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Retinoblastoma protein expression in nasopharyngeal carcinoma

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Background : *Alterations in the retinoblastoma (RB) gene and its protein products have been detected in numerous solid tumor malignancies. Loss of RB function is believed to contribute to neoplastic transformation.*

Objective : *To study the alteration of* pRB *in nasopharyngeal carcinomas (NPC), we designed to determined* pRB *status by immunohisto-chemical analysis in paraffin-embedded tissue sections.*

Materials and methods : *Since a variety of RB gene mutations have been described which cause absence or alterations of* pRB *expression, thirty - eight formalin fixed, paraffin-embedded tissue sections were examined for the pRB expression, and the results correlated to the patients' characteristics.*

Results : *There was alteration of* pRB *expression in 16 of the 38 (42.1%) tissue sections. Although there was no statistically significance ($p < 0.05$) between the expression alteration and the patients' characteristics, our data demonstrated that the defective expression*

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in 3 of 12 (25%) in the low stage (I and II) and the higher defect was found in 13 of 26 (50%) in the high stage (III and IV).

Conclusions : *Our data not only showed the early* pRB *alteration but also the important role in NPC multistage tumorigenesis. Further study to determine of the pathogenesis of Epstein-Barr virus (EBV) in NPC should provide an important explanation for NPC tumorigenesis.*

Key words : *Retinoblastoma expression, Nasopharyngeal carcinoma.*

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ปัญหาของการทำวิจัย : *มีรายงานความผิดปกติของยีนและโปรตีน RB ในโรคมะเร็งหลายชนิด เชื่อว่าการสูญเสียการทำงานของยีน RB มีความสำคัญต่อการเกิดเป็นโรคมะเร็ง*

วัตถุประสงค์ : *เพื่อศึกษาหาความผิดปกติของโปรตีน RB ในโรคมะเร็งโพรงหลังจมูก คณะผู้วิจัยทำการตรวจหาโปรตีน RB โดยวิธี immunohistochemistry ในชิ้นเนื้อพาราฟิน*

วัสดุและวิธีการวิจัย : *เนื่องจากมีรายงานว่าการเกิดการกลายพันธุ์ของยีน RB ทำให้เกิดความผิดปกติในการตรวจพบโปรตีน RB คณะผู้วิจัยจึงทำการย้อมหาโปรตีน RB ในชิ้นเนื้อพาราฟิน 38 ราย และทำการศึกษาหาความสัมพันธ์กับคุณสมบัติของผู้ป่วย*

ผลการวิจัย : *พบว่ามีความผิดปกติของการตรวจพบโปรตีน RB 16 ราย ใน 38 ราย (คิดเป็นร้อยละ 42.1) แม้จะไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ระหว่างการตรวจพบโปรตีน RB และความสัมพันธ์กับคุณสมบัติของผู้ป่วย แต่ข้อมูลของคณะผู้วิจัยได้แสดงให้เห็นว่ามีความผิดปกติของการตรวจพบโปรตีน RB 3 ราย ใน 12 ราย (คิดเป็นร้อยละ 25) ในกลุ่มระยะต้น ๆ (I และ II) และพบความผิดปกติของการย้อมพบโปรตีน RB 13 ราย ใน 26 ราย (คิดเป็นร้อยละ 50) ในกลุ่มระยะรุนแรง (III และ IV)*

สรุป : *ข้อมูลของคณะผู้วิจัยได้แสดงให้เห็นว่าไม่เพียงแต่จะพบว่ามีความผิดปกติของการตรวจพบโปรตีน RB ในระยะต้นเท่านั้น แต่พบว่าความผิดปกติของการตรวจพบโปรตีน RB มีความสำคัญต่อระยะต่าง ๆ ของโรคมะเร็งโพรงหลังจมูก การศึกษาหาพยาธิกำเนิดจากการติดเชื้อไวรัส Epstein-Barr ของโรคมะเร็งดังกล่าวเป็นสิ่งที่ควรศึกษาต่อไป*

Nasopharyngeal carcinoma (NPC) is one of the most common cancers in southern China and Southeast Asia.[1-2] It is unique among cancers of the head and neck in that it affects young patients, frequently has an undifferentiated histological appearance, and is associated with Epstein-Barr virus (EBV) infection.[3] Several genetic alterations in NPC pathogenesis involve multiple genetic changes associated with the activation of oncogenes and the inactivation of tumor suppressor genes.[4-5]

The retinoblastoma gene (*RB*) was originally identified as the tumor suppressor gene involved in hereditary retinoblastoma.[6] It encodes a 110 to 116 kDa nuclear protein known as the retinoblastoma protein (pRB).[7] The pRB acts as a transcriptional regulator of genes involved in DNA synthesis and cell-cycle control.[8] The *RB* gene is mutated or othewise inactivated in a wide array of human tumors, which in turn results in absent or defective pRB expression.[9-10] Several studies which have identified allele loss in the vicinity of the *RB* gene in head and neck cancer,[11] but this loss was not necessarily associated with absence of the pRB.[12-13] A few studies have showed that there are no *RB* gene alterations in NPC,[14] but little is known about pRB expression in this cancer.

To evaluate the presence or absence of the protein product in tumor cells, our study conducted immunohistochemistry assays for pRB. Immunostaining is advantageous in its ability to demonstrate the absence of the protein regardless of the mechanism for that absence. Specifically, homozygous deletion, and hemizygous deletion accompanied by mutation of the remaining allele, usually results in no protein production. In this study, the pRB expression was examined in formalin-fixed, paraffin-embedded tissue sections of 38 NPC cases. Results were interpreted in relationship to tumor histology, staging, and patients' sex and age.

Materials and Methods

Tumor specimens:

Thirty-eight formalin fixed, paraffin-embedded nasopharyngeal cancer specimens were obtained from the Department of Pathology, Faculty of Medicine, Chulalongkorn University. All tumors were examined by one pathologist to determine histological differentiation of tissues according to WHO classification. All cases were determined for TMN stage according to the American Joint Committee for Cancer Staging and End Results Reporting (AJCC). All patients' data were reviewed and characteristics are shown in Table 1.

Immunohistochemistry:

RB immunostains were performed on formalin fixed, paraffin-embedded tissue sections using purified mouse anti-human pRB monoclonal antibody (Mab) clone PMG G3-245 which was obtained from PharMingen (San Diego, CA). As a detection system the Vector (Burlingame, (A) ABC Elite kit was used. The antigen retrieval method was modified from a procedure described by Shi et al.[15] After methanol hydrogen peroxide blocking, slides were washed in distilled water and immersed in a Coplin jar filled with Tris-HCl : 0.01 M acetate buffer (pH 1). They were then incubated at 100°C in a microwave oven for 5 min x 4 times. After heating, they were transferred immediately to a distilled water rinse followed by 2 phosphate buffer saline (PBS) (pH 7.6) washes. For immunohistochemical analysis, the PMG G3-245 Mab were used at concentrations of 1.0 mg/mL. Incubation time was 2 hours at room temperature, followed by Vector ABC

Table 1. Patients' characteristics.

Characteristics	Total	Male	Female
1. Number	38	27	11
2. Age (year)	51.9 (20 - 72)	53.6 (20 - 72)	47.7 (21 - 70)
3. WHO Classification			
Type I - Keratinizing squamous cell carcinoma	-	-	-
Type II - Non – keratinizing carcinoma	16	11	5
Type III - Undifferentiated carcinoma	22	16	6
4. TNM stage			
Stage I	3	3	-
Stage II	9	7	2
Stage III	11	6	5
Stage IV	15	11	4

Elite kit protocol. Counter stain was performed by using methyl green.

Interpretation:

The pRB expression in a tissue section of a neoplasm was considered normal if definite nuclear staining could be identified in all areas of the tumor. Frequently, staining intensity was not uniform, and not all nuclei, either normal or neoplastic, were stained. The pRB expression in a tumor was considered aberrant if either the whole section or major focal areas within the section showed no nuclear staining in neoplastic cells and if definite nuclear staining was seen in immediately adjacent non-neoplastic cells. Cytoplasmic staining, which was noted in some specimens, was disregarded. The pRB expression was assessed by estimating the staining of nucleus, and the maximum staining intensity was graded as -(absent), +/- (very weak),+ (weak but distinct), 2+(moderate) or 3+(strong) (Figure 1.). Distinct nuclear immunostaining was interpreted as a positive (+, 2+, and 3+) staining result for pRB.[16]

Statistical Analysis:

Statistical significance among categorical data was evaluated using the Chi-square (x^2) test, and confidence level as percentage and $p < 0.05$ was considered statistically significant.

Results

Thirty-eight cases of NPC were evaluated for pRB expression by the an immunohistochemical method using the PMG G3-245 Mab. Among these 38 cases, 27 were males with an average age of 53.6 year old and 11 were females with an average age of 47.7 year old. Sixteen cases were classified as WHO

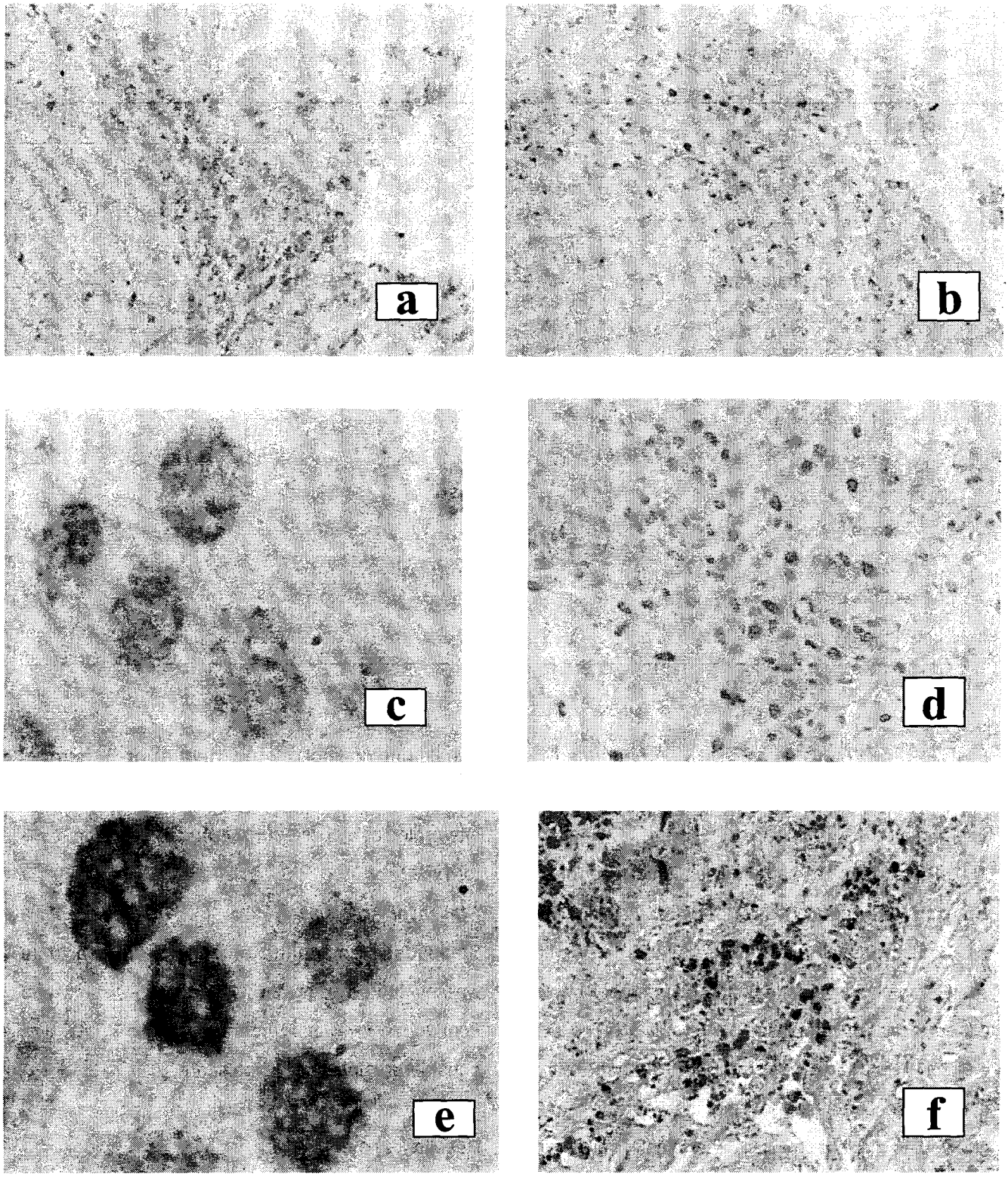

Figure 1. Immunohistochemical staining of NPC using PMG3-245 Mab.

a). Tumor with very weak (+/-) pRB staining (magnification x 100). b). and c). NPC tissues showing weak (+) pRB expression (x 100) and oil magnification (x 1000) in respectively. Moderate (2+) pRB expression at low (x 100) and oli power (x 1000) in d). and e). in respectively. f). Tumor with strong (3+) pRB staining (x 100).

type II, and 22 cases were WHO type III. Three, 9, 11 and 15 cases were graded as TMN stage I, II, III and IV, respectively. The pRB expression was detectable in 22 of the 38 (57.9%) cases. According to our data (Table 2), there was no statistically significant difference ($p > 0.05$) of the pRB expression between sex [male: pRB positive was 15 of 27 (55.6) cases; female: pRB positive was 7 of 11 (63.6%) cases], age [$\leq$ 40 year old: pRB positive was 7 of 10 (70%) cases; > 40 year old: pRB positive was 15 of 28 (53.6%) cases], WHO classification [type II: pRB positive was 9 of 16 (56.2%) cases; type III: pRB positive was 13 of 22 (59.1%) cases], and TMN stage [stage I-II, low stage group, pRB positive was 9 of 12 (75%) cases; stage III-IV, high stage group, pRB positive was 13 of 26 (50%) cases]. However, from the data, the alteration of pRB expression was an early event in NPC, since the defective pRB expression was found in 3 of 12 (25%) cases during stages I-II and the abnormal pRB expression was demonstrated higher level in 13 of 26 (50%) cases regarded to the high stage (stage III and IV) cancer (Figure 2.). In addition, the total alteration of the pRB expression was 16 of 38 (42.1%) cases. This data suggests the possible important role in the development of NPC.

Table 2. The pRB expression in NPC (n=38).

Chracteristics	pRB expression		P - value
	negative	positive	
1. Total (n = 38)	16 (42.1%)	22 (57.9%)	
2. Sex : Male (n = 27)	12 (44.4%)	15 (55.6%)	P > 0.05
Female (n = 11)	4 (36.4%)	7 (63.6%)	
3. Age (year) :			
$\leq$ 40 year old (n = 10)	3 (30%)	7 (70%)	P > 0.05
> 40 year old (n = 28)	13 (46.4%)	15 (53.6%)	
4. WHO classification			
Type II (n = 16)	7 (43.8%)	9 (56.2%)	P > 0.05
Type III (n = 22)	9 (40.9%)	13 (59.1%)	
5. TNM stage			
Stage I - II (n = 12)	3 (25%)	9 (75%)	P > 0.05
Stage III - IV (n = 26)	13 (50%)	13 (50%)	

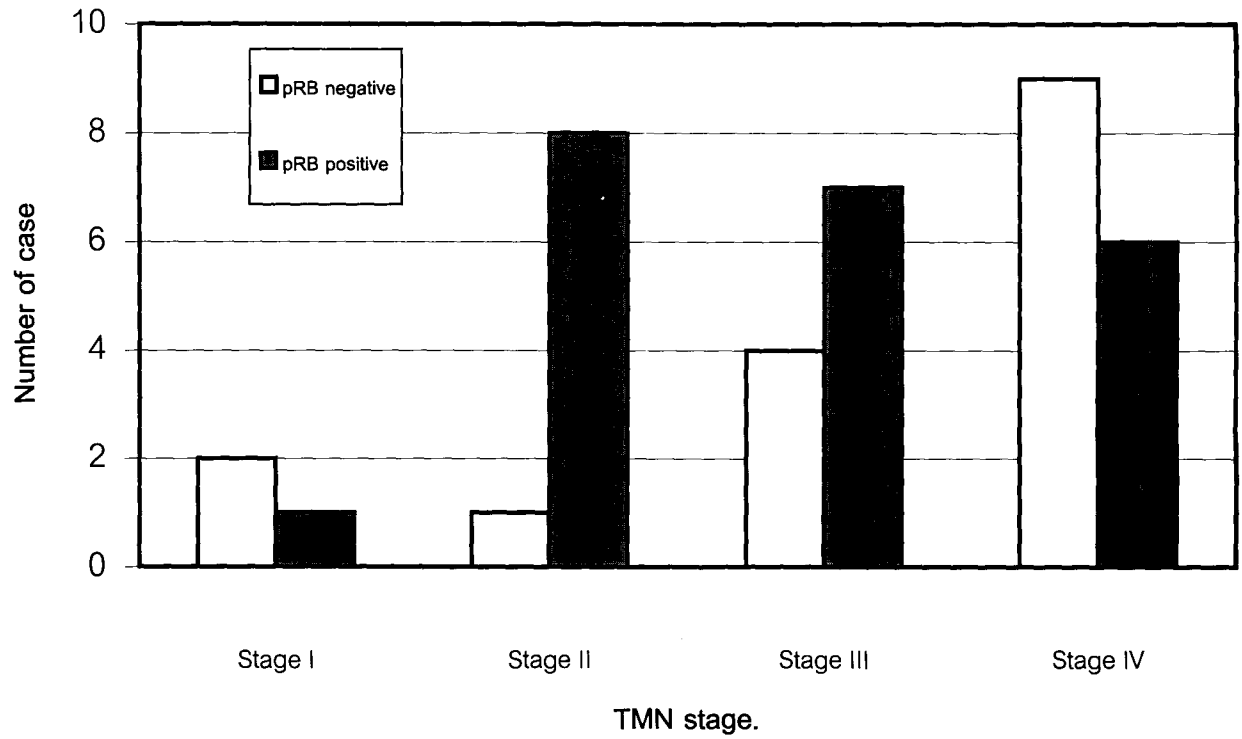

Figure 2. The pRB expression in TMN stage. The low stage group (stage I and II) demonstrates the tendency of pRB positive while the hight stage (stage III and IV) shows the trend of pRB negative.

Discussion

The *RB* gene is located on chromosome 13, includes 27 exons, and encompasses approximately 200 kb. Because of its large size detailed molecular genetic analysis is difficult. Loss of *RB* function in tumor cells has been determined by loss of heterozygosity (LOH), Southern blot, Northern blot, Western blot or immunoprecipitation analysis. However, the detection of *RB* gene using these molecular techniques has been limited by time-consuming from its large size and contamination with non-neoplastic tissues.[16] Immunohistochemical analysis of pRB expression offers a practical alternative for studying its status, but this method also has limitations. The vast majority of reported *RB* mutations result in a complete absence of the pRB expression which is reflected as a lack of nuclear staining in immunohistochemical analysis.[9-10] Furthermore, in a small number of cases, *RB* gene mutation may result in the production of a truncated protein or a full-length mutant protein.[17] In these cases immunohistochemical analysis may not be able to distinguish mutant protein from wild-type protein.[18] Consequently, in a relatively small percentage of cases, nuclear reactivity may be observed even in the presence of a mutation. Nonetheless, with the proper staining, immunohistochemical analysis of the pRB expression generally provides an accurate reflection of *RB* status.

The *RB* gene was first defined as a tumor suppressor gene and was originally described in retinoblastoma, a rare childhood tumor of the eye.[6, 9] More recently, alterations in RB have been observed

in a variety of human neoplasms.[19] There are reports that the pRB alteration is associated with poor prognosis in some cancers, such as carcinomas of the urinary bladder,[20] lung,[21] breast,[22] prostate[23] and liver.[24] However, there are few studies which have examined the *RB* gene and pRB expression in NPC.[14,25] Clinical and experimental data suggest EBV as an important etiologic agent for NPC.[3] EBV is a DNA virus belonging to the Herpes virus group, it encodes a number of transformation associated proteins, designated EBV nuclear antigen 1-6 (EBNA-1-6). EBNA-5 protein (alternatively designated EBNA-LP) can form a molecular complex with the retinoblastoma protein.[26] Since the mutational or other alterations of *RB* gene are not common in nasopharyngeal carcinogenesis,[14,25] the mechanisms of impairment of pRB function involved by EBV proteins might be the important role. We determined to examined the pRB expression in formalin-fixed, paraffin-embedded tissue sections of 38 NPC cases and its intensity of expression was correlated with the characteristics of the patients. Although our data could not demonstrate statistical significance between the pRB expression and patients' characteristics, the tendency of the alteration of its expression was shown to be higher in the more than 40 year old group and the high TMN stages (III and IV). Our data demonstrated alteration of expression during the early stage [stage I: the alteration of pRB expression was found in 2 of 3 (66.7%); stage II: the alteration of pRB expression was shown in 1 of 9 (11.1%)]. However, the pRB alteration in stage I is higher than in stage II in our study due to too small number of cases in stage I. Usually, the stage I cancer find hardly in developing country where the people decide to look for the diagnosis lately. In addition, the altered pRB expression we demonstrated in 16 of 38 (42.1%) cases. Our study suggested that the role of the pRB protein in NPC was an early important event and may be related to the severity of the cancer. Our finding was different from the previous study[14] that concluded the rare *RB* gene alterations in NPC. Possible explanations may be the small number of cases (7 cases) of that study, the different method used, and the different patients' characteristics. The more important explanation might involve the pRB inactivation mechanisms. First is the disruption by pRB phosphorylation, second by viral oncoprotein binding to pRB, and finally by mutation of *RB* genes.[19] Since EBV appears to be an important etiological factor,[3] the viral oncoprotein binding to pRB might be the main mechanism to inactivate pRB functions. Further study of EBV pathogenesis of NPC should be an important approach to understand the multistage carcinogenesis of this cancer.

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