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DLG1 polarity protein expression associates with the disease progress of low-grade cervical intraepithelial lesions

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ABSTRACT

Human Discs large tumour suppressor (DLG1) participates in regulating cell polarity and proliferation, suggesting an important connection between epithelial organization and cellular growth control. However, it was demonstrated that DLG1 could acquire oncogenic attributes in some specific contexts. In this work, we evaluated the expression of DLG1 and its contribution to the progress of cervical lesions in order to investigate a potential role of this polarity protein in human oncogenic processes.

We analyzed cervical biopsies from women with low-grade squamous intraepithelial lesion (LSIL) diagnosis ($n = 30$), for DLG1 expression by immunohistochemistry. These results were correlated with the clinical monitoring of the patients during a 24-month follow-up period. Our data indicate that while all LSIL patients with a DLG1 staining pattern similar to normal tissues are significantly more likely to regress ($n = 23$, *Pattern I*), all LSIL biopsy specimens showing a diffuse and intense DLG1 staining likely progress to high-grade lesions ($n = 4$, *Pattern II*). Finally, all persistent LSIL analyzed showed an undetermined DLG1 staining, with a diffuse distribution without a strong intensity ($n = 3$, *Pattern III*). We found a significant association between the expression pattern of DLG1 and the evolution of the lesion ($p < 0.00001$).

This work contributes to the knowledge of DLG1 biological functions, suggesting that its expression may have an important role in the progression of early dysplastic cervical lesions, giving prognostic information.

1. Introduction

DLG1, the human homologue of *Drosophila* discs large tumour suppressor, is a multi-PDZ (PSD, DLG1, ZO-1) domain-containing protein that belongs to the family of molecular scaffolding proteins known as membrane associated guanylate kinases (MAGUKs). DLG1 is a component of the Scribble polarity complex which participates in regulating cell polarity and proliferation, suggesting a key connection between epithelial organization and cellular growth control (Assemat et al., 2008; Roberts et al., 2012). An abnormal expression of DLG1 has been reported in several cancer types, with a loss of DLG1 expression associated with complete lack of cell polarity and tissue architecture during the latest stages of malignant progression, thereby defining DLG1 as a potential tumour suppressor (Facciuto et al., 2012; Sugihara et al., 2016).

In addition, the most compelling evidence of DLG1 oncosuppressor activity in humans was its identification as a target of several viral oncoproteins: Human papillomavirus (HPV) E6, Human T cell leukemia virus type 1 Tax, and Adenovirus type 9 E4-ORF1 (James and Roberts, 2016). However, recent studies have demonstrated that DLG1 can acquire oncogenic attributes in some specific contexts; highlighting the importance of DLG1 deregulation in human carcinogenesis (Roberts et al., 2012).

On the other hand, cervical cancer is one of the most common cancers affecting women worldwide, and is responsible for an estimated 530,000 new cases with a mortality approaching 50% each year (Parkin, 2006; Lowndes, 2006; de Freitas et al., 2014). Furthermore, over 85% of these episodes occur in developing countries due to inadequate prevention and control programs (Frazer, 2004; WHO/ICO, 2010). It has been confirmed that high-risk HPV persistent infection is the main pathogenic factor for the development of cervical cancer (zur Hausen, 2002). The main clinical stages in cervical carcinogenesis include HPV

Abbreviations: DLG1, human Discs large; HPV, Human papillomavirus; MAGUKs, membrane-associated guanylate kinase homologues; PDZ, PSD-95/DLG/ZO-1 domains; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CIN, cervical intraepithelial neoplasia; IHC, immunohistochemistry; PAP, Papanicolaou; MAGI-2, WW and PDZ domain containing protein 2.

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infection, persistence, progression to precancerous lesions and invasion. Thus, invasive cervical cancer is preceded by a progressive spectrum of cervical intraepithelial neoplasia (CIN), also termed squamous intraepithelial lesion (SIL) (Darragh et al., 2012). Therefore, it is one of the few preventable human cancers and its prevention is based on vaccination and early diagnosis of the precancerous lesions (Arbyn et al., 2011; Bratic et al., 2016). SIL is a heterogeneous group graded as either low-grade (LSIL) or high-grade squamous intraepithelial lesions (HSIL), depending on the severity of their histological epithelial abnormalities (Doorbar et al., 2012). However, not all HPV infections give rise to cervical dysplasia and promote its progression to carcinoma. On the contrary, it is well known that most LSIL are transient and regress spontaneously to normal epithelium in 1–2 years (Schiffman et al., 2007), and only a small fraction of these lesions are expected to progress to HSIL and, eventually, to invasive cervical cancer (Quint et al., 2013). Thus, the identification of biomarkers that can predict the chance of progression or regression of these early lesions, could represent a major contribution to the clinical management of patients at high risk to progress to cancer.

A more complete understanding of the factors involved in progression to cancer may lead to new paradigms for the efficient monitoring of cervical diseases. In regard to this, the proper establishment and maintenance of cell polarity is essential for normal epithelium physiology and loss of this polarity and tissue organization is a hallmark of cancer development (Martin-Belmonte and Perez-Moreno, 2011). In this work, we focused on the analysis of the expression of DLG1 polarity protein and its contribution to the progress of cervical lesions, in order to elucidate a potential role of the polarity proteins in cervical oncogenic processes.

In previous studies we and other groups evaluated the differential expression of DLG1 during the progression to malignancy in cervical lesions by immunohistochemistry (IHC) (Watson et al., 2002; Cavatorta et al., 2004; Lin et al., 2004). Interestingly, while a marked reduction of DLG1 levels in invasive carcinomas was described, a high overexpression and changes in DLG1 distribution at earlier stages of the cervical carcinogenic process were observed (Cavatorta et al., 2004). Specifically, LSIL samples showed a marked variability in their DLG1 staining with two different archetypes according to the DLG1 expression patterns. One of such archetypes comprised samples where both DLG1 level and distribution, within the different strata of the squamous epithelium, were quite similar to those observed in normal epithelium. The other subset of LSIL biopsies presented an over expression of DLG1 throughout the full thickness of the epithelium, with a preferentially cytoplasmic localization similar to the pattern obtained for HSIL specimens (Cavatorta et al., 2004). Therefore, given the characteristics of DLG1 and its likely involvement in both tumour suppression and oncogenic processes, it is interesting to investigate whether these different patterns of expression may provide information about the potential progression and/or regression of LSIL. For this, we analyzed the immunohistochemical detection of DLG1 polarity protein and its association with the course of SIL. We evaluated uterine cervical biopsies from women with LSIL diagnoses, and the DLG1 immunohistochemistry results were correlated with the clinical monitoring during the follow-up period.

2. Materials and methods

2.1. Patient samples

All experiments were undertaken with the written informed consent of each subject, and we received study approval from the ethics committee of the Hospital Provincial del Centenario, Rosario, Argentina. Fifty-two patients with an established diagnosis of LSIL via a colposcopy-directed biopsy and without previous history of cervical lesion were randomly selected from the Lower Genital Tract Diseases and Gynecologic Oncology Section, Service of Gynecology, Hospital Provincial del Centenario, Rosario, Argentina.

All tissue samples were fixed in 10% buffered formalin, routinely processed and embedded in paraffin. Biopsies were collected from the bank of paraffin-embedded tissue sections of the Cátedra de Anatomía y Fisiología Patológicas and the histological diagnoses were established using morphologic criteria based on H&E stained sections.

Patients were grouped according to their outcome during the 24-month follow-up and clinical information was obtained from the patients' medical record. The final outcome of LSIL was classified as regression, persistence and progression according to the following criteria: *i*) Regression: concomitant negative cervical smear (Papanicolaou test, PAP test) and/or negative biopsy observed at any time during the follow-up and confirmed at 24-month follow-up; *ii*) Persistence: LSIL was diagnosed on the basis of either 1 or more cytologies and biopsies of LSIL diagnosis during the follow-up; *iii*) Progression: appearance of a histologically confirmed high-grade lesion at any time during the follow-up.

Gynecology oncologists followed up the LSIL patients by cytology and colposcopy exams with check-up every 6 months for at least 2 years. When a PAP test presented a higher-grade lesion or suspicious colposcopy images were observed, the gynecologist confirmed this with a new biopsy. If the lesion progressed during the follow-up, the patient received proper treatment.

2.2. Immunohistochemistry

The procedures were performed as previously described (Cavatorta et al., 2004; Gardiol et al., 2006). Briefly, sections (5 μm) were cut from paraffin-embedded tissue blocks and mounted on pretreated glass. After deparaffinizing with xylene, the slides were rehydrated using a graded alcohol series. Endogenous peroxidase activity was blocked by immersing sections in 3% hydrogen peroxide in methanol for 20 min. Sections were placed in 0.1 M citric acid (pH 6) and heated for 12 min on high power using a conventional microwave oven, to facilitate antigen retrieval. After blocking nonspecific binding by addition of normal horse serum (Vectastain ABC Kit; Vector, Burlingame, CA, USA) for 40 min, sections were incubated overnight with the primary anti-DLG1 mouse monoclonal antibody diluted at 1:20 (clone 2D11, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. For detection, samples were treated successively with biotinylated secondary antibody for 30 min and with avidin–biotin peroxidase complex for a further 30 min at room temperature (DAKO, Denmark). The reaction was developed using a diaminobenzidine chromogenic substrate kit for peroxidase (Vector), and sections were counterstained with haematoxylin. Negative controls were processed as described, except that primary antibody was omitted. Specimens were visualized and photographed using a Zeiss Primo Star light microscope (Zeiss, Germany).

2.3. Evaluation of immunohistochemical staining results

The DLG1 staining was graded as either “no overexpression” (similar to normal samples, called *Pattern I*), “overexpression” (moderate to strong cytoplasmic staining intensity with diffuse distribution, called *Pattern II*), or “undetermined” (diffuse distribution without a strong intensity, called *Pattern III*).

2.4. Statistical analysis

Statistical analysis of categorical variables was performed by Fisher's test. A p-value of 0.05 or less was considered to indicate statistical significance.

3. Results

This study began with 52 patients of reproductive age (mean, 24 years) with a cervical biopsy specimen that confirmed the LSIL diagnosis. Twenty-two cases were discarded because some of them did not

have residual lesion upon subsequent paraffin block cuts and others did not complete the follow-up process. Therefore, 30 patients with a LSIL biopsy were finally included in this analysis. Out of these patients, 23 (76.7%) regressed, 3 (10%) persisted, and 4 (13.3%) progressed to HSIL, according to their outcome within the 2-years of follow-up. This concurs with current data in the literature that estimates that only 10% to 15% of LSIL evolve to high-grade lesions (Schiffman et al., 2007; Wright et al., 2007).

DLG1 immunostaining results showed that from the 23 LSIL cases that regressed to a normalization of the epithelium during their follow-up routine, the DLG1 expression in the initial biopsy presented a pattern quite similar to that observed for normal tissue in our previous report (Cavatorta et al., 2004). In normal samples DLG1 was expressed in the basal, parabasal and intermediate layers of the stratified cervical epithelium but it was absent in the uppermost-differentiated cellular strata. DLG1 was localized preferentially in the cytoplasm of basal cells, but in the parabasal and intermediate areas it was also present at regions of cell-cell contact. Nuclear localization of DLG1 was additionally observed (Fig. 1a). These results indicate that LSIL with a DLG1 staining pattern similar to that of normal tissue (*Pattern I*) are significantly more likely to regress. However, in these cases the basal cells showed a marked decrease of DLG1 staining when compared with normal samples. Fig. 1b and c show the DLG1 staining both in the initial and final biopsies, corresponding to a representative LSIL patient (Fig. 1b) who regressed to a normal cervical epithelium (Fig. 1c). This final sample, consistent with a normalization of the epithelium, exhibited the typical cell border DLG localization in suprabasal layers of normal epithelium (Fig. 1c).

On the other hand, the four patients (4/30, 13.3%) that experimented a progression to a high-grade lesion, expressed a diffuse and very intense DLG1 staining throughout the full thickness of the epithelial strata, with a predominant cytoplasmic distribution and loss from cell contacts (*Pattern II*). This result indicates an up-regulation of DLG1 levels together with a sub-cellular relocalization, archetype that corresponds to that observed for HSIL specimens in our previous study (Cavatorta et al., 2004) (Fig. 2a). Fig. 2b and c show the DLG1 staining both in the initial and in the final biopsy respectively, corresponding to a representative LSIL condition (Fig. 2b) that progressed to HSIL (Fig. 2c) during the follow-up. In this case both specimens presented a very strong DLG1 immunostaining throughout the epithelium.

Finally, when we analyzed the 3 LSIL persistent specimens, the pattern of the DLG1 staining was diffuse comparing with the normal tissue but they did not show a clear overexpression like those progressing to HSIL (*Pattern III*, undetermined) (Fig. 3).

It is important to notice that this study examined a low number of samples. However, the data from this pilot investigation clearly indicate that while all LSIL patients with a DLG1 staining pattern similar to normal tissues rarely progress, all LSIL biopsy specimens showing a diffuse and very intense DLG1 staining are significantly more likely to progress to high-grade lesions. In fact, we found a significant association between the expression of DLG1 and evolution of the lesion ($p < 0.00001$) (Table 1).

4. Discussion

Malignant transformation in many carcinomas is associated with failure in the establishment and maintenance of epithelial cell polarity. These features are seen in early dysplastic cervical lesions and during their progression to cancer (Watson et al., 2002; Cavatorta et al., 2004; Lin et al., 2004). In the current study, we focused on the involvement of DLG1 polarity protein in cervical cancer development analyzing biopsies from women with diagnoses of LSIL by IHC. The results have been related to the diagnosis and monitoring of patients during the 24-month follow-up. Remarkably, our findings showed that all LSIL biopsy specimens from patients who progressed to HSIL presented an overexpression of DLG1 throughout the full thickness of the epithelium, with a preferentially cytoplasmic localization. Accordingly, all LSIL biopsy specimens that showed a DLG1 expression pattern similar to that observed in normal tissue regressed spontaneously to normal epithelium.

Some possible explanations for these observations are related to the fact that the mechanisms that control DLG1 expression at transcriptional (transcription factors) or post-transcriptional (splicing, phosphorylation and ubiquitinylation) levels are de-regulated during transformation by still unknown pathways (Facciuto et al., 2012). It is also possible that in neoplastic cells, the normal protein interactions that localize DLG1 at cell borders were disrupted, and novel unidentified binding partners of DLG1 could then promote its redistribution to the cytoplasm resulting in a change of function. In fact, the accumulation of DLG1 in the cytoplasm may have an oncogenic significance, since it was shown that specific sub-cellular pools of DLG1 could gain oncogenic functions in the presence of viral oncoproteins (Frese et al., 2006; Krishna Subbaiah et al., 2012). In this sense, and in agreement with the present study, we have recently reported that high risk HPV-18 E6 and E7 oncoproteins induce a clear increase in DLG1 abundance and a

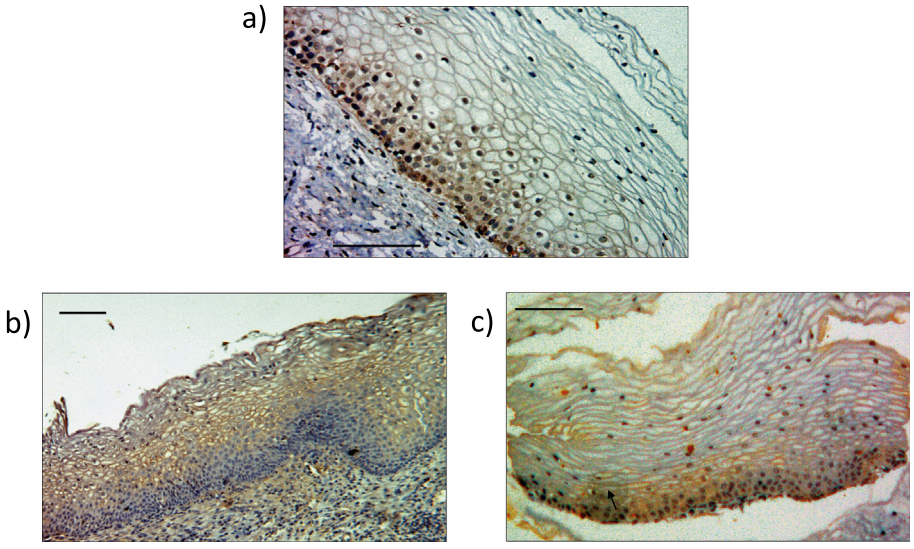

Fig. 1. Detection of *Pattern I* DLG1 staining in the squamous epithelium of the cervix. a) Expression of DLG1 in normal cervical epithelium. b) DLG1 staining of an area of LSIL in a patient who regressed to a negative result of the PAP test and negative biopsy. c) DLG1 immunostaining in the final biopsy showing regression to normal epithelium. Scale bars = 100 μm.

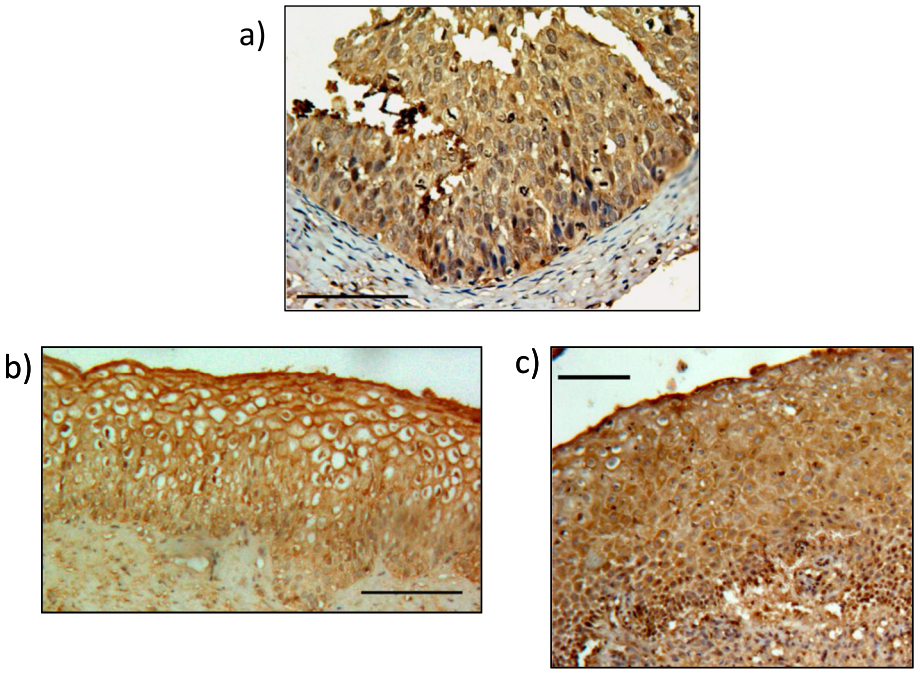

Fig. 2. Detection of *Pattern II* DLG1 staining in the squamous epithelium of the cervix. a) Expression of DLG1 in HSIL. b) DLG1 staining of an area of LSIL in a patient who progressed to HSIL. c) DLG1 immunostaining in the final biopsy showing progression to HSIL. Scale bars = 100 μm.

striking cellular redistribution from the cell contacts to the cytoplasm using organotypic models that in vitro mimic the epithelial tissue (Valdano et al., 2016). Thus, even though DLG1 has been recognized as a potential oncosuppressor, the mislocalization of DLG1 found in premalignant cervical lesions could have acquired oncogenic traits, contributing to the early stages of cancer development. This concept can be extrapolated to non-viral-associated epithelial tumours, where DLG1 overexpression and mislocalization were also reported (Facciuto et al., 2012).

This study is in line with previous reports about the value of p16INK4a (p16) expression as a prognostic marker for LSIL lesions. The increased level in p16 protein, a key oncosuppressor regulating cell proliferation, has been identified as a diagnostic indicator for transforming HPV infections. Moreover, a recent report has shown that another MAGUK protein that maintains the architecture of cell junctions, MAGI-2 (WW and PDZ domain containing protein 2), is elevated in prostate cancer, underlining the possible role of these PDZ proteins in oncogenic processes (Goldstein et al., 2016).

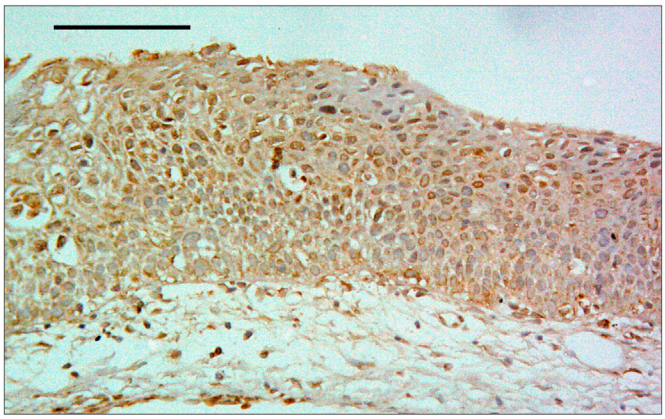

Fig. 3. Detection of *Pattern III* DLG1 staining in the squamous epithelium of the cervix. DLG1 immunostaining of an area of LSIL in a patient who presented a persistent LSIL diagnosis during the follow-up period. Scale bars = 100 μm.

As mentioned before, cervical cancer is associated with high-risk HPV infections and there are some reports showing that different HPV types differentially target DLG1 protein (Thomas et al., 2005). In this regard, there are some possible limitations in this study since no HPV typing data were available. Therefore, the potential correlation among DLG1 staining, specific HPV type and progression of LSIL could not be determined. However, previous studies using a low number of samples (Cavatorta et al., 2004; Lin et al., 2004) showed that DLG1 staining in cervical lesions did not vary in relation to the associated HPV types. This issue suggests that in cervical samples HPV type may not influence the level and cell/tissue distribution of DLG1.

LSIL are lesions of uncertain behavior, and no histological criteria allow a differentiation between cases that progress or return, though complementary assays to predict the future may contribute to a prophylactic intervention in patients at high risk, avoiding unnecessary treatment. Despite the importance of such tools for clinical practice, the study and implementation of prognostic markers as part of the gynecological triage are scarce. In this regard and even though the number of samples included in this study is limited, the results obtained suggest that DLG1 staining would be a promising candidate. While previous published investigations described DLG1 expression in cervical biopsies (Watson et al., 2002; Cavatorta et al., 2004; Lin et al., 2004), this is the first report that associates the staining of DLG1 at the moment of LSIL diagnosis with the eventual progression or regression of the lesion during the 24 months clinical follow-up. Nevertheless, further studies with larger cohorts are encouraged to confirm our present findings and to identify it potential use as a prognostic biomarker.

Table 1
DLG1 Immunostaining vs Follow-up Status. After applying Fisher's exact test it is concluded that there is a significant association ($p < 0.00001$) between DLG1 expression and the evolution of the lesion.

	DLG1 immunostaining		
Follow-up status	Pattern I	Pattern II	Pattern III
Regression	23	0	0
Persistence	0	0	3
Progression	0	4	0
Number of samples	23	4	3

In conclusion, this work contributes to understand the involvement of DLG1 polarity protein in human cancer progression, showing that DLG1 expression patterns may associate with disease progress in cervical lesions.

Conflict of interest

The authors declare no conflict of interest.

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