Role of Retinoblastoma Protein Family (Rb/P105 And Rb2/P130) Expression in the Hystopathological Classification of Borderline Ovarian Tumors

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Abstract

Background

Borderline ovarian tumors (BOT) are uncommon but not rare epithelial ovarian neoplasms, intermediate between benign and malignant categories. Emerging knowledge supports the notion that subtypes of borderline ovarian tumors comprise distinct biologic, pathogenetic, and molecular entities, precluding a single unifying concept for BOT. Therefore, the identification of valuable markers for the diagnosis and classification of these tumors is in need. Among the molecular candidates, the Retinoblastoma (Rb) family members Rb/p105 and Rb2/p130 seem to play a pivotal role in ovarian cancer. In particular, Rb/p105, when in the unphosphorylated form, acts as a growth suppressor and plays a pivotal role in the negative control of the cell cycle and in tumor progression; whereas, the phosphorylated form (p-pRB) activates genic transcription and cellular proliferation. While Rb/p105 is ubiquitously confined to the nuclei of cycling and quiescent cells, Rb2/p130 activity is also regulated by intracellular localization. According to this premise, Rb family members could represent a novel marker in diagnosis and classification risk for patients with borderline ovarian tumors (BOT).

Aims

In this study, we evaluated the immunohistochemical expression and subcellular localization of proteins of the retinoblastoma (Rb) gene family: Rb/p105 and Rb2/p130 in 65 ovarian borderline tumors (26 serous, 19 sero-mucinous and 20 mucinous subtypes).

Results

Statistically significant differences were found in nuclear and cytoplasmic expression of Rb/p105 and Rb2/p130 according to different examined histotypes. In detail, the nuclear expression of Rb/p105 and Rb2/p130 was more frequently detected in serous (84.6%) than sero-mucinous (42.1%) and mucinous (50%) types. Conversely, the cytoplasmic expression of Rb2/p130 was not detected in serous tumors and frequently observed in mucinous subtypes (80%).

Conclusions

Our findings suggest that Rb proteins do not play a key role in the tumor progression of serous borderline tumors since they are always located in the nucleus and no cases showed a cytoplasmic localization. By contrast, the observed higher cytoplasmic expression of Rb2/p130 in mucinous BOTs (intestinal) types, is suggestive of Rb proteins involvement in the cancerogenesis pathway of mucinous ovarian tumors. Our results also suggest that mucinous BOTs of intestinal type, exhibiting low nuclear and high cytoplasmic levels of Rb2/p130 might potentially be considered a high risk category of malignant evolution. Further

studies on larger series are needed in order to clarify how BOTs could be stratified in different prognostic groups according to their Rb proteins immunohistochemical profile.

Background

Borderline ovarian tumors (BOTs) represent one of the controversial topics in gynecologic pathology [1]. They are a heterogeneous group of tumors that account for 10–20% of all ovarian epithelial neoplasms. The most common BOT histotypes are serous (50%) and mucinous (45%) with less common subtypes (5%) including sero-mucinous, endometrioid, clear cell, and borderline Brenner tumors [2]. The 97% of all stages of BOT have a good prognosis with a mean 10-years survival [4], although recurrences and malignant transformation can occur in a very small proportion of cases [5]. In contrast to serous BOTs, that are rarely characterized by evolution in a low grade serous carcinoma whereas are often associated to peritoneal implants and relapses [5], mucinous carcinoma frequently develops from benign and borderline mucinous tumors [6, 7]. Similarly sero-mucinous BOTs are often the land of endometrioid or clear cell carcinoma and usually represent a morphologic continuum in the middle of benign and malignant counterparts.

BOTs often occur in young women, however the absence of stromal invasion warrant a better prognosis compared to ovarian carcinoma [8, 9]. Nevertheless, since the standard treatment of BOTs is usually surgery, the fertility of these women may be affected [10]. Identifying the genetic background for diagnosis and prognosis should avoid a radical resection and help in developing new targeted therapies, especially in younger woman with desire of childbearing. Thus, a better understanding of the clinical phenotype and pathogenesis of BOTs would contribute to their earlier detection and is essential for development of more effective treatments.

Previous studies support the idea that the serous and mucinous BOT have distinct carcinogenic pathways. For example, the expression of p21 and MDM2 differs between mucinous and serous forms [11]. In other studies, a higher rate of p53 mutation was observed in mucinous compared to serous BOTs and p21 and bcl-2 overexpression appeared specific to serous forms and different between serous benign, serous BOTs and serous carcinoma [12–14].

The retinoblastoma gene family consists of three members and their products are: Rb/p105, Rb2/p130 and RbL1/p107, together known as “pocket proteins” family [15, 16]. Their most important target is the E2F-family of transcription factors which control the expression of genes that mediate G1-S transition [15, 16]. The localization of these proteins into the nucleus or around the nuclear membrane has been shown at the molecular level during the different phases of the cell cycle [17, 18]. In detail, during the cell progression through the S into the G2/M phases of division cycle, pRB undergoes phosphorylation, while in the late M phase, pRB is rapidly dephosphorylated. When pRB is in the unphosphorylated form, it acts as a growth suppressor by repressing transcription of E2F. By contrast, the phosphorylated pRB status (p-pRB) leads to activation of E2F-responsive genes and entry into the S phase. While Rb/p105 is ubiquitously confined to the nuclei of cycling and quiescent cells, Rb2/p130 activity is also regulated by

intracellular localization. The phosphorylation status of Rb2/p130 itself, therefore, is important in the regulation of cell cycle [19]. The hyperphosphorylated form of pRb2/p130 is cytoplasmic and typical of cells progressing into G1 phase [20]. Alteration of Rb family members is frequently involved in gynaecological cancers [21–23]. We previously showed that the loss of Rb2/p130 or its cytoplasmic expression occurs in 40% of ovarian tumors and is inversely correlated with tumor grade [24]. However, little is known about Rb proteins expression in borderline ovarian tumors.

In this study, we utilized immunohistochemistry to evaluate the expression pR/p105 and pRb2/p130 family members in a large, single-institution, series of mucinous, sero-mucinous and serous BOTs.

Materials And Methods

Data collection

Sixty-five ovarian BOTs were retrospectively collected from patients who underwent salpingo-oophorectomy for ovarian cancer in the Division of Gynaecologic Oncology of the Fondazione Policlinico Universitario A.Gemelli IRCSS, Rome, Italy between 2010 and 2016; all selected patients did not receive chemotherapy or radiotherapy before surgical enucleation. All the subjects gave a written informed consent before enrollment. Twenty specimens were defined as mucinous BOTs, 19 were classified as sero-mucinous, whereas 26 cases were classified as serous BOTs. Histological classification of tumors was carried out according to the WHO system, and disease staging was established according to the International Federation of Gynaecologist and Obstetricians (FIGO) criteria.

Immunohistochemistry

After surgical resection, tissues were immediately fixed in 10% formalin and then paraffin-embedded for immunohistochemical analysis. The immunostaining was performed using a streptavidin-biotin complex immuno peroxidase method (DakoCytomation). Detection for the retinoblastoma gene family members was performed using purified mouse anti-human retinoblastoma protein (Rb) monoclonal antibody (BD Pharmingen) diluted 1:50 and mouse monoclonal antibody Rb2 p130 (clone 130-P215; Novus Biologicals, Inc.) diluted 1:25.

Paraffin blocks of each specimen were sectioned at 3 μ m, mounted on slide and dried overnight at 37 °C. All sections were dewaxed in xylene and dehydrated in descending graded alcohols to Phosphate-Buffered Saline (PBS; pH 7.4).

Antigen retrieval was performed by microwaves in 10 mM Citrate buffer (pH 6), at 750 W for 10 min (two cycles of 5 min each), followed by cooling at room temperature for at least 20 min prior to incubation with the antibodies. Sections were treated with 0.4% H₂O₂ methanol solution (15 min at room temperature to inhibit endogenous peroxidase activity), quickly rinsed in water and then in PBS.

Sections were then placed in a humidified chamber and incubated with primary antibody at room temperature for 40 min. The sections were then washed in PBS (two times for 5 min each).

Antigen detection was carried out by exposure to a biotinylated universal secondary antibody for 10 min followed by a streptavidin-peroxidase complex working solution for 10 min.

After another PBS wash, the antigen antibody complex was visualized by staining with chromogen 3,3'-diaminobenzidine/ tetrachloride solution (DAB, Vector) for 5 min. The sections were rinsed in deionized water; cell nuclei were counterstained with hematoxylin and dehydrated in graded alcohols followed by xylene.

Specimens of human colon cancer, follicular cyst and fallopian tube served as positive controls for pRb and pRb2, respectively. For negative control, slides were simultaneously incubated with PBS in the absence of the primary antibody. The results were independently reviewed by three experienced pathologists (GFZ, GA, AS), who were blinded to clinical outcome at the time of evaluation. Discrepancies in the evaluation (< 10% of cases) were resolved by re-observation of the cases using a multi-headed microscope.

Quantitative scoring of protein expression was based on the staining intensity and the percentage of positive cells as follows: negative (0%); lower positive (1–30%) or upper positive (> 30%) cells. A cut-off of 30% was considered statistically significant and, therefore, functionally operative.

Statistical Analysis

The associations between Rb proteins staining and other clinic-pathological parameters were analysed using contingency table methods and tested for significance using the Fisher's exact χ^2 test. All calculation were performed using the Statistical Package for Social Science (SPSS 17.0 software, Chicago, IL) and the result was considered statistically significant when the P value was less or equal to 0.05.

Results

Clinico-pathological features

The series included 20 mucinous, 19 sero-mucinous and 26 serous BOTs. The mean and median age of the patients were 44.7 and 44 years (range 20–72), respectively. All mucinous and sero-mucinous selected tumors (39) were Stage 1, without evidence of implants or recurrences, whereas serous tumors included stage 1 (n = 20), stage 2 (n = 1) and stage 3 (n = 5) cases and follow-up data were available for all 65 patients. Five serous BOTs were classified as BOTs with microinvasive foci. One mucinous BOT showed foci of intraepithelial carcinoma and an area of malignant invasive mucinous carcinoma with expansive pattern of growth.

Fifty-nine cases were limited to the ovary, without peritoneal implants and 6 cases (all serous BOTs) were associated to peritoneal implants (only 1 of invasive type). Sixty-one cases did not relapse; the remaining 4 developed peritoneal recurrences. By the time this study was undertaken, no patients had died of the disease.

Clinico-pathologic characteristics of patients are summarized in Table 1. The expression levels of Rb/p105 and Rb2/p130 were determined by immunohistochemistry.

Table 1
Clinical data of the patients with BOTs

HISTOLOGIC TYPES	N° (% of Cases)
Serous subtype*	26 (40.0)
Sero-Mucinous subtype	19 (29.2)
Mucinous subtype**	20 (30.8)
STAGE	N° (% of Cases)
1	59 (90.8)
2	1 (1.5)
3	5 (7.7)
IMPLANTS	N° (% of Cases)
Not Implants	59 (90.8)
Implants without invasion	5 (7.7)
Implants with invasion	1 (1.5)
RECURRENCES	N° (% of Cases)
No	61 (93.8)
Yes	4 (6.2)
CLINICAL OUTCOME	N° (% of Cases)
Dead	0
Alive	65 (100)
<i>* 5 serous BOTs showed micro-invasive foci</i>	
<i>** 1 mucinous BOT showed foci of intraepithelial carcinoma and an area of invasive mucinous carcinoma with expansive pattern of growth</i>	

Correlation of Rb/p105 with clinic-pathological parameters in BOTs.

The expression of Rb/p105 according to clinico-pathological parameters is shown in Table 2. Notably, the immunoreactivity for Rb/p105 was only nuclear in our series. Moreover, the nuclear Rb staining in intestinal type mucinous BOTs tended to concentrate at the bases of the papillary projections (Fig. 1).

Table 2
Nuclear Distribution of Rb/p105-negative and Rb/p105-positive cases according to tumoral characteristics

Rb/p105 Nuclear	Total N°	Rb/p105 negative (0%) N° (%)	Rb/p105 positive (1–30%) N° (%)	Rb/p105 positive (> 30%) N° (%)	P
HISTOLOGIC TYPES	65	25 (38.4)	33 (50.8)	7 (10.8)	
Serous	26	4	22 (84.6)	0 (15.4.)	< 0.0001
Sero-Mucinous subtype	19	11 (57.9)	6 (31.6)	2 (10.5)	
Mucinous subtype	20	10 (50.0)	5 (25.0)	5 (25.0))	
STAGE	59	25 (42.3)	27 (45.8)	7 (11.9)	0.04
1	6	0	6 (100)	0	
2 + 3					
IMPLANTS	59	24 (41.4)	27 (46.5)	7 (12.1)	NS
Absent	6	1 (14.3)	6 (85.7)	(0)	
Present					
RECURRENCES	61	24 (39.4)	31 (50.8)	6 (9.8)	NS
Absent	4	1 (25.0)	2 (50.0)	1 (25.0)	
Present					
<i>Numbers in parentheses represent the percentage of specimens achieving that particular score. NS, not significant.</i>					

The nuclear expression of Rb/p105 was observed in 40 (61.6%) out of the 65 patients, whereas it was not detectable in the remaining 25 cases (38.4%). The expression of nuclear Rb/p105 was more frequently detected in serous (22 cases; 84.6%) than in serous mucinous (8/19, 42.1%) and mucinous (10/20, 50%) types, and this difference was statistically significant ($P < 0.0001$). Positive staining was observed in 34 (57.6%) out of 59 stage 1 and in all 6 (100%) stage 2/3 cases and this difference was slightly significant ($p = 0.04$). No statistically significant correlation was observed between the nuclear expression of Rb/p105, implants and recurrences (Table 2).

Correlation of Rb2/p130 expression with clinico-pathological parameters in BOTs

Nuclear Rb2/p130 expression was detected in 33 (50.8%) of the 65 cases whereas it was absent in the remaining 32 cases (40.2%) (Fig. 2). The expression of nuclear Rb2/p130 was more frequent in serous

(21/26, 80.8%) than in sero-mucinous (10/19, 52.6%) and intestinal (2/20, 1.0%) types, and these differences were statistically significant ($P < 0.0001$). Positive staining was observed in 27 (45.7%) out of the 59 stage 1 and in all 6 (100%) stage 2/3 cases and this difference was significant ($p = 0.03$). No statistically significant correlation was observed between the nuclear expression of Rb2/p30, implants and recurrences (**Table 3A**)

Table 3 (A)

Nuclear distribution of Rb2/p130-negative and Rb2/p130-positive cases according to tumoral characteristics

Rb2/p130 Nuclear	Total N°	Rb2/p130 negative (0%) N° (%)	Rb2/p130 positive (1-30%) N° (%)	Rb2/p130 positive(>30%) N° (%)	P
HISTOLOGIC TYPES					<0.0001
Serous	26	5 (19.2)	7 (26.9)	14 (53.9)	
Sero-Mucinous subtype	19	9 (47.4)	5 (26.3)	5 (26.3)	
Mucinous subtype	20	18 (90.0)	2(10.0)	0	
STAGE					0.03
1	59	32 (54.3)	12 (20.3)	15 (25.4)	
2+3	6	0	2 (33.3)	4 (66.7)	
IMPLANTS					NS
Absent	59	31 (53.4)	12 (20.7)	15 (25.9)	
Present	6	1 (14.3)	2 (28.6)	4 (57.1)	
RECURRENCES					NS
Absent	61	30 (49.2)	12 (19.7)	19 (31.1)	
Present	4	2 (50.0)	2 (50.0)	0	

Numbers in parentheses represent the percentage of specimens achieving that particular score. NS, not significant

Table 3 (B)

Cytoplasmic distribution of Rb2/p130-negative and Rb2/p130-positive cases according to histologic types

Rb2/p130 Cytoplasmatic	Total N°	Rb2/p130 negative (0%) N° (%)	Rb2/p130 positive (1-30%) N° (%)	Rb2/p130 positive(>30%) N° (%)	P
HISTOLOGIC TYPES					<0.0001
Serous	26	26 (100)	0	0	
Sero-Mucinous subtype	19	17 (89.4)	1 (5.3)	1 (5.3)	
Mucinous subtype	20	4 (20.0)	7 (35.0)	9 (45.0)	

Numbers in parentheses represent the percentage of specimens achieving that particular score

The cytoplasmic expression of Rb2/p130 was detected in 18 (27.7%) cases whereas it was not evident in the remaining 47 cases (72.3%). Unlike nuclear Rb2/p130 expression, the cytoplasmic expression of Rb2/p130 was not detected in serous tumors, rarely detected in sero-mucinous (2/19, 10.6%) and frequently observed in mucinous subtypes (16/20, 80%) (Fig. 3); this difference was statistically significant ($P < 0.0001$). The cytoplasmic expression of Rb2/p130 according to clinico-pathological parameters is shown in **Table 3B**. In Fig. 4 we have shown Rb/p130 immunohistochemistry in an intestinal type mucinous ovarian tumor composed of benign, borderline and malignant areas. Notably, the Rb2/p130 expression moves from nuclear expression in the benign counterpart, to nuclear-cytoplasmic in the BOT counterpart and cytoplasmic in the malignant counterpart (Fig. 4, A/B/C).

Discussion

Borderline ovarian tumors (BOTs) represent about 15–20% of all ovarian malignancies and differ from invasive ovarian cancers by many characters. On the one hand, they are characterized by cellular proliferation and nuclear atypia but, on the other hand, they usually do not show infiltrative growth pattern. Recent knowledge supports the notion that subtypes of borderline ovarian tumors comprise distinct biologic, pathogenetic, and molecular entities, precluding a single unifying concept for BOT. Serous borderline tumors (SBT) share molecular and genetic alterations with low-grade serous carcinomas and can present at higher stages with peritoneal implants and/or lymph node involvement, which validates their borderline malignant potential. All other (non-serous) subtypes of BOT commonly present at stage I confined to the ovary(ies) and are associated with overall survival approaching that of the general population.

The retinoblastoma (Rb) gene family includes Rb2/p130, RB/p105 and p107 genes, which encode nuclear proteins (pRB) acting as negative regulators of cell proliferation, when in their dephosphorylated status.

Alteration of Rb family members is frequently involved in gynaecological cancers [21–23].

Dong et al., in a series of 168 specimens, demonstrated high pRb expression in 41% percent of the benign, 50% of the borderline and 71% of the malignant tumors; in this study, protein accumulation increased progressively with poorer differentiation and there was a trend for high pRb expression to be associated with advanced stage of disease [25]. Additionally, Milde-Langosch et al. demonstrated a correlation between higher pRb expression and shorter survival suggesting thus that pRb expression could play a role in early tumorigenesis, while in later stages, the tumor is independent from pRb [26].

Other authors reported significantly lower pRb levels in low malignant potential ovarian tumors (LMP) than in carcinomas and in this latter group, a reduction of pRb expression with increasing grade, advancing stage and bulk residual disease; in their study a low pRb to Ki-67 ratio appeared as an indicator of poor survival in uni- and multivariate analysis, along with histologic type and FIGO stage [27].

We were the first to demonstrate that loss of Rb2/p130 or its cytoplasmic expression occurs in 40% of ovarian tumors and is inversely correlated with tumor grade [24]. This has been confirmed by Worley MJ et al. who evaluated the immunohistochemical Rb2/p130 expression in a series of benign, borderline (SBT), and malignant ovarian tumors (low-grade (LGSC) and high-grade (HGSC) serous carcinoma), demonstrating a significant decrease in Rb2/p130 expression during the progression from cystadenoma to SBT to LGSC. They reported no loss of expression in benign forms, whereas 10% of SBTs, 47% of LGSCs and 16% of HGSCs had loss of expression [28].

Differences in additional molecular markers support the idea that the serous BOTs are histologically and clinically distinct from the mucinous BOTs. For example, the expression of p21 and MDM2 differs between mucinous and serous LMP tumors [11]. In other studies, a higher rate of p53 mutation was observed in mucinous relative to serous BOTs and p21 and bcl-2 overexpression appeared specific to serous BOTs and different among benign, borderline and malignant forms [12, 13].

In the present study, the observed differences in the expression of pRb/p105 and pRb2/p130 between serous, sero-mucinous and mucinous BOTs supports the concept that these tumors follow different pathogenic pathways. In our series, the nuclear expression of Rb/p105 and pRb2/p130 was highly detected in serous (84.6%) compare to sero-mucinous (42.1%) and mucinous (50%) types. On the other hand, the cytoplasmic expression of Rb2/p130 was not detected in serous tumors but frequently observed in mucinous subtypes (80%). Our findings suggest that both pRb and pRb2/p130 do not play a key role in the tumor progression of serous borderline tumors since these proteins remain located in the nucleus and never showed a cytoplasmic localization.

By contrast, the observed higher cytoplasmic expression of Rb2/p130 in mucinous BOTs, is suggestive of the involvement of Rb proteins in the carcinogenesis of mucinous ovarian tumors. To further support our hypothesis, in Fig. 4 we have shown pRb2/p130 immunohistochemistry in an intestinal type mucinous ovarian tumor composed of benign, borderline and malignant areas. Notably, the Rb2/p130 expression moves from nuclear expression in the benign counterpart, to nuclear-cytoplasmic in the BOT counterpart and cytoplasmic in the malignant counterpart.

Despite no statistically significant relationships between pRb immunohistochemistry and prognosis have been observed, our results may suggest that mucinous BOTs, exhibiting low nuclear and high cytoplasmic levels of Rb2/p130, may potentially be considered the BOT histotype with a higher carcinogenic risk. In fact, loss of pRb2/p130 expression has been previously reported to inversely correlate with tumor grade and to be a poor prognostic indicator in several human cancers [14, 14, 16, 18]. Moreover, its cytoplasmic localization, which implicates a loss of function, has been observed in several tumor types, including lymphoma and gastric cancer [29].

On the other hand, the normal pRb2/p130 nuclear localization, as more frequently observed in our series for serous and sero-mucinous BOT histotypes, enables its oncosuppressive function through the interaction with the E2F4 and E2F5 transcription factors.

Conclusion

In conclusion, we have demonstrated a specific histology-related Rb proteins profile of serous, sero-mucinous and mucinous borderline tumors.

Our findings indicate a clear role of pRb2/p130 protein in the tumor progression of intestinal-type mucinous BOTs thus suggesting a possible role of Rb proteins as prognostic factors in ovarian cancer.

Further studies on larger series are needed in order to clarify how BOTs could be stratified in different prognostic groups according to their pRb immunohistochemical profile.

Abbreviations

BOT

Borderline Ovarian Tumor; RB:Retinoblastoma; pRb:Retinoblastoma protein product or hypophosphorylated form; p-pRb:hyperphosphorylated form.

Declarations

• Ethics approval and consent to participate:

- This study was approved by institutional ethics review board. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from the participants included in the study.

- **Consent for publication:**

- not applicable

- **Availability of data and materials:**

- all the data and materials supporting the conclusions were included in the main paper.

- **Competing interests:**

- the authors declare that they have no conflict of interest

- **Funding:**

the present research received no fundings

- **Authors' contributions:**

Study conceptualization and methodology design: VM and GFZ; Formal analysis and the original draft preparation: GA, AS, GS, PA; Review and editing: GA, As, AG.

- **Acknowledgements:**

- not applicable

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Figures

Figure 1

Immunohistochemical staining of Rb/p105 expression in the cells nuclei (A) Colon Carcinoma as positive control for pRb (X400). (B) Brenner BOT as negative control for pRb (X400). (C) Mucinous BOT of intestinal type showing a concentration of positive nuclei at the base of the papillar projection ("Arrow"; X200). (D) Sero-mucinous BOT with intermediate staining of positive nuclei (X200). (E) Follicular cyst as an example of internal positive control for pRb (X200). (F) Serosus BOT with lower pRb staining (X200).

Figure 2

Immunohistochemical staining of Rb2/p130 expression (A) Tube as internal positive control for Rb2/p130 (X100) (B) Mucinous benign tumor with positive nuclei for Rb2/p130 (X200). (C) Serous BOT with nuclear Rb2/p130 staining (X200). (D) Sero-mucinous BOT with intermediate staining of positive nuclei (X200). (E) Mucinous BOT showing cytoplasmic Rb2/p130 staining (X200).

Figure 3

Rb2/p130 cytoplasmic expression in BOTs The graph shows cytoplasmic distribution of Rb2/p130 expression according to histologic types, with focus on the percentage of stained neoplastic cells

Figure 4

Rb/p30 expression in Mucinous BOTs A-B) Rb/p30 expression shows a mix of patterns, in a mucinous BOT including combination of benign, borderline and invasive carcinoma. In particular Rb/p130 expression is nuclear in benign area (A, X200) and nuclear-cytoplasmic in borderline component (B, X200); cytoplasmic positivity has been observed in invasive carcinoma (B, X200).