantification was done using Rotorgene v4.6 and v5 software. The relative quantification method was employed by extrapolation from a standard curve and calculation of expression levels as ratios to β-actin.

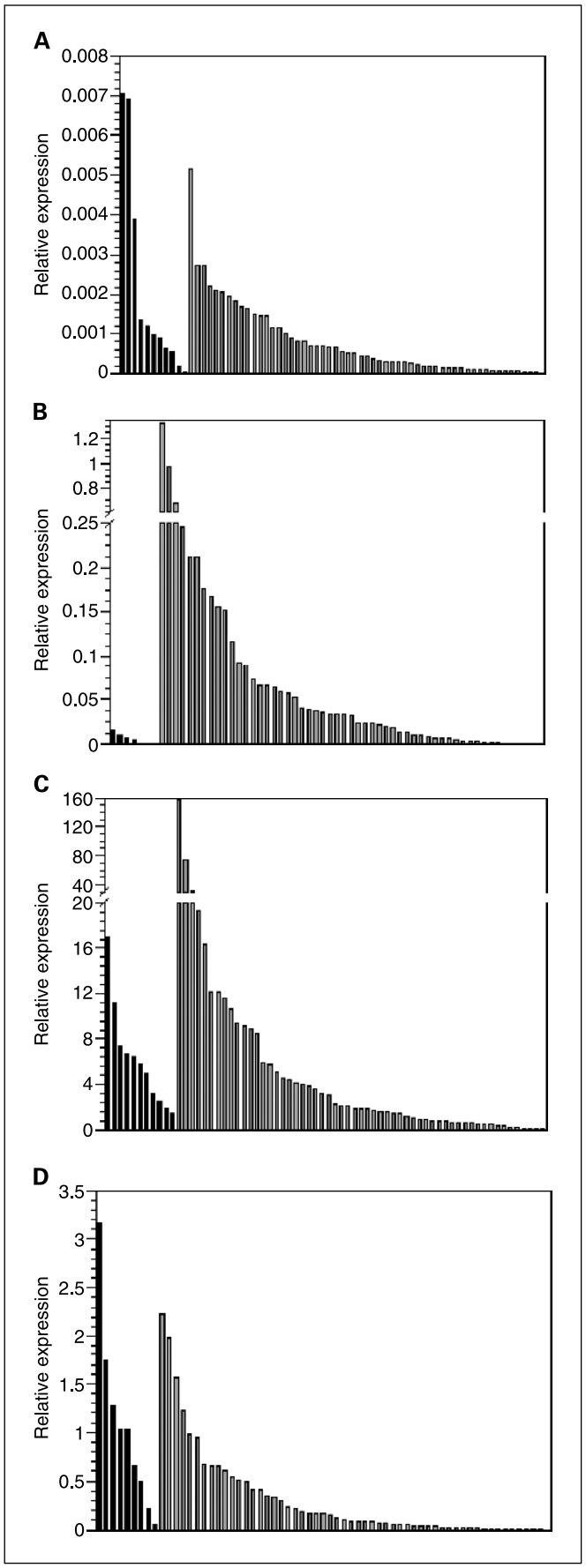
Cycling conditions were set as follows: reverse transcription at 50°C for 30 minutes, followed by a 15-minute polymerase activation at 95°C, and 40 cycles of denaturation at 94°C for 15 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 30 seconds; after a final extension at 72°C for 1 minute, product melting was set across a 72°C to 99°C temperature ramp, with 5-second steps of 1°C. Data were corrected for inter-run variation using a "normalizer" sample that was included in all runs.

Statistical analysis. All statistical analyses were conducted in SPSS v12 (SPSS Inc., Chicago, IL). Nonparametric comparisons between unmatched samples were made using the Mann-Whitney (U) test for two samples or the Kruskal-Wallis (χ^2) test for more than two samples. Nonparametric correlations were tested using the Spearman rank (r_s) test. Regression models were made using linear regression analysis. Survival curves were produced using the Kaplan-Meier method and tested with the log-rank test. Survival models were estimated using Cox proportional hazards regression analysis.

Results

IgLON expression levels differ between normal ovaries and EOC. In order to evaluate the RNA expression levels of the four IgLON family members in the panel that comprised 11 normal human ovaries and 57 ovarian tumor samples, a real-time quantitative reverse transcription-PCR approach was followed. The results are shown in Fig. 1. For *OPCML*, *LSAMP*, and *NEGR1*, the majority of tumors exhibit reduced expression as compared with the normal ovary samples. In contrast, tumor samples have increased *HNT* expression in relation to nonmalignant ones. The distribution of values in all four genes is not normal, hence, nonparametric approaches were chosen for statistical analysis.

Associations of IgLON expression with EOC histologic types. The expression data prompted us to take a closer look at each gene separately in the different histologic types and make comparisons to expression in the normal ovaries. In all four IgLONs, there are statistically significant differences between normal ovaries and tumor subgroups (Fig. 2). More specifically, *OPCML* expression is reduced in clear cell carcinomas ($U = 19$, $P = 0.039$); *LSAMP* expression is reduced in endometrioid tumors ($U = 28$, $P = 0.012$); and *NEGR1* expression is reduced in serous ($U = 59$, $P = 0.014$), clear cell ($U = 9$, $P = 0.009$), and endometrioid ($U = 7$, $P = 0.001$) tumors. In contrast, *HNT* expression is elevated in serous carcinomas as compared with normal ovaries ($U = 113$, $P = 0.008$).



Correlations in expression between IgLONs. As all the four genes that constitute the focus of this study are structurally and functionally related, a question that arises is whether their individual expression levels are interlinked. The following statistically significant correlations of expression between IgLONs were found: *LSAMP* and *NEGR1* (r_s = 0.553, P < 0.001); *OPCML* and *NEGR1* (r_s = 0.389, P = 0.001); *OPCML* and *LSAMP* (r_s = 0.356, P = 0.003). In all these cases, the correlations are positive.

Associations between IgLON expression and clinicopathologic variables. In an attempt to establish whether expression of IgLON family members could be a function of age as a continuous variable, the expression of each gene was tested against age. However, evidence to support associations was not found, either by correlation testing or by regression analysis.

Neither was stage found to differentiate IgLON expression; nonetheless, there is a statistically significant difference in *LSAMP* expression among tumors of different grades (χ^2 = 6.73, P = 0.035). In fact, as can be seen in Fig. 3, this difference is even more significant when comparing grade 3 tumors with grades 1 and 2 tumors: undifferentiated carcinomas have lower levels of *LSAMP* than moderately or well-differentiated ones (U = 148, P = 0.017). In comparison to the large study of Clark et al. (16), grade 1 tumors are underrepresented in our relatively small sample cohort. The low number of grade 1 cases in our cohort has prevented us from comparing expression in these samples against grades 2 or 3, which is a more customary comparison.

IgLON expression and survival. The impact of IgLON expression levels on the survival of the patients was tested at two levels. Firstly, correlation between expression of each gene and overall survival was investigated. This highlighted *LSAMP* as having a negative correlation (r_s = −0.296, P = 0.025).

Patients were then divided into high and low expressers for each gene, using the median values of the normal samples as cutoff points. Survival curves were plotted and high expressers were compared with low expressers (data not shown). However, we were not able to show statistical significance for any of the four genes on this division.

To address whether the expression level of an *IgLON* gene can predict clinical outcome, we tested the expression variables, as well as the clinicopathologic ones, for prognostic value by Cox regression analysis. As can be seen in Table 2, in univariate analyses, log-transformed *LSAMP* expression, as well as stage, were found to be negative predictors of overall survival. Both variables were still found to be significant predictors of overall survival when tested together by multivariate analysis.

Discussion

When the link between a member of the IgLON family, *OPCML*, and EOC was put forward (14), questions were raised regarding the role of this gene in ovarian physiology and ovarian cancer pathology. The realization that the family as a whole might be associated with ovarian or other types of cancer also

Fig. 1. IgLON expression in EOC and normal ovaries. The relative expression levels of the four IgLON genes, *OPCML* (*A*), *HNT* (*B*), *LSAMP* (*C*), and *NEGR1* (*D*) were investigated in a panel of EOC samples and normal human ovaries using a quantitative reverse transcription-PCR approach. *Black,* normal samples; *gray,* tumor samples.

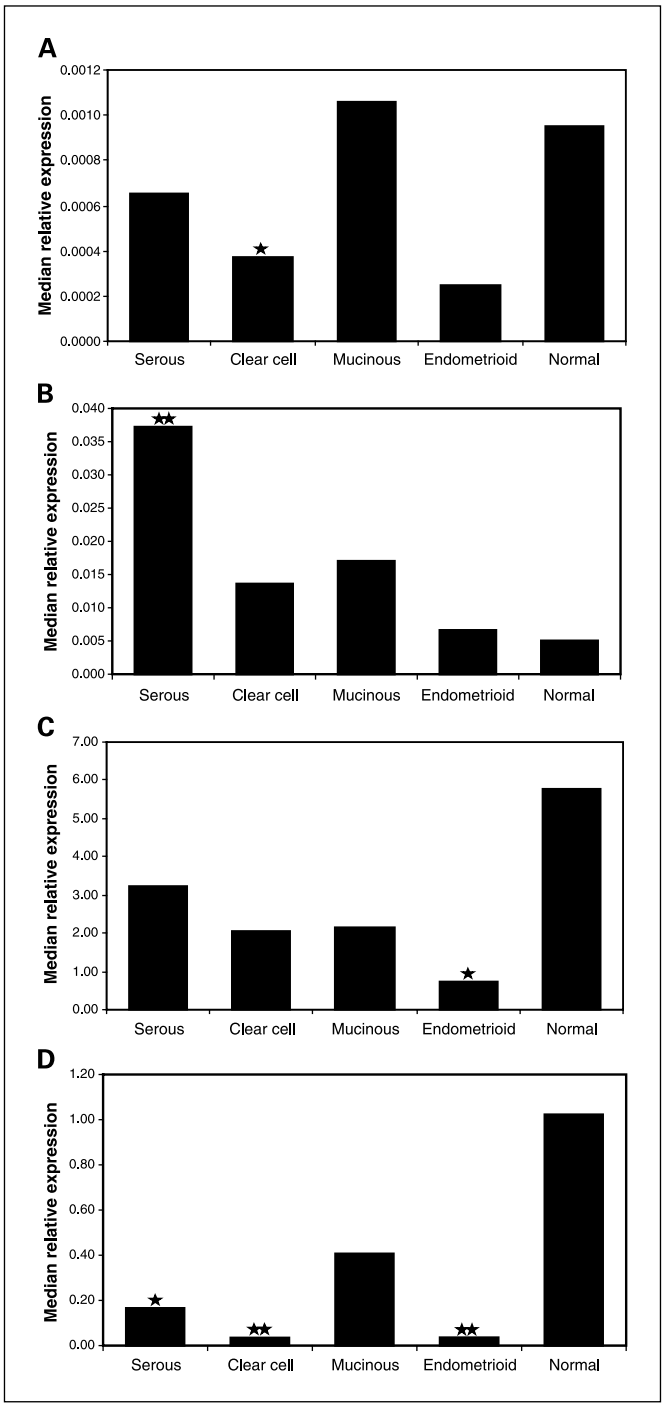


Fig. 2. IgLON expression according to histologic type. The EOC sample panel was categorized by histologic type, i.e., into serous, clear cell, mucinous, and endometrioid tumors. The median relative expression levels of *OPCML* (*A*), *HNT* (*B*), *LSAMP* (*C*), and *NEGR1* (*D*) are shown for these subgroups as compared with normal ovary samples. *, $P < 0.05$; **, $P < 0.01$.

emerged. This probability was subsequently strengthened by the finding of another IgLON, *LSAMP*, being associated with clear cell renal cell carcinomas as a putative tumor-suppressor gene (15). Could there be a case for the IgLON family being of importance in cancer?

To answer this question, one needs a powerful global approach that examines the family as a whole. This study has been based on a well-characterized panel of ovarian tumors and normal ovaries, which has come under scrutiny for the expression of each IgLON separately, but also for expression patterns and associations deduced in a statistically sound manner.

Here we present evidence that, at least with respect to EOC, it is not just *OPCML*, but the other members as well, which may be of relevance. We have shown that the expression of all the IgLONs differs significantly between normal ovaries and EOC at the RNA level. The expression levels of *OPCML*, *LSAMP*, and *NEGR1* are all reduced in EOC, whereas that of *HNT* is increased. Confirmation of these trends at the protein level will merit investigating whether, apart from OPCML, there could be a case for LSAMP and NEGR1 acting as tumor suppressors in sporadic EOC. It has been shown that the cause of the reduction in expression in the case of *OPCML* in EOC is mainly epigenetic (14), and there is evidence that the same applies for *LSAMP* in clear cell renal cell carcinomas (15). Thus, it seems likely that the reduction in *LSAMP* and *NEGR1* expression in EOC may be attributed to epigenetic mechanisms. In addition, we have found a significant positive correlation, the strongest in this study, between the expression of *LSAMP* and that of *NEGR1*. Consequently, it could be hypothesized that both genes need not be genetically or epigenetically silenced in EOC; the reduction in expression of the one could result in a reduction in expression of the other, potentially through a mechanism of coordinated transcriptional regulation. Although epigenetic modification is an age-related variable which affects gene expression, we have not found an association between altered IgLON expression and age in this study.

We have shown that in EOC, the importance of each IgLON might be specific to histologic type. *OPCML* expression is significantly reduced in clear cell carcinomas, *LSAMP* in endometrioid, and *NEGR1*, exhibiting the most striking reduction in expression, in serous, clear cell, and endometrioid tumors. In contrast, *HNT* is significantly elevated in serous carcinomas. With regard to mucinous carcinomas, it should be noted that the lack of statistical significance could be accounted for by the under-representation of this type of tumor in the panel.

Malignant transformation in ovarian cancer is characterized by a differentiation process, in contrast with most other tumor

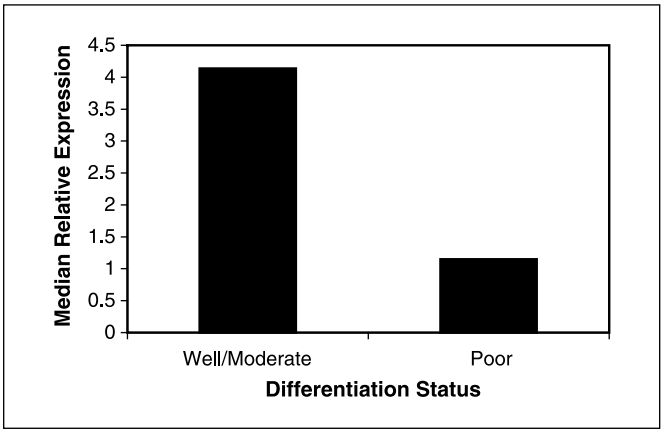


Fig. 3. *LSAMP* expression according to differentiation status. The EOC sample panel was divided into tumors of poor differentiation, i.e., of grade 3 histology, and tumors that are moderately or well-differentiated, i.e., of grades 1 or 2 histology. *Columns,* median relative expression of *LSAMP* in each of these tumor subgroups.

Table 2. Survival models

	Variable	Odds ratio	95% Confidence interval		Significance
			Lower	Upper	
Univariate	*OPCML**	0.84	0.50	1.41	0.506
	*HNT**	1.15	0.84	1.57	0.376
	*LSAMP**	1.63	1.10	2.41	0.015
	*NEGR1**	1.30	0.86	1.96	0.221
	stage†	8.86	2.10	37.32	0.003
	grade‡	22.06	0.04	13126	0.343
	age at diagnosis	1.03	1.00	1.06	0.020
	age last recorded	1.01	0.98	1.04	0.400
Multivariate	*LSAMP**	1.85	1.14	3.02	0.013
	stage†	10.21	2.40	43.77	0.002

NOTE: The expression and clinical variables were tested by univariate regression analysis. The two variables that were found to be statistically significant were further tested by multivariate analysis.
*Log-transformed.
†Early (i.e., stages I and II) versus late (i.e., stages III and IV).
‡High (i.e., grade 3) versus rest (i.e., grades 1 and 2).

types, where the converse occurs (17). Normal ovarian epithelium is not fully differentiated, retaining certain mesenchymal characteristics; upon transformation, differentiation drives the epithelium toward certain histologic morphologies, depending on the type of the tumor. Because IgLONs have partially distinct patterns of histologic subtype expression, they could be part of a different pathway that guides this differentiation process.

Interestingly, *LSAMP* expression is significantly higher in moderately and well-differentiated tumors. This indicates that the level of *LSAMP* expression is likely to be a biological function of differentiation. It could be speculated that the significance of reduction of *LSAMP* expression in the process of malignant transformation of the ovarian surface epithelium could lie in the cells acquiring a less differentiated phenotype, which is considered to be of higher malignant potential.

Furthermore, *LSAMP* was found to be a negative prognostic factor in both univariate and multivariate models of EOC in addition to being negatively correlated with overall survival. In both cases, the impact of *LSAMP* on survival is not high. This link may seem paradoxical, given that its overall tumor levels are significantly lower than in normal ovaries. However, the role of residual *LSAMP* in the tumors might be antithetical to its original functions in the normal ovary. In terms of the multifactorial output described as survival, ovarian tumors could be disadvantaged by not expressing *LSAMP* and losing its novel functions. The true reason behind this paradoxical finding remains as yet undetermined.

The analysis of the present study, albeit deriving from a relatively small cohort and requiring confirmation in a larger study, has pointed to a very interesting and novel association of the IgLON family with ovarian cancer. In order to integrate *OPCML* and its relatives into models of ovarian carcinogenesis, we need to establish their functions in the normal ovary, their localization within the ovarian environment and the pathway that links them to ovarian cancer. In addition, the functional importance of homo- and heterodimers needs to be addressed. Finally, profiling the expression of the IgLONs in other types of neoplasia might reveal that this hitherto mysterious family has a wider importance in cancer.

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