·Basic Research·

Expression levels of autophagy related proteins and their prognostic significance in retinocytoma and retinoblastoma

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Abstract

•AIM: To discuss the prognostic significant of autophagy related proteins (ARPs) in retinoblastoma (RB) and to find the molecular marker to distinguish retinocytoma (RC) and RB by investigating the different expression profiling of microtubule-associated protein light chain 3 (LC3B) and other ARPs in RC and RB.

•METHODS: Specimens with retinocytoma region (RCR) or mainly composed with Flexner-Winterstein rosettes (FWR) were screen out from 219 paraffin-embedded RB samples and respectively taken as RCR group and FWR group. Others were taken as undifferentiated (UD) group. Immunochemistry (IHC) of LC3B and electronic microscopy was used to identify autophagy. The IHC scores of LC3B and other ARPs, such as Beclin, PTEN, p27, $p16^{INK4a}$, mTOR and BCL-2 were compared and correlation analysis was applied to find potential proteins which may involve in autophagy regulation. The prognostics significance of LC3B was evaluated by comparing the high risk features (HRFs) in 3 groups of total 219 samples.

•RESULTS: Twenty-one specimens with RCR and 36 specimens mainly composed with FWR were screen out. RCR cell had a high level of LC3B and lots of autophagic vacuoles. Beclin, PTEN, p27 had positive correlation with LC3, and $p16^{INK4a}$ had negative correlation, while the expression of mTOR and BCL-2 in RCR and RB region did not show any difference. Cases with RCR had lower rate of HRFs than undifferentiated cases.

•CONCLUSION: ARPs had different expression pattern between RCR and other pathological types of RB, and could be ideal markers to distinguish RC from RB. Our finding indicated cases with RCR had favorable prognosis just like those with FWR.

•KEYWORDS: autophagy related protein; retinoblastoma; retinocytoma; LC3B; prognosis

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INTRODUCTION

Retinoblastoma (RB) was the most common primary malignant intraocular tumor in children, which had various pathological types, including undifferentiated type, Homer-Wright rosettes (HWR), Flexner-Winterstein rosettes (FWR) and retinocytoma (RC). Among which, RC was the special one. It was first reported in 1982 by Gallie *et al* [1] and got bogged down debating whether it represented well differentiation as Flexner-Winterstein rosettes or tumour regression as phthisis bulbi. In the past, the researches about RC were mainly case report or clinical investigation. Research in basic science proved that retinocytoma region (RCR) had lower levels of genomic instability than RB [2]. RCR had also been found to undergo clonal progression from benign to malignant RB [3]. Thus it reached a consensus that RC was the benign lesion of RB. While according to clinical researchers, only 6%-20.4% cases of RB both had RCR and undifferentiated regions [2,4,5]. It seemed that as a kind of precancerosis, RC did not widely coexist with RB. We assumed lacking reliably molecular markers may be the main cause.

Autophagy involves cell degradation of unnecessary or dysfunctional cellular components through the actions of lysosomes. During the formation of autophagy, microtubule-associated protein light chain 3 (LC3) was one of the essential factor, which was proportion to autophagosomes and taken as the marker of autophagy. In recent years, many studied had shown that autophagy has a biphasic effect on tumours [6]. On one hand, autophagy plays an anti-oncogene role during the initial stage of tumourigenesis. Igusai proved that deleting Atg7 (autophagy related protein 7) results in benign liver tumours[7]. It was also

found that a loss of autophagy leads to genomic instability and aneuploidy, which promotes tumourigenesis [8]. Some proteins such as Bcl2 and PI3K/AKT are regarded as suppressors of autophagy and are amplified or overexpressed in many human tumours [9]. On the other hand, copious evidences also suggest that autophagy plays a protective role in malignant tumours. For example, autophagy contributes to the survival of malignant cells in hypoxic and nutrient-deficient environments [10]. Moreover, in some cases, autophagy mediates therapeutic resistance, and combining autophagy inhibitors with cytotoxic agents or radiotherapy has a synergistic effect[11-13].

Interestingly, some study found RC, the benign lesion of RB was more resistant to chemotherapy than RB [14]. Compared with autophagy, we can find both of them having relation with tumorigenesis and drug resistance. Furthermore, many researchers had found LC3B had relation with the prognosis of several kinds of tumour [15-17]. For those reasons, we assumed autophagy may play different roles in RC and RB. The purposes of this research were to identify whether autophagy related proteins (ARPs) had different expression patterns between RCR and undifferentiated RB regions and whether ARPs could be a potential maker to identify RCR and be a prognostic indictor. By compared the expression level of LC3B and other ARPs, we also try to find some clues about the mechanisms underlying the down-regulation of autophagy in RB. Finally, we found that autophagy existed in RCR and samples with RCR had a lower high risk features (HRF) rate. ARPs were excellent marks to identify RC or RCR.

SUBJECTS AND METHODS

Samples Selection Informed formal consent of the research was send to hospital medical ethics committee and the study was approved by the committee. Formalin-fixed, paraffin-embedded blocks were collected over a 3-year period and anonymously obtained from the Zhongshan Ophthalmic Center of Sun Yat-sen University. Pure RC sample was rare, thus cases both having RCR and undifferentiated RB regions were screen out and taken as RCR group. Cases were mainly composed by FWR (more than 20%) were used as FWR group. Others which mainly constituted by undifferentiated RB cells, were taken as undifferentiated (UD) group. Identification of RCR region were based on bland nuclei, a fibrillar eosinophilic stroma, scattered fleurettes, no mitoses by haematoxylin and eosin staining and Ki67 (-), which was described by Dimaras *et al* [2] and Indovina *et al* [18].

Immunohistochemistry Specimens of RCR group and FWR group were cut into sections, 4 μm slices thickness, which were deparaffinised in xylene and re-hydrated. Then those slices were incubated in 0.3% hydrogen peroxide (H_2O_2, Sigma, St. Louis, USA) for 30min to block endogenous peroxidase activity. Antigen retrieval was achieved using a 0.01 M citrate buffer, pH6, for 45s in a pressure cooker. For immunohistochemistry, the sections were stained with anti-LC3B (1:75) (3868, CST, Boston, USA), anti-$p16^{INK4a}$ (1:75) (sc-81613, Santa Cruz, California, USA), anti-Ki67 antibodies (1:200) (sc-23900, Santa Cruz, California, USA), anti-Beclin (1:100) (sc-11427, Santa Cruz, California, USA), anti-PTEN (1:100) (sc-133242, Santa Cruz, California, USA), anti-p27 (1:200) (WH0001027M1, Sigma, St. Louis, USA), anti-mTOR (1:200) (ab2732, Abcam, Cambridge, USA), and anti-Bcl2 (1:100) (sc-509, Santa Cruz, California, USA). Signals were detected using the Dako-CytomationEnVision+anti-rabbit or anti-mouse secondary system. The slides were then incubated in DAB (3,3N-Diaminobenzidine Tertrahydrochloride) solution (Vector Laboratories DAB substrate Kit for peroxidase) for approximately 1min and counterstained briefly in Harris hematoxylin. The slides were differentiated, dehydrated, cleared and mounted in neutral balsam (DPX). Images were collected on a BX51 microscope (Olympus, Japan). The sections were semi quantitatively evaluated for the level of antibody staining. LC3B, $p16^{INK4a}$, PTEN, Beclin, Bcl2, mTOR, and p27 were graded for the percentage of positively stained cells on a scale of 0-2: grade 0, no staining; grade 1, staining below 70%; grade 2, staining of 70%-100%. Three separate regions of each specimen in FWR group were randomly chose. In RCR group, 3 RCR and adjacent undifferentiated RB regions of each specimen were randomly chose respectively. Immunochemistry (IHC) scores of LC3B among different regions were recorded and compared with other ARPs. All fields were screened and evaluated by two experienced pathologists blinded to the clinical background of each specimen.

The expression level of LC3B was compared among RCR, adjacent undifferentiated region and samples of FWR group to detect autophagy in different pathological types of RB. Then the IHC scores of other ARPs, such as $p16^{INK4a}$, PTEN, Beclin, Bcl2, mTOR, and p27, were compared with the scores of LC3B to find the potential proteins which may involve in the regulation of autophagy in RB.

Electronic Microscopy It was used to determine whether autophagic vacuoles existed in LC3B positive cells. Based on hematoxylin and eosin (H&E) staining, two cases were selected. Tissues were cut into 2 mm^3 pieces, which were deparaffinised in xylene twice for 15min and then dehydrated in a series of graded alcohol. After soaking in phosphate buffer for 10h, the tissue pieces were post-fixed in 2% osmium tetroxide for 2h, dehydrated in ethanol and propylene oxide and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and observed by electronic microscopy (Hitachi 600).

Table 1 General information of RB patients with different pathological types

Parameters	RCRG	FWRG	UDG	*P*
No.	21	36	162	—
Age (mo)	22.71±17.69	13.59±13.46	31.56±28.32	*P*=0.000[a]
Gender				
F	7	12	63	*P*=0.758 [b]
M	14	24	99	
Eye				
OD	11	21	78	*P*=0.533[b]
OS	10	15	84	
High risk feature (%)	7 (33.4)	8 (22.3)	70 (43.2)	*P*=0.108[b]; RCR-UDG *P*=0.000; RCR-DG *P*=0.047
Massive invasion (%)	5 (23.8)	4 (11.2)	27 (16.7)	
Post-laminar invasion (%)	2 (9.6)	1 (2.8)	24 (14.8)	*P*=0.044[b]
Surgical transaction orperiorbital and periorbital invasion (%)	0	3 (8.4)	19 (11.7)	
IIRC group				
Group C (%)	3 (14.3)	2 (5.6)	7 (4.4)	
Group D (%)	11 (52.4)	21 (58.4)	45 (27.8)	*P*=0.000[b]
Group E (%)	7 (33.4)	13 (36.2)	110 (67.9)	

[a]One- way ANOVA; [b]Kruskal-Wallis test.

High Risk Feature All 219 cases were divided into 3 groups, RCR group, FWR group and UD group. HRF included massive choroid invasion, post-laminar invasion, reaching the line of surgical transaction or periorbital invasion which were compared among the 3 groups [19].

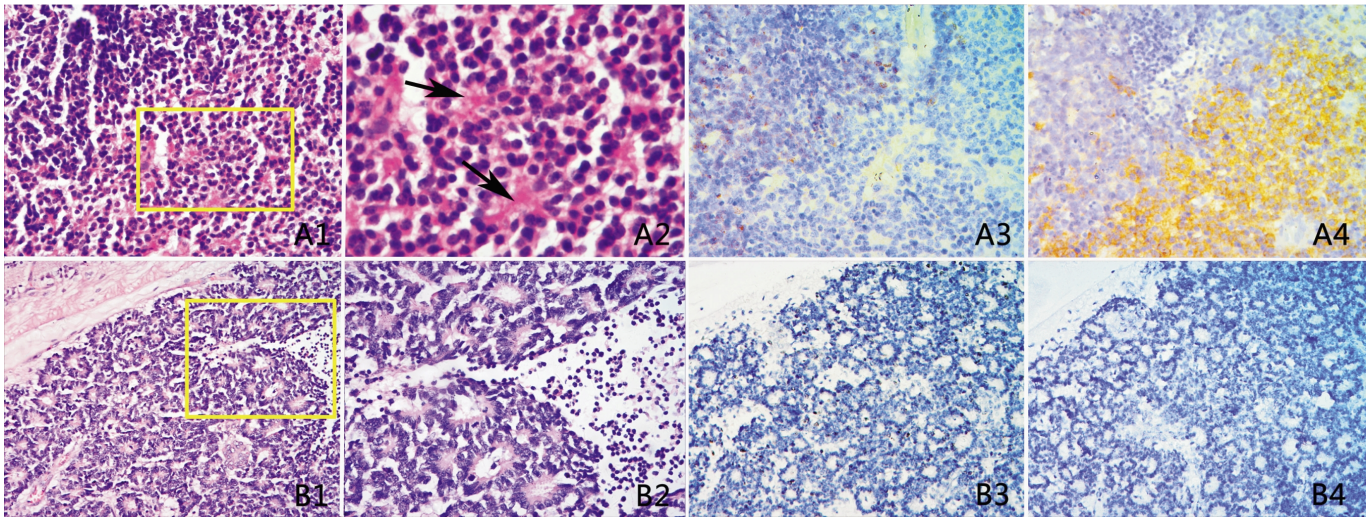
Statistical Analysis The Kruskal-Wallis test was used to analyse the distinctive expression of ARPs between RCR and undifferentiated RB regions. The correlations between LC3B and the other ARPs (Beclin, PTEN, p27 and $p16^{INK4a}$) were analysed by Spearman's rank test. Kruskal Wallis test was applied to compare the rates of HRF among different groups. In all analyses, $P<0.05$ was accepted as statistically significant. These analyses were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA).

RESULTS

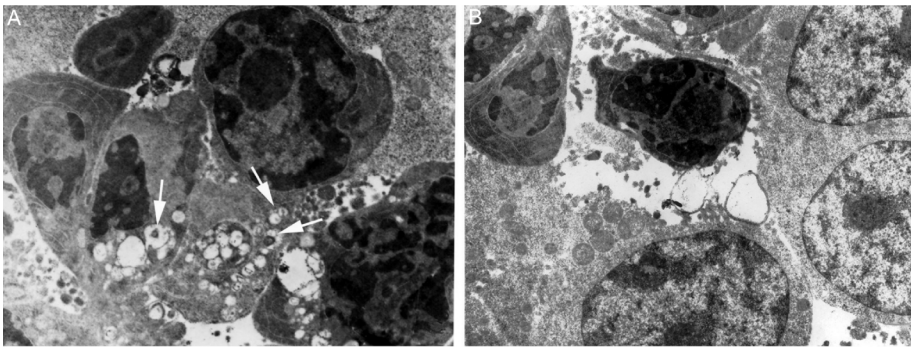
A total of 21 samples (9.6%) were collected from 219 patients in the last 3y. All patients' general information were summarised in Table 1. Differences of gender and eye among 3 groups did not show statistical significance. Patients of FWR group were younger than the other two groups. UD group had the highest rate of HRF, second was RCR group. It should be noted that most HRF in RCR group were massive choroid invasion. Other HRFs, such as RB cells reached to the line of surgical transaction or infiltrated into orbit, were not found in any cases of RCR group, while the same lesions in UD group were more than 11.7%. Specially, any degrees of optic nerve invasion were not being found when RCR located before optic papilla. Accordingly, cases of group E in UD group were more than that in RCR group and FWR group.

Autophagy Detection in Retinocytoma Region LC3B were strong positive in RCR, while the adjacent undifferentiated RB regions were weak positive (5/21) or completely negative (16/21, Figure 1). The difference of IHC scores between the two regions was significant ($P<0.01$). Two specimens were examined by electron microscopy and numerous autophagic vacuoles were found in one end of the RCR cells, which had the differentiated structures of fleurette-like inner segment (Figure 2). While in the undifferentiated RB regions of both specimens, autophagic vacuoles could not be found. By combining the two methods, it was confirmed that autophagy was a common phenomenon in RCR. All cases in FWR group were LC3B negative (Figure 1).

LC3B and Other Autophagy –Related Proteins in Retinocytoma Region and Matched Undifferentiated Retinoblastoma Regions To investigate the potential proteins which may involved in the regulation of autophagy, we studied a series of ARPs, such as Beclin, Bcl2, PTEN, mTOR, p27 and $p16^{INK4a}$. In RCR, IHC staining of Beclin1 was weakly to strong positive and in the adjacent RB region the expression level decreased, excepting 7 (33.3%) specimens (Figure 3). The difference of IHC scores of Beclin between RCR and RB was statistically significant ($P<0.05$). PTEN had a similar expression pattern; the expression level of which in RCR was higher than that in adjacent RB region. Still there were 5 (23.8%) exceptions (Figure 3). The difference of IHC scores of PTEN between RCR and undifferentiated RB regions was statistically significant ($P<0.05$). We also found that Beclin and PTEN had a complementary



Figures 1 Pathological characteristics of RCR and Flexner-Winterstein rosettes H&E staining showed the structures of RCR (A1 low right), undifferentiated RB region (A1 upper left) and Flexner-Winterstein rosettes (B1) (200×). A2 and B2 were partial enlarged detail of yellow square frame in A1 and B1 (400×). Ki67 was positive in RB region (A3 upper left) and Flexner-Winterstein rosettes (B3), while negative in RCR (A3 low right) (200×). The expression pattern of LC3B was opposite to Ki67 (A4, B4) (200×).



Figures 2 RCR cells shown lots of autophagosome RCR cells had elongated cytoplasmic structures and numerous vacuoles, some of which contained a mass of membranous debris (A, arrow). Cells in the RB region lacked autophagic vacuoles (B).

relationship: in one specimen, if Beclin1 had not difference between RCR and undifferentiated RB regions, PTEN must have, and vice versa.

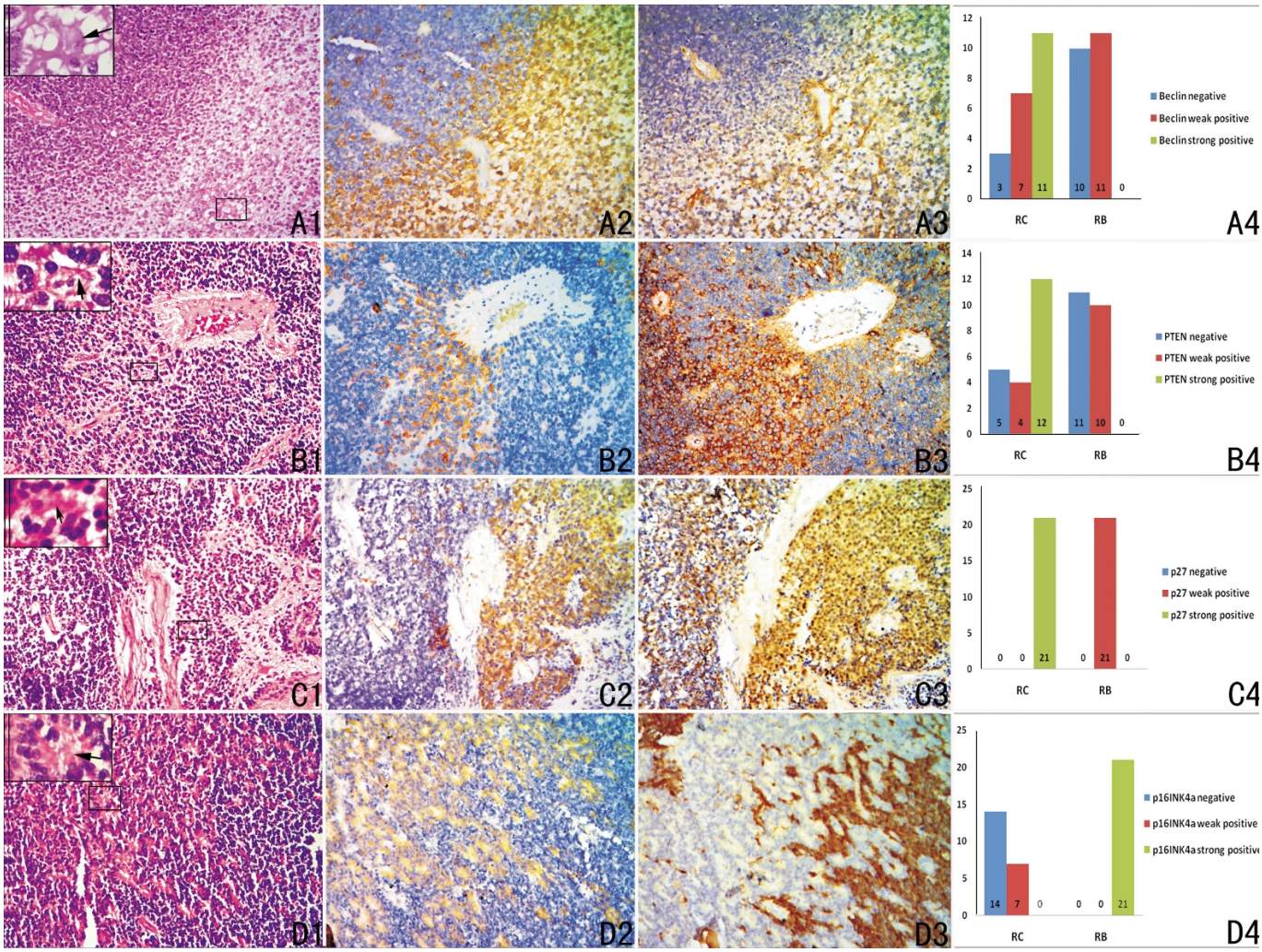
P27 and $p16^{INK4a}$ showed significantly difference in RCR and undifferentiated RB regions. In RCR, p27 was strong positive, but in the adjacent undifferentiated RB region, the expression level turned to weak (Figure 3). More importantly, in RCR, p27 was positive both in the cytoplasm and nucleus, but in undifferentiated RB regions, only the nucleus were positive. $p16^{INK4a}$ was strong positive in the RB region and nearly negative in RCR (Figures 3). In RB region, $p16^{INK4a}$ was mainly located in the cytoplasm instead of the nucleus. The differences of both p27 and $p16^{INK4a}$ between RCR and undifferentiated RB regions, were statistically significant (both $P<0.01$).

Bcl-2 was negative in all specimens, while mTOR was moderately positive in all samples; both of the two proteins completely did not show distinct between the RCR and undifferentiated RB regions (data not shown).

Correlation Between LC3B and Other Autophagy – Related Proteins in Retinoblastoma To understand the mechanism underlying the down-regulation of autophagy in RCR, we analysed the correlation between LC3B and other ARPs (Beclin, PTEN, p27 and $p16^{INK4a}$). As shown in Figure 4, when the expression level of LC3B improved, the IHC scores of Beclin, PTEN and p27 tended to increase, and the scores of $p16^{INK4a}$ decreased (Figure 4). By statistical analysis, we found that LC3B was positively correlated with Beclin ($P=0.561$; $P=0.000$, Spearman's rank test), PTEN ($P=0.665$; $P=0.000$, Spearman's rank test) and p27 ($P=0.823$; $P=0.000$, Spearman's rank test), but negatively correlated with $p16^{INK4a}$ ($P=0.852$; $P=0.000$, Spearman's rank test).

DISCUSSION

Two ubiquitylation-like modifications were essential for the formation of autophagy, Atg12-conjugation and LC3 [19]. LC3 had two subunits, LC3-I and LC3-II, which have been discovered to coexist during the formation of autophagy [20]. LC3-I was activated by Atg7 and converted to LC3-II in the presence of Atg10 [20]. Only the amount of LC3-II was closely correlated with the number of autophagosomes and widely used to monitor autophagy [21]. While the IHC staining of LC3B could not tell LC3-I from LC3-II, and it was usually

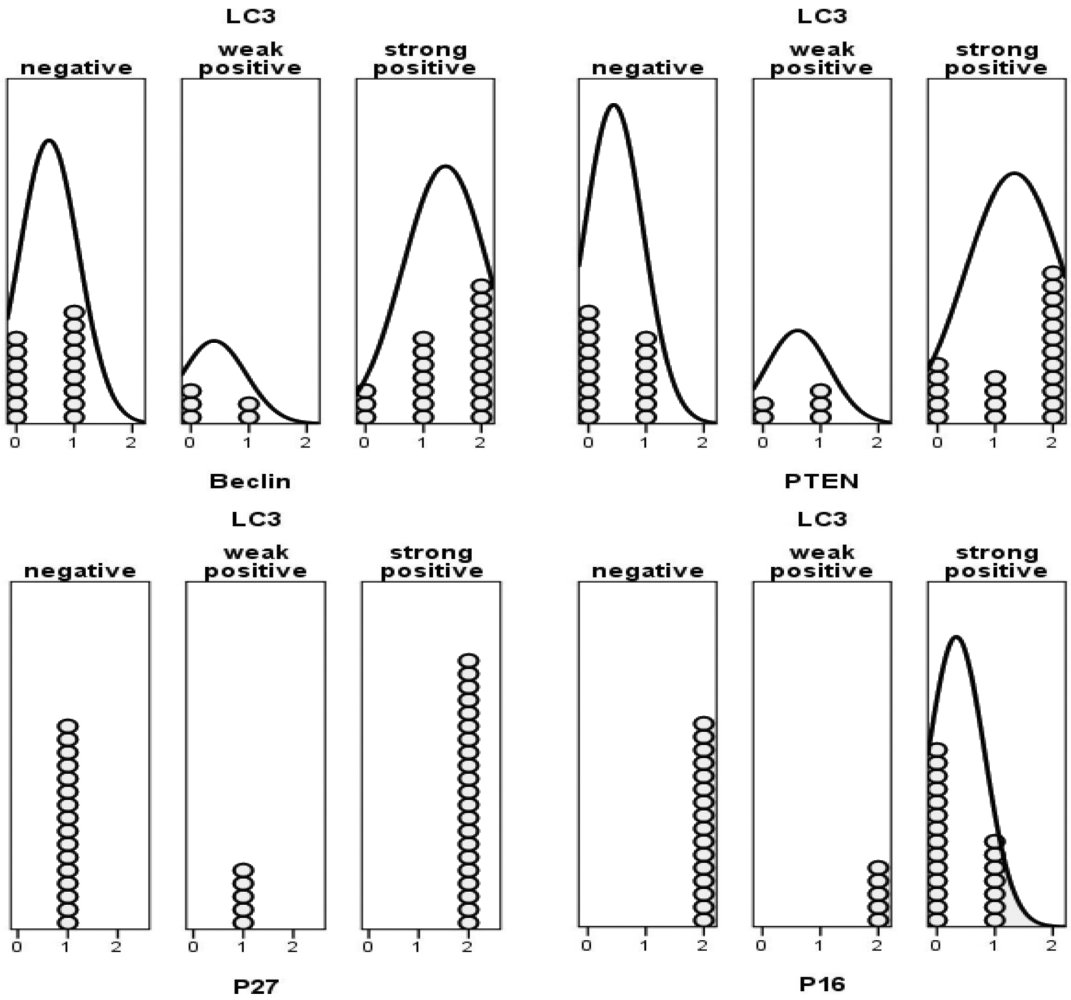


Figures 3 Different Expression of LC3B and autophagy related proteins in RCR and undifferentiated RB regions H&E staining shown RCR (200×), and it was a magnified images (400×) in the square frame (A1-D1). LC3B was positive in RCR and negative in RB region (A2-D2) (200×). Beclin (A3), PTEN (B3) and p27 (C3) displayed an expression pattern consistent with that of LC3B (200×), while p16INK4a displayed opposite pattern (D3) (200×). The bar chart showed the different positive rate of each protein (A4-D4).

combined with other methods to monitor autophagic activity[22]. Electron microscopy has been recognised as the gold standard [22]. In our study, it was proved that RCR had a high level of autophagy by combining with LC3B staining and electron microscopy. As we had mentioned, autophagy has been observed in different normal and tumor cells following anticancer drugs resistance. So one of the clinical significance of our research was it can explain why RC cells were resistant to drugs.

Moreover, recent reports had demonstrated that autophagy and apoptosis have common regulators. For example, Beclin1 was the negative regulation factor of autophagy, BCL-2 can activated autophagy by down-regulated Beclin1. At the same time, the down-regulation of Beclin1 inhibited apoptosis by reducing the release of cytochrome C from mitochondrial[22,23]. In clinical, some focus of RC can stably lie in retina more than 10y and could rapidly undergo malignant transformation at any time. To those patients, inducing RC cells shift from autophagy to apoptosis maybe an effective method.

As we had mentioned, RC had been proved to be the benign lesion of RB. While it hard to say RC was the transition stage of RB in general, because only 6%-20.4% of RB cases were found coexisted with RCR [2-4]. The contradiction maybe due to some focus of RCR lacked typical structures and were too small to be picked out. Solve this problem was crucial for the researcher of RB. If the coexistence of RC and RB was a universal phenomenon, it implied RB was the disastrous consequence of RC malignant transformation, and blocking the process would be a promising and effective treatment. If it was not, it implied those patients were a special subtype of RB, and it would have important clinical implications for finding out the clinical features and prognosis of those patients. The first step to solve the problem was to identify RC easily and clearly. At present, the identifications of RC were mainly based on structures of H&E staining, such as smaller and less hyperchromatic nuclei, abundant cytoplasm and intercellular matrix, mitotic figures absent, necrosis typically absent, differentiation into fleurettes [24]. In clinical,



Figures 4 Correlation between LC3B and other regulatory proteins involved in RB Each point represents one specimen and the columns represent different immunostaining grades of LC3B. The tip of each trend line represents the mean value of each column. The labels 0, 1 and 2 on the X axis represent negative, weakly positive and strongly positive, respectively.

the identification criteria sometimes were hard to understand and lead to some chaos. The most common mistake was arbitrarily taken fleurettes as the diagnostic criteria which would lead to missed diagnosis or misdiagnosis. Above all, finding a sensitive and specific marker to distinguish RB from RC allowed of no delay. Our results proved the expression lever of LC3B, p27 and $p16^{INK4a}$ in RB region were obvious different from that in RC region. Those proteins were the excellence marker to identify RC. Our finding will be immensely useful to the future researches.

For basic theoretical researches, our findings also provided some clues for the mechanism of RB development. As we known, the development of RB was described as a process of M1, M2, M3, to Mn and the mutant of RB1 alleles were the first two Ms [25,26]. In 2010, Indovina proved both RC and RB had the same mutation of RB1 alleles [18]. At present, So the fade of autophagy could be the key step in the evolution from M2 to Mn. More over the function of pRB in autophagy was still under debate. Jiang *et al* [27] found that transducing the RB1 gene into RB-defective human cancer cells induced autophagy. However, Ciavarra and Zacksenhaus [28] reported that an RB1 gene deficiency during myogenic differentiation induced autophagy. Both RC and RB had similar mutations of RB1 [2,29]. Our research provided some clues to the direct influence of RB1 on autophagy and supported the view of Zacksenhaus Eldad.

There were some limitations on our study. First, RCR could not completely equal to RC. Because RC was an independent benign disease, while RCR was a well differentiated regions of RB. Second, it was not an intervention study and we did not perform dynamic assays. All the limitations were due to RC was rare and enucleation of the eye with RC was not advised by most ophthalmologists. Till now, paraffin specimen is still unique and suitable tool for studying the relationship between RB and RCR, because it included both regions which were excellent self control for each other. Based on paraffin specimen, researchers got the conclusion that RC was a precancerous lesion [2]. In our study, we screen out 21 samples having RCR. The sample sizes, as we known, exceeded others before.

Truly, our research presented no direct evidences to indicate the mechanisms underlying the down-regulation of autophagy

in RB, but by compared the different expression patterns of several ARPs in RCR and its corresponding undifferentiated RB regions, some potential clues were found in our research. It was reported that Beclin1 and PTEN can induce autophagy by inducing the formation of Beclin 1-Vps34-Vps15 complexes or by inhibiting PI3K-I respectively [30,31]. A lower expression of LC3B and Beclin1 were found in other cancers [32-34]. Notably, in ours researches Beclin1 and PTEN shown complementary expression which indicated there may be a supplementary mechanism between them. Phosphorylation of p27 at Thr 198 stabilises p27 and limits it to the cytoplasm, which induced quiescent cells autophagy and promoted cell survival[35,36]. It was in accordance with our results. Thus, we hypothesised that intracytoplasmic p27 restricted the malignant transformation of RC by influencing autophagy.

The expression pattern of $p16^{INK4a}$ was completely opposite to other ARPs. Traditionally, $p16^{INK4a}$ was regarded as a tumour suppressor protein. While in many highly malignant tumours, it has been found that, some proteins, such as α-β-γ actin, α-β tubulin, CDK4/6 and AE1, bound to $p16^{INK4a}$ and formed a stable complex to prevent it located in nuclear [37-39]. The increased expression level of $p16^{INK4a}$ in the cytoplasm was considered to be a feedback regulation in response to RB protein dysfunction [40]. RCR and RB have the same alteration of RB1 alleles, but why the cytoplasm of RCR did not express $p16^{INK4a}$ firstly? Was the accumulation of $p16^{INK4a}$ just a passive feedback reaction? We believe the answer was no. Given that LC3B and $p16^{INK4a}$ have an inverse expression pattern, both of them maybe the excellent candidate for identify RC. We also hypothesise that $p16^{INK4a}$ may participated in the autophagy suppression and it merits further study. It seemed that the other two ARPs, Bcl2 and mTOR, had no relation with down-regulation of autophagy.

In conclusion, a series of ARPs were found to have different expressing pattern between RCR and undifferentiated RB regions and could be taken as the biomarkers, especially LC3B and $p16^{Ink4a}$. Our research also paved the way for future researches of RB. In clinical, it implied anti-autophagy may be a hopeful therapy; in basic research, it implied down-regulation of autophagy in RB maybe the crucial step in the malignance transformation of RC. All of those may boost larger series of researches to explore this novel and promising fields.

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