Immunohistochemical Study of Retinoblastoma Protein (Prb) Expression in Cervical Carcinomas and Their Premalignant Lesions

Arwa Mujahid Abdullah Al-Shwaikh[*] MSC

Dr. Ismail Ibrahim Latif[**] Ph.D.

Abstract

The retinoblastoma gene was the first tumor suppressor gene identified that was altered not only in retinoblastomas but has been described in a wide variety of human neoplasms. The retinoblastoma gene encodes a nuclear phosphoprotein that in its hypophosphorylated stat plays an important role in regulation the cell cycle, thus preventing from tumor formation. This study was designed to determine pRB expression in a group of cervical tissues comprised of normal, premalignant and malignant lesions. A total of 60 cervical tissue samples were included in this study. 50 archival tissue biopsy samples comprised a risk group for cervical neoplasia; these were selected from histopathology files of Al-kadhimya teaching hospital, Al-Ulwiya teaching hospital, Al-Yarmouk hospital, Medical City department of teaching laboratories, and from four private laboratories. The remaining 10 normal cervical postmortem tissue biopsies were obtained from the institute of forensic medicine and considered as control group. Immunohistochemistry was done for detection pRb expression. The results of this study found that there were significant differences of pRb expression between CINII/III or ISCC and normal cervical tissue ($p<0.01$, $p<0.05$ respectively). There was no significant difference between CIN I and CIN II/III ($p>0.05$). There were no significant differences between CIN I or CIN II/III and ISCC in relation to pRb expression ($p>0.05$).This results suggest that Rb protein expression may be important in the pathogenesis of cervical carcinoma.

Key words: Cervical carcinoma, tumor suppressor protein (pRb), immunohistochemistry.

Introduction

Cervical carcinoma is the second leading cause of the death in women worldwide [1]. In Iraq, the neoplasms of cervix uteri ranked the 6th among the commonest ten cancers in female during the period 1976-1985, whereas during the period 1995-1997 it ranked the tenth within the leading

Al- Nahrain University \ College of Medicine \Department of Medical Microbiology *

Diyala University\ College of Medicine\ Department of Anatomy **

cancers in females [11, 12]. From the overview of cervical cancer situation we found that cancer of the cervix uteri constitutes 1.4% of the total number of cancer reported in 1995, 1996 and 1997, respectively [11].

Many epidemiological studies over the last 20 years have established a strong association between the high risk of human papillomavirus (HPV type16 and 18) and cervical cancer, up to 95% of cases [21]. However viral infection alone is not sufficient to initiate malignant transformation. Additional genetic changes must occur in order for malignant transformation [3].

pRb is a nuclear protein of 105 to 110 KDa and its locus is located on chromosome 13q 14 [13,16]. Rb protein play a pivotal role in eukaryotic cell cycle regulation. Hypophoshorylated Rb binds to transcription factors of the E2F family and represses the transcription of particular cell cycle genes. When cell progress in mitosis from G0 through G1 to S-phase, Rb gets hyperphosphorylated by G1 cyclin-cyclin dependent kinases, releasing the transcription factor E2F, which in turn activates genes involved in DNA synthesis and cell cycle progression [4]. Rb remains phosphorylated during S, G2, and M, until late M, when it is dephosphorylated by a specific phosphatase [16].

Materials and Methods

***Tissue Samples**:* A total of 60 tissue samples from uterine cervix were included in this study. 50 out of 60 samples comprised a risk group in our study were obtained from archival paraffin embedded blocks selected from the histopathology files of Al-Kadhimiya Teaching Hospital, Al-Ulwiya Teaching Hospital, Al-Yarmouk Hospital, Medical City Department of Teaching Laboratories, and from four private laboratories. The remaining 10 samples (autopsies) which comprised a normal control group were obtained from the Institute of Forensic Medicine.

Ethical approval for use of all specimens was obtained and the histopathologic diagnosis was confirmed by review of freshly prepared hematoxylin and eosin-stained slides by certified pathologists and classified according to criteria outlined by the World Health Organization.

Materials:

- Mouse Anti-Human Rb protein (DAKO); Clone: Rb1; Isotype: IgG1, kappa; Code number: M7131; Specificity to retinoblastoma gene product.
- Research Kit detection system; Code number OA315 (DakoCytomation, Denmark).

Procedure of Immunohistochemistry (IHC): The procedure of immunohistochemical was carried out in accordance with manufacturer's instructions (DakoCytomation, Denmark). The process include: baking the slides in a hot air oven at 60^{o}C overnight and then washed in xylene. After gradual

hydration through graded alcohols, the sections were rinsed in distilled water. After that the antigen retrieval [6, 15] and endogenous peroxidase quenching were done. For localization of Rb protein, the sections were incubated for 60 min at 37^{o}C with a 1:50 dilution of the monoclonal antibody of pRb then placed at 4^{o}C overnight. After that the slides were visualized by the visualization reagent and incubated at 37^{o}C for 30 min. 100 μl freshly prepared DAB-substrate chromogen solution was placed onto the section and incubated in darkness at room temperature for 10 min. Then the slides were rinsed gently with wash buffer and counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted.

Quality Control: Sections from a colon cancer patient that were known to be immunoreactive for pRb antibody were used as positive controls for pRb. Further, the admixed nonneoplastic inflammatory cells within the tissue were considered as internal positive control for pRb detection [17].The negative control was obtained by replacing the primary antibody with PBS buffer [15]. Both positive and negative controls were included for each run of immunohistochemistry.

Evaluation of the Immunostaining:

Slides were examined by light microscope at X400 magnification. Cells were noted as positive when they showed nuclear immunoreactivity (brown precipitate). Cytoplasmic staining was disregarded. Only the neoplastic region of each tissue section was evaluated. The percentage of positive cells in each tissue section was estimated on a semi-quantitative. Scale where O: less than 10%, 1: 10-50% and 2: more than 50% of the total tumoural area. After the

examination of the slides by two independent observers, a global agreement was reach regarding the result [18].

Statistical analysis:

Anova analysis program was used to calculate the difference in the mean of pRb immunostaining among different histological type of cervical lesions. Analysis was performed using SPSS program. A *p* value of less than 0.05 was considered statistically significant.

Results

By using immunoassay techniques, we investigated pRb immunodetection pattern in normal tissues, CIN I, CIN II/III and ISCC. The pattern of pRb immunostaining and the histological diagnosis is summarised in Table (1).

pRb immunoreactivity was observed in 7/10 (70 %) of normal cases. All positive cases showed +1 (10-50 %) pRb immunoreactive cells. The staining was found in parabasal cell layers of the squamous cervical epithelium. In these cases, the squamous epithelial cells in other cell layers were completely negative for pRb protein expression. In CIN I, pRb staining was seen in 8/9(88.9%) of cases. The percentage of pRb positive cells was higher in CIN I than in normal cervical epithelium, 5/9 samples were given a value of +1(10-50% of positive cells). The pRb staining was confined in basal third of squamous epithelial of CIN I lesions. In CIN II/III, pRb staining was seen in 13/14(92.9) of cases, 6/14 samples were given a value of +1(10-50%) and 7/14 samples showed +2(>50%). CINII/III cases showed pRb-positive cells in basal two-third or full thickness of cervical epithelium, Figure: (1B and 1C). In ISCC 23/27(85.2%) of cases exhibited immunoreactivity for pRb, 11/27(40.7%) were given a value of +1 and 12/27(44.4%) showed +2(>50) pRb immunoreactivity, Figure: (1D).

There were significant differences of pRb expression between CINII/III or ISCC and normal cervical tissue ($p<0.01$, $p<0.05$ respectively). There was no significant difference between CIN I and CIN II/III ($p>0.05$). And there were no significant differences between CIN I or CIN II/III and ISCC in relation to pRb expression ($p>0.05$) Table (2).

Table (1): Expression of pRb in relation to histological type of the cases examined.

Histological type	pRb positive (%)			Total (%)
	0 (<10)	+1 (10-50)	+2 (>50)	
Normal	3/10 (30)	7/10 (70)	0 (0)	7/10 (70)
CIN I	1/9 (11.1)	5/9 (55.6)	3/9 (33.3)	8/9 (88.9)
CIN II/III	1/14 (7.1)	6/14 (42.9)	7/14 (50)	13/14 (92.9)
ISCC	4/27(14.8)	11/27(40.7)	12/27(44.4)	23/27 (85.2)

Table (2): The difference of pRb immunostaining among different histological type of cervical lesions.

Histological type	CIN I	CIN II/III	ISCC
Normal	0.192 (NS)	0.004 (HS)	0.026 (S)
CIN I	□	0.146 (NS)	0.539 (NS)
CIN II/III	□	□	0.241 (NS)

ISCC	□	□	□

*HS = highly significant (p<0.01), S = significant (p<0.05), NS = non significant (p>0.05) (Anova test, LSD)

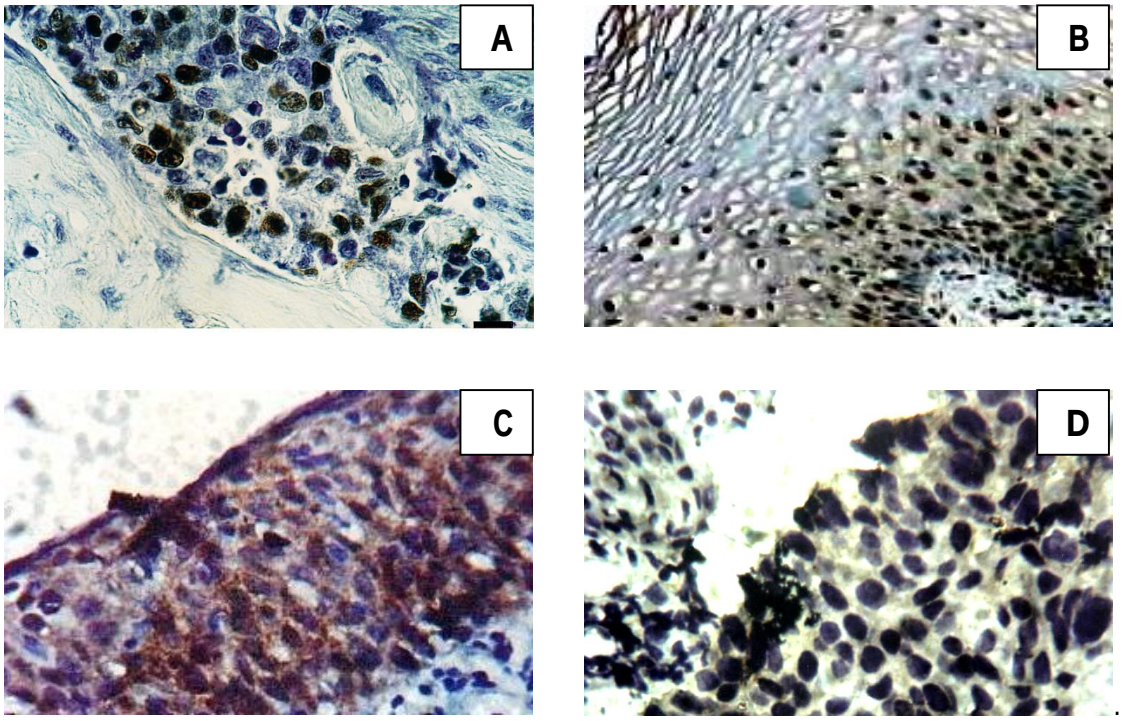

Figure (1): Immunohistochemical staining of Rb protein. Staining by DAB chromogen (dark brown), counterstained with Mayer's heamatoxylin. [A] Colorectal carcinoma, positive control for pRb (X400). [B,C] pRb immunoreactive cells involving the basal two-third of CINII (B, X400) and entire of the thickness of CINIII lesions (C, X400) . [D] ISCC, showing 75 – 100% pRb immunorective cell (X400).

Discussion

The protein product of the *Rb* gene (pRb) is a nuclear phosphoprotein that plays an important role in regulating the cell cycle [14]. Therefore loss of normal Rb function may allow cells to proliferate in uncontrolled manner, not only to initiate event in tumorigenesis, but also as a step associated with malignant progression and progressive out come [18, 17].

This study showed overexpression of pRb in majority of premalignant and malignant lesions of the uterine cervix as compared with normal cervical aquamous epithelium (Table 1). Statistical analysis showed significant differences in pRb expression between normal and CIN II/III lesions, also between normal and ISCC (Table 2).

On the protein level, previous analysis of pRb expression in cervical tissues ranging from normal to invasive carcinoma revealed a statistical difference in staining pattern of pRb between normal / reactive cervical biopsies and CIN lesions ($p<0.05$) [2]. Expression of pRb protein product (pRb) in normal ectocervical squamous epithelium and normal esophageal mucosa (78% and 100% respectively) was reported in previous studies [2, 22]. Our study showed a similar finding with 70% pRb expression which was limited to the basal third layer of normal cases.

The primary antibodies used in this study were mouse anti-human Rb protein (Dako Cytomation, Denmark), which was recognized both the unphosphorylated and phosphorylated form of pRB may inhibit proliferation of mature differentiating epithelial cells, rather than controlling cell-cycle progression of their dividing progenitor [18].

Our study, pRb immunoreactivity was found in (88.9%, 8/9) CIN I and (92.9%, 13/14) CIN II/CIN III as compared to previous analysis in which (100%, 43/43) cervical intraepithelial lesions showed positive pRb immunoreactivity [17]. While another study showed (94.4%, 34/36) CIN lesions (17 CIN I and 17 CIN II/III) showed positive pRb immunoreactivity [2]. Higher proportions of pRb positive cells were observed in CIN II/CIN III lesions as compare to normal cervical epithelium (Table 2) and this comparison was statistically significant ($p<0.01$).

Increased pRb expression in premalignant and malignant lesions in general may be due to increase proportions of proliferating cells [2, 22]. This is supported by the fact that hyperphosphorylated pRb is increased during G2/M phases [9].

The pRb immunoreactive tumor cells were found in 23 out of 27 (85.2%) cases of invasive cervical carcinomas in this study. One study has reported pRb expression in all 74(100%) primary carcinomas of cervical analyzed [19], while another study showed pRb expression in 64/65 (98.5%) (Norniani *et al.,* 2003)[17]. Our results were consistent with (Chetty *et al.,* 1997) [5]who found pRb expression in 43/50 (86%).

We found(14.8%) of ISCC in our series had low or loss pRb expression. Low expression of pRb may be related to pRb inactivation resulting from complex formation with high-risk HPV E7 oncoprotein and it's degradation [7,8]. Alternative explanation includes *Rb* gene mutations, but this has been shown to be infrequent in cervical tumors [10]. It has been proposed that mutation in *Rb* gene play a limited role in these tumors and may represent the late event in carcinogenesis [17]. In our study, no attempt was made to study the influence of stage of the invasive cervical carcinoma on pRb expression, because the number of specimens evaluated in this study is too small to allow statistically significant conclusion to be drawn. Noraini *et al.* (2003) found that pRb-positive cells

were found in much higher percentages in well differentiated ISCC (81.8%) compared to moderately and poorly differentiated cases (64.3%, 7.1%, respectively)[17].

In a study carried by Wang and Lu (2004), found that the loss of nuclear pRb expression in small cell carcinoma is not evident may suggest that Rb protein is deregulate through two different mechanisms in HPV-related tumors. pRb deregulation is achieved through accelerated protein degradation as evidenced by a negative nuclear staining or pRb is inactivated by the HPV E7 oncoprotein at the functional level and thus the nuclear pRb protein detectable immunohistochemically [20].

In this study, the majority of the pRb positive cases showed a heterogeneous staining pattern. The intensity of the nuclear staining varied from cells to cells with variable staining proportion of cells having an unstained nucleus. Similar finding have been observed and reported by (Zur Hausen *et al*, 1999)[22].This variation in staining probably resulted from asynchronous progression of the cells through the cell cycle[17] .

We found that some ISCC cases showed nuclear and cytoplasmic pRb staining but most published reports indicate nuclear staining in a variety of tumours and normal tissues, considering cytoplasmic localization as an artifact or related to Rb gene mutation. In addition, some of the actively dividing cell (mitotic cells) showed cytoplasmic immunoreactivity with or without staining of the chromosomes, indicating that in the absence of nuclear envelope pRb diffuses to other cellular location [18]. However, additional studies should be carried out to support these observations.

References

[1] Abd El All, H., Rye, A. and Duvillard, P.,: 53 immunohistochemical expression of Egyption cervical carcinoma. Pathology Oncology Research, 1999, 5 (4), : 280–284.

[2] Amortegai, A.J., Meyer, M.P., Elborne, V.L. and Amin, R. M.,: 53, Retinoblastoma gene product, and cyclin expression in human papillomavirus DNA-positive cervical intraepithelial neoplasia and invasive cancer. Mod. Pathol. 1995, 8 (9), 907-912.

[3] Bishop, J.M.,: Molecular themes in oncogenesis. Cell, 1991, 64, 235-248.

[4] Chakrabarti, O. and Krishna, S., : Molecular interaction of "high risk" human papillomaviruses E6 and E7oncoproteins: implications for tumor progression .J.Biosci, 2003,28(3), 337–348.

[5] Chetty, R., Bramdev, A., Aguirre - Arteta, A., Pegoraro, R.J. and Sataar, N., : Relation between retinoblastoma and p53 proteins in human papilloma virus 16/18 positive and negative cancers of the uterine cervix. J. Clin. Pathol, 1997, 50 (5), 413 – 416. (Abstract).

[6] Cook, D.J., : Immunological techniques. In: Cellular Pathology. Palliste, C.J. (editor), Butterworth Heinemann,2000, Chapter 10, 190.

[7] Fiedler, M., Muller-Holzner, E., Viertler, H.-P., Widschwendter, A., Laich, A., Pfister, G., Spoden, G.A., Jansen-Durr, P. and Zwersch, W., 2004: High level HPV-16 E6 oncoprotein expression correlates with reduced pRb-levels in cervical biopsies. The F.A.S.E.B. Journal. (Abstract).

[8] Gonzalez, S.L., Stremlau, M., He, X., Basile, J.R. and Munger, K., : Degradation of the papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from prpteasomal degradation of E7. Journal of Virology, 2001,75 (16), 7583 – 7591.

[9] Goodrich, D.W., Wang, N.P., Qian, Y.W., Lee, E.Y.H.P. and Lee, W.H., : The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell, 1991,67, 293-302.

[10] Herrington, C.S., Graham, D., Southern, S.A., Bramdev, A. and Cheitty, R., : Loss of retinoblastoma protein expression is frequent in small cell neuroendocrine carcinoma of the cervix and is unrelated to HPV type. Hum. Pathol, 1999, 30 (8), 906 -910.

[11] Iraqi Cancer Board., : Results of Iraqi Cancer Registry 1995 -1997.Ministry of Health (editor), Baghdad, Iraq, 1999,11-13, 34, 32–39.

[12] Iraqi Cancer Registry Center, : Results of Iraqi Cancer Registry 1976-1985. Ministry of Health (editor), Baghdad, Iraq, 1987,18, 35, 59.

[13] Kim, J.-R., Kim, S.-Y., Kim, M.-J. and Kim, J.-H., : Differential expression of protein related to START checkpoint of the cell cycle in human stomach, lung, cervix and liver cancers. Experimental and Molecular Medicine, 1997A, 29 (2), 123 – 128.

[14] Kim, J.W., Lee, C.G., Han, S.M., Kim, K.S., Kim, J.O., Lee, J.M., Kim, I.k. and Namkoong, E.E.,: Loss of heterozygosity of the retinoblastoma and p53 genes in primary cervical carcinoma with human papilloma virus infection.Gynecol. Oncol, 1997B.67, 215-221.

[15] Miller, R.T., (2001): Technical immunohistochemistry: achieving reliability and reproducibility of immunostaions. Propath. Laboratory, Inc. (Available online at http://www.propathlab.com).

[16] Munger, K. and Howley, P.M., : Human papillomavirus immortalization and transformation functions. Virus Research, 2002,89, 213 – 228.

[17] Noraini, M.D., Siti-Aisah, M.A. and Kwan, S.W., : An immunohistochemical study of retinoblastoma gene product in normal, premalignant and malignant tissues of the uterine cervix.

Malaysian Tournal of Medical Sciences, 2003,9 (2), 52 – 59.

[18] Salcedo, M., Taja, L., Utrea, D., Chavez, P., Hidalgo, A., Perez, C., Benitez, L., Castaneda, C., Delgado, R. and Gariglio, P., : Changes in retinoblastoma gene expression during cervical cancer progression. Int. J. Exp. Path., 2002, 83, 275 – 285.

[19] Skomedal, H., Kristensen, G.B., Lie, A.K. and Holm, R., : Aberrant expression of the cell cycle protein TP53, MDM2, p21, cdk4, cyclin D1, RB and EGFR in cervical carcinomas. Gynecol Oncol, 1999, 73(2), 223-8.

[20] Wang, H.L. and Lu, D.W., : Detection of human papillomavirus DNA and expression of p16, Rb, and p53 protein in small cell carcinomas of the uterine cervix. The American Journal of Surgical Pathology, 2004,28 (7), 901 – 908.

[21] Young, L.S., Bevan, I.S., Johnson, M.A., Blomfield, P.I., Bromidge, T., Maitland, N.J. and Woodman, C.B.J., : The polymerase chain reaction. A new epidemiologic tool for investigating cervical human papilloma virus infection. Br. Med. J. 1998, 298, 14-18.

[22] Zur Hausen, A., Sarbia, M., Heep, H., Willers, R. and Gabbert, H.E., : Retinoblastoma-protein (pRB) expression and prognosis in squamous cell carcinoma of the oesophagus. Int. J. Cancer (Pred. oncol.), 1999, 84, 618-622.