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Correlations between claudin-1 and PlGF expressions in retinoblastoma

H.-F. WANG[1], S.-F. ZHENG[2], Y. CHEN[3], Z.-Y. ZHOU[4], J. XU[1]

[1]Department of Ophthalmology, People's Hospital of Weifang, Weifang, Shandong, China
[2]Eye Hospital, Yulin Traditional Chinese Medicine Hospital, Yulin, Shaanxi, China
[3]Otorhinolaryngological Department, People's Hospital of Weifang, Weifang, Shandong, China
[4]Department of Ophthalmology, the Affiliated Municipal Hospital of Qingdao University, Qingdao, Shandong, China

Abstract. – OBJECTIVE: To investigate the expressions of claudin-1 and placental growth factor (PlGF) proteins in retinoblastoma (RB) and their relationships with the differentiation of RB, the infiltration of optic nerve and choroid and clinical stages.

PATIENTS AND METHODS: Immunohistochemical (IHC) method was used to detect the expressions of claudin-1 and PlGF proteins in 56 cases of RB paraffin-embedded tissue samples. The x^2-test and Fisher exact test were used to compare the qualitative variables. The Pearson correlation coefficient was used to detect the correlation of the expression of claudin-1 with that of PlGF in RB tissues.

RESULTS: 1) Among RB tissues, the positive expression rates of claudin-1 in clinical stage I tumors and clinical stage III tumors were 69.2% and 38.9%, respectively, and claudin-1 was not expressed in all clinical stage II tumors (p=0.002). In case of optic nerve invasion, the lowly positive expression of claudin-1 was detected, and the difference was significant (p=0.001). 2) The positive expression rate of PlGF proteins in RB was 73.8%, which was higher in tumors with optic nerve invasion than in tumors without the invasion; the expression was significantly different (p=0.001). In addition, the positive expression rate of PlGF in tumors with choroidal invasion was 74.1%. 3) The expression of claudin-1 in RB was negatively correlated with the presence of choroidal invasion (r=0.52, p≤0.0001) and optic nerve infiltration (r=0.49, p=0.0003). There was a significant positive correlation between the expression of PlGF and the presence of optic nerve invasion (r=0.30, p=0.009). In addition, there was a significant positive correlation between the expression of claudin-1 and that of PlGF (r=0.41, p=0.006).

CONCLUSIONS: The expression level of claudin-1 is negatively correlated with the differentiation of RB cells, optic nerve infiltration and clinical stages, while the expression of PlGF was positively correlated with the optic nerve infiltration and clinical stages of RB. The role of claudin-1 may be opposite to that of matrix metalloproteinase-2 (MMP-2) in the development of RB.

Key Words
Retinoblastoma, Claudin-1, PlGF, Immunohistochemistry, Pearson correlation coefficient.

Introduction

Retinoblastoma (RB) is a malignant tumor consisting of embryonic tumor cells of metrocytes in the retina originating from neuroepithelia. It is the most common primary intraocular malignant tumor in childhood with a relative incidence rate of 3% among all pediatric tumors[1]. In developed countries, due to early diagnosis, RB is a disease rarely threatening life, but in developing countries, clinical diagnosis is conducted in the late stage, so the mortality rate is still high[2]. RB patients account for nearly 30% of the population, and the diagnosis rate of myopia among children younger than 3 years old was 80%. If diagnosed with RB after they grow into 6 years old, it could be regarded as a rare case[3]. Leukocoria is the main manifestation of children with RB, and its prognosis is affected by many risk factors, the most important of which is the extent of RB invasion into the orbit and optic nerve[4].

Recent investigations[5,6] have shown that the abnormal expressions of claudin-1 and placental growth factor (PlGF) proteins are closely related to RB invasion and extracorporeal metastasis. The main function of claudin-1 is that it is involved in the tight junction (TJ) process of cells and plays an important role in tumor invasion and metastasis. However, there are rare reports on the expression of it in RB. PlGF is a member of the vascular endothelial growth factor (VEGF) family, which can induce the proliferation and migration of endothelial cells and the apoptosis of anti-endothelial cells, increase vascular permeability and

Corresponding Author: Jing Xu, MD; e-mail: jing_xuwf@163.com

enhance the biological activity of VEGF at a low concentration. PlGF is an important angiogenic factor promoting the angiogenesis of a variety of tumors. In this study, expressions of claudin-1 and PlGF in tissue sections of RB were detected via the immunohistochemical (IHC) method. The expressions of claudin-1 and PlGF in RB and their correlations with the differentiation of tumor cells, the invasion and metastasis of optic nerve and choroid, and different clinical stages, were observed.

Patients and Methods

Patients

Among tumor outpatients in the Department of Ophthalmology of various large general hospitals in the city, a prospective case study was conducted, in which samples of affected eyes of patients receiving ophthalmectomy due to RB were collected. From January 2010 to January 2015, paraffin-embedded glass slides stained by hematoxylin and eosin (HE) were observed in the Pathology Department of our hospital using an optical microscope, followed by classification, and RB was classified according to the International Classification of Retinoblastoma (ICRB)[7]. All RB patients received the removal of the affected eyes without any prior treatments. Patients and controls with the history of ophthalmic surgery, trauma or other diseases were excluded as this might confuse the results. This study included 56 children newly diagnosed with RB with at least one eye with advanced disease.

Historical data of all children were complete, including age, gender, nature of publicity (morbidity, course of disease and duration), family history and kinship, previous information on any test results and treatment methods. Under the general anesthesia, full pupil dilatation was conducted, and the indiophthalmoscope was used for fundus examination. Fundus photography was performed using a fundus camera (Genesis D, Kowa Medicals, Tokyo, Japan), which provided clinical study results. Stages were recorded according to the ICRB[8]. The ocular ultrasonography was performed to determine the tumor size and confirm the presence of intraocular calcification. The head computed tomography was performed to detect any intracranial dilatation and trilateral RB. Magnetic resonance imaging could not be used as a detection method unless there was no visualization of the optic nerve during clinical examination.

Ethics Statement

All patients who participated in the study signed the written informed consent prior to surgery. The study was approved by the Medical Research Ethics Committee of People's Hospital of Weifang, Shandong, China.

Histopathological Analysis

The histopathological diagnosis and cell differentiation status of HE-stained glass slides were examined. The specimens were well differentiated (accompanied by Fleurettes and/or Flexner-Wintersteiner chrysanthemum-like structures), poorly differentiated (without these structures) or moderately differentiated (isolated Homer-Wright-type chrysanthemum clusters)[9]. Only when the tumor cells were identified beyond the lamina, it was considered that the optic nerve invasion existed, and when the tumor cells were observed passing through the Bruch membrane, it was considered that the choroidal invasion existed. When the tumor cells destroyed the Bruch membrane without exceeding the choroidal thickness, it was considered that the minimal choroidal invasion existed. Up to three microscopic clusters and a large number of choroidal lesions suggested that any choroidal involvement is not minimal[10].

IHC Staining

4 μm formalin-fixed and paraffin-embedded tissue samples of the studied cases were prepared. Primary antibodies were used for IHC staining; rabbit anti-human polyclonal Claudin-1 antibodies (Dako Cytomation; Dako Canada, Mississauga, Ontario, Canada) with a dilution of 1:50; rabbit polyclonal PlGF antibodies (Dako Cytomation; Dako Canada, Mississauga, Ontario, Canada) with a dilution of 1:30; RB-9031-R7 (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a dilution of 1:200. According to the avidin-biotin immunoperoxidase complex technique used by Hsu et al[11], a hypersensitive detection kit (Biogenex, Fremont, CA, USA) was used to fix the prepared tissue sections on poly-L-lysine-coated glass slides at 37°C overnight. The sections were dewaxed and rehydrated through graded alcohol series. Then, the solution was boiled in an autoclave for 20 minutes while maintaining the pressure, and the sections were soaked in 10-3 m sodium citrate buffer [potential of hydrogen (pH) 6.0] under the condition with claudin-1. The slides were placed in a target recovery solution at a high (pH 9.9) under the condition with PlGF, and antigens were recovered overnight at

40°C. After quenching and blocking for 5 min in 3% hydrogen peroxide, the sections were incubated at 4°C for the treatment with claudin-1 overnight and PlGF for 1 h, followed by the addition of biotinylated anti-mouse immunoglobulin and streptavidin-conjugated streptavidin. Finally, 3,3'-diaminobenzidine was used as the substrate or chromogen to form an insoluble brown product. Hematoxylin was used to re-stain claudin-1 and PlGF. Gastrointestinal stromal tumors and angiosarcomas were used as positive controls for claudin-1 and PlGF, respectively. The negative control had a primary antibody that was replaced by a buffer.

Explanation of IHC Staining

The IHC analysis of claudin-1 and PlGF was performed by pathologists with no knowledge of clinicopathological data. The cytoplasmic expressions of claudin-1 and PlGF were assessed. The analysis was performed using a computer image analysis software (Special SIS starter 3.2, Olympus, Germany) connected to an Olympus microscope (Model BX51, Olympus, Tokyo, Japan). Claudin-1 was evaluated by the percentage of positively stained tumor cells in at least 5 regions at a magnification of 400 times according to a previously used scoring method. When more than 30% of the cells showed significant positive immunostaining, the sample was considered positive. PlGF staining was also assessed on the basis of the percentage of positive cells, and it was classified as follows: if more than 10% of the cells were stained, the sample was positive; if there was no detectable staining or <10% cell staining, the sample was negative[11].

Statistical Analysis

All data were encoded and analyzed using Statistical Product and Service Solutions 13.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD). Qualitative variables were described as numbers and percentages. One-way ANOVA test followed by LSD (Least Significant Difference) Post-Hoc Test was performed for comparison among multiple groups. The x^2-test and Fisher exact test were used to compare qualitative variables. Horizontal $p \leq 0.05$ was considered to be a meaningful threshold. $p \leq 0.01$ represented that the difference was quite significant. Pearson correlation coefficient (r) was used to examine the correlation of the expressions of claudin-1 and PlGF with different clinicopathological variables.

Results

Clinicopathological Features

The study included 24 boys (42.9%) and 32 girls (57.1%). The mean age of them when being diagnosed was (21.83±9.76) months (range: 3-48 months). 3 patients were found to have a positive family history; 4 patients reported that their parents are close relatives. Leukocoria was the most common manifestation of 50 patients; 2 of the remaining 6 patients suffered from strabismus, 2 had protopsis, and tumors were accidentally found in the other 2 patients. 49 patients (87.5%) had bilateral lesions. According to the ICRB (all unilateral and two eyes of bilateral patients), 56 eyes were removed in group E. In group B, 2 of the remaining 7 eyes appeared in group A received topical treatment, and 5 eyes in group B underwent chemical induction and focal consolidation laser therapy. Growth patterns in clinical stage I and II were observed in 31 patients, and the growth pattern of stage I was observed in 21 patients, while that of stage II was observed in the remaining 4 patients. There were 27 patients with moderately differentiated tumors, 20 with poorly differentiated tumors and 9 with well-differentiated tumors. In 35 patients, the differentiation expanded to the choroid, and it appeared in the optic nerve in 26 patients (it did not spread to the surgical section after the stratification). The clinical and pathological data of the study cases are shown in Table I.

IHC Expression of Claudin-1 in RB

Positive claudin-1 immunoreactivity was observed in 48.2% RB patients. Cytoplasms of all the positive samples were stained. No IHC staining was observed in the adjacent unobserved retina. For the growth pattern of RB, 69.2% of all patients at clinical stage I, and 38.9% at clinical stage III presented positive expression of claudin-1, while patients at clinical stage II exhibited no expression. A low positive expression growth curve was detected in patients (p=0.002) (Table II). In 26 RB patients with infiltration of the optic nerve, 7 patients showed positive; for claudin-1, there were 73.3% of the patients without infiltration. Low positive expression of claudin-1 was detected in the presence of optic nerve invasion (p=0.001) (Table II).

In addition, negative expression of claudin-1 with HS was found in patients with choroidal infiltration ($p \leq 0.01$), in which 34.3% of the patients with RB and choroidal infiltration showed negative

Table I. Clinicopathological features of RB children (n=56).

Variable	RB case [n (%)]	Variable	RB case [n (%)]
Average age (month) ± (SD)	21.83±9.76	Infiltration of the choroid	
Gender		• Negative	21
• Boy	24	• Positive	35
• Girl	32	A small amount	24/35
Tumor side		A large amount	11/35
• Ocellus	49	Infiltration of the optic nerve	
Right eye	16	• Negative	30
Left eye	33	• Positive	26
• Binoculus	7	Degree of infiltration	
Tumor growth pattern		• No infiltration outside the retina	14
• Clinical stage I	21	• Infiltration only on the choroid	16
• Clinical stage II	4	• Infiltration only on the optic nerve	7
• Clinical stage III	31	• Infiltration on the choroid and optic nerve	19
Family		Degrees of differentiation	
• Single-parent family	7	• Good differentiation	9
• Two-parent family	46	• Moderate differentiation	27
• No parent	3	• Poor differentiation	20
Residence registration			
• Urban registration	34		
• Rural registration	22		

n: case number.

claudin-1 immunoreactivity. However, only 91% of the patients without choroidal infiltration showed negative expression. As an additional study on this relationship, choroidal invasion was further stratified into "small amount" or "large amount". It was found that 9% of the patients with choroidal invasion showed negative expression of claudin-1, whereas 45.8% of the patients with a small amount of choroidal infiltration showed negative expression of claudin-1. The expression of claudin-1 with a small amount of choroidal invasion was statistically significant (p=0.05) (Table III).

In order to study the relationship between claudin-1 and tumor differentiation, the samples were divided into two groups. Group I included moderately and well differentiated tumors, while Group II included poorly differentiated tumors. In Group I, 44.4% of the samples showed positive

Table II. IHC expressions of claudin-1 and different clinicopathological variables of RB patients (n=56).

Clinical pathological feature	n (%)	Positive expression(n)	Negative expression (n)	Positive expression rate (%)	p
Clinical stage					
Clinical stage I	21	5	16	23.80%	0.002
Clinical stage II	4	4	0	100%	
Clinical stage III	31	18	13	41.93%	
Degree of differentiation					
Good and moderate differentiation	36	20	16	55.56%	0.98
Poor differentiation	20	7	13	35%	
Infiltration of the optic nerve					
Negative	30	8	22	26.67%	0.001*
Positive	26	19	7	73.08%	
Infiltration of the choroid					
Negative	21	4	17	19.05%	≤0.01
Positive	35	23	12	65.41%	

n: case number, (*): Fisher exact test.

Table III. IHC expressions of claudin-1 and infiltration of the choroid in RB patients (n=35).

Clinical pathological feature	n (%)	Positive expression(n)	Negative expression (n)	Positive expression rate (%)	p
Infiltration of the choroid					
A small amount	24	16	11	66.67%	0.05
A large amount	11	7	1	63.64%	

n: case number.

expression of claudin-1 and 65% of the samples showed positive expression in Group II. There was no significant difference between the two groups in the expression of claudin-1.

IHC Expression of PlGF in RB

Positive PlGF immunoreactivity was detected in 73.8% of the RB patients. In the cytoplasm of tumor cells, IHC staining of PlGF was observed, and PlGF was also expressed in adjacent vasculatures that were not involved in the retina. PlGF expression was more frequent in tumors with optic nerve invasion than that in tumors without optic nerve invasion. This finding was statistically significant (p=0.01) (Table IV). PlGF was positively expressed in most tumors with choroidal invasion (74.1%). However, there was no significant difference between patients with negative choroidal invasion and those with positive choroidal invasion (p=0.43) (Table V) or between patients with a large amount of choroidal invasion and those with a small amount of choroidal invasion (p=0.38) (Table IV). There were no statistically significant differences among PlGF-expressed tumor growth patterns (p=0.51) or among varying degrees of tumor differentiation (p=0.26) (Table V).

IHC Expressions of Claudin-1 and PlGF in RB Correlation of Claudin-1 and PlGF with Different Clinicopathological Variables

Pearson correlation coefficient was used to detect the correlation of claudin-1 expression and PlGF expression with different variables: age, gender, tumor growth pattern, choroidal or optic nerve invasion, degree of differentiation and degree of presence. The expression of claudin-1 was detected to be negatively related to the presence of choroidal invasion (r=0.52, $p \leq 0.0001$) and optic nerve infiltration (r=0.49, p=0.0003). There was a significant positive correlation be-

Table IV. IHC expressions of P1GF and different clinicopathological variables of RB patients (n=56).

Clinical pathological feature	n (%)	Positive expression(n)	Negative expression (n)	Positive expression rate (%)	p
Clinical stage					
Clinical stage I	21	5	16	23.80%	0.51
Clinical stage II	4	0	4	0%	
Clinical stage III	31	8	23	25.81%	
Degree of differentiation					
Good and moderate differentiation	36	7	29	19.44%	0.26
Poor differentiation	20	6	14	30%	
Infiltration of the optic nerve					
Negative	30	11	19	36.67%	0.013*
Positive	26	2	24	7.69%	
Infiltration of the choroid					
Negative	21	5	16	23.81%	0.43
Positive	35	8	27	22.86%	

n: case number, (*): Fisher exact test.

Table V. IHC expressions of claudin-1 and infiltration of the choroid in RB patients (n=35).

Clinical pathological feature	n (%)	Positive expression(n)	Negative expression (n)	Positive expression rate (%)	p
Infiltration of the choroid					
A small amount	24	7	15	21.67%	0.38
A large amount	11	1	12	9.09%	

n: case number.

tween PlGF expression and the presence of optic nerve invasion (r=0.30, p=0.009). In addition, a significant positive correlation was found between claudin-1 expression and PlGF expression (r=0.41, p=0.006) (Table VI).

Discussion

RB is the most common primary intracranial tumor in children. Its most important characteristic is the invasion and metastasis of extraocular tissues, which seriously endangers the vision and life of children. Claudin-1 expression is closely related to the evolution of RB tumors, and multi-factors are involved in the progressive process of occurrence, development and invasion, but the specific mechanism is not yet clear. Recent studies have shown that the loss of adhesion between cells will lead to the loss of connectivity between cells and the invasion and metastasis of tumor cells. It has been found that the adhesive structure has four kinds of connection methods, namely TJ, adhesive tape, gap junction and desmosomes. In recent years, it has been found that the abnormal expression of claudin-1 destroys the integrity of the closely connected cells, which is related to the occurrence, progression and prognosis of malignant tumors.

In this study, the innovative combination of prospective study with IHC method was applied in RB for the detection of the expression of claudin-1 proteins. It was found that 69.2% of all patients at clinical stage I, and 38.9% at clinical stage III presented positive expression of claudin-1, while patients at clinical stage II exhibited no expression. The expression was significantly different (p=0.002). In 26 RB patients with optic nerve infiltration, 7 patients showed positive expression of claudin-1, and for claudin-1, there were 73.3% of the patients without infiltration. The lowly positive expression of claudin-1 was detected in the case of optic nerve invasion, and the expression was significantly different (p=0.001). In addition, the negative expression of claudin-1 with HS was found in patients with choroidal infiltration, and the expression was significantly different ($p\leq$0.01), in which 34.3% of the RB patients with choroidal invasion showed negative claudin-1 immunoreactivity, suggesting that the expression of claudin-1 proteins is related to the clinical stage of RB, the degree of tumor cell differentiation, and the presence of optic nerve infiltration and choroidal infiltration. The study showed that the expression of claudin-1 was down-regulated in RB. The reason might be that the destruction of the integrity of closely connected cells made the RB in the retina more prone to optic nerve infiltration and choroidal infiltration, thus promoting the occurrence and progression of malignant tumors. In this report, it was found that the down-regulation of claudin-1 expression was significantly associated with tumor growth patterns, optic nerve infiltration and choroidal invasion, which is consistent with the study results that the abnormal expression of claudin-1 protein is closely related to the occurrence, progression and prognosis of malignant tumors in a lot of studies[12,13]. At the same time, it has been found recently that there is a close correlation between the claudin-1 expression and histopathological features with poor prognosis (including optic nerve and choroidal infiltration), which is parallel to the study results. Compared with that in patients with the smallest amount of invasion, the expression of claudin-1 in patients with a large amount of choroidal invasion showed a statistically significant reduction.

Table VI. IHC expressions of claudin-1 and PIGF in RB patients (n=56).

	Expression of claudin-1		
	Negative (n)	Positive (n)	p
Expression of PIGF	14 15	5 22	0.01 (HS)

n: case number.

RB is a rapidly growing tumor that frequently exceeds its blood supply, leading to extensive necrotic areas. The microscopic features of RB include concentric arrangement of sleeve-like proliferating living cells around the vessel. In the periphery of the sleeve-like cells, the cells of RB become necrotic. The growth rate of RB is more dependent on the ability of tumors to induce neovascularization, rather than the intrinsic growth rate of tumor cells[14]. Angiogenesis is a key process in tumor progression, which not only provides tumors with necessary oxygen, nutrients and growth factors for growth, but also provides a circulation pathway that allows tumor cell metastasis[15]. Fidler[16] has reported that the potential for angiogenesis of RB is associated with invasive growth and metastasis, and these two factors are associated with poor prognosis. Tumor angiogenesis is regulated by a variety of positive and negative regulatory molecules[17]. When the net effect of the positive regulatory factors exceeds the effect of negative factors, pre-existing blood vessel recruitment and neovascularization occur in the tumor and exist in the so-called process of "angiogenesis"[18]. The most important determinant of angiogenesis is the expression of PlGF in tumor cells[19].

PlGF is a heparin-binding glycoprotein[20] involved in the formation of vascular tumor stroma[21]. It is known that PlGF messenger RNA is expressed in recurrent tumor cells, and that VEGF secreted by tumor cells affects nearby endothelial cells and is used as a paracrine regulator[22]. *In vitro* researches[23] have revealed that PlGF stimulates the division and migration of endothelial cells. In this study, the positive expression rate of PlGF in RB was 73.8%, and the study results are consistent with Zhou et al findings[24]. Wang et al[25] detected the positive PlGF immunoreactivity in 72.5% and 64.7% of RB, respectively. However, Areán et al[26] found a higher expression rate (98%). The prognostic value study of PlGF expression in RB showed the opposite results. Together with Zhou et al[24], we found that PlGF expression was significantly positively correlated with optic nerve infiltration. In addition, Yuan and Song[27] found that PlGF expression is associated with invasion and metastasis. In contrast, Areán et al[26] observed that PlGF expression is not significantly correlated with optic nerve infiltration. Together with Arean et al[27], it was found in this study that there was no statistical significance between PlGF and choroidal infiltration. In present studies, PlGF is not statistically correlated with tumor differentiation.

Conclusions

We showed the direct relationship between claudin-1 and PlGF reactivity in RB for the first time, and their relationships with tumor differentiation, invasion and clinical stage were concluded from the comprehensive statistical analysis. Claudin-1 and PlGF play different roles in the invasion and metastasis of RB, and their mechanism may be different, but their expression may be related. The normal expression of claudin-1 may play a role in maintaining cell morphology. However, in RB tissues, claudin-1 is lowly expressed, and the integrity of the connection between cells will be lost. PlGF overexpression promotes the growth and metastasis of RB tumor cells, and the two interact with each other, inducing the invasion of RB and promoting the progression of tumor cells. In this study, PlGF expression in RB was regulated, which might be associated with optic nerve and choroidal invasion to a certain degree. We suggest that the use of anti-neovascular therapy in RB may be effective in targeting immature neovascularization within the tumor and reduce tumor invasion. The study confirmed the positive relationship between claudin-1 and PlGF in RB for the first time, and also proposed for the first time that the low expression of claudin-1 and the high expression of PlGF in RB may be the indexes for optic nerve and choroidal invasion and high-risk progression of RB. Further research is necessary, and its application and guidance for clinical treatment still require a lot of time for more in-depth studies and clinical practice. At the same time, the accuracy and practicality of the test method still need to be improved to effectively develop the treatment strategy.

Conflict of Interests

The authors have no conflicts of interest to declare.

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