Balkan Med J 2014;31:202-7 © 2014

Immunohistochemical Expression of Apoptosis Regulators in Squamous Cell Carcinoma of the Cervix and Their Association with Human Papillomavirus 16/18 Subtypes

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Background: Human papillomavirus (HPV) infection is an important aetiological factor in squamous cell carcinoma (SCC) of the cervix. Limited studies have been focused on the differences between carcinogenesis of SCCs with and without HPV infection.
Aims: The main goal of this study is to determine the expression of some of the apoptotic pathway regulators, including P53, Bax and Bcl2 in SCCs with and without high risk HPV 16/18 infection.
Study Design: Cross sectional study.
Methods: A total of 42 paraffin-embedded blocks with the histopathological diagnosis of invasive SCC with determined HPV 16/18 status were selected; half of them were HPV positive and the rest were negative. Afterwards, immunohistochemistry stained slides for p53, Bcl2 and Bax were evaluated with H-score, multiplicative and Additive Quick score by two pathologists; in cases of controversy about the results, the mean results were recorded.
Results: Mean results and percentage of expression of our three markers were significantly higher in the HPV 16/18 infected group than in uninfected individuals: Respectively, the mean score for Bcl2, Bax and p53 staining according to H-scoring method was 68.5, 234, 106.4 in the HPV 16/18 infected group and 4.5, 218.8, 5.07 in the uninfected group; and the Multiplicative Quick score was 4, 14.6, 8.2 in the HPV 16/18 infected group and 3, 12.3, 3.5 in the uninfected group.
Conclusion: High risk HPVs possibly act in favour of apoptotic pathway inactivation. The significant difference in apoptotic pathway between SCCs with and without high risk HPVs suggests a different early carcinogenesis pathway.
(*Balkan Med J* 2014;31:202-7).
Key Words: Bax, Bcl2, cervix, human papilloma virus, p53, squamous cell carcinoma

Squamous cell carcinoma (SCC) of the cervix is one of the most frequent female genital tract cancers, which causes a high level of malignancy-related mortality, affecting a large population worldwide. All of the effects of Human papilloma virus (HPV) infection, which is an important aetiological factor in the development of cervical carcinoma, remain to be clarified completely (1). Compared to studies focused on high risk HPVs in squamous cell carcinoma of the cervix, there are few studies about the mechanisms of carcinogenesis in cervical SCCs lacking HPV.

There are about 40 subtypes of HPV affecting the genital tract, some of them have a high association with SCC of the cervix (2). Although more than 20 types of HPV have been found in these tumours, 4 types are responsible for more than 80% of cases (3, 4) and HPV 16 is the most frequent. HPV 16 and 18 are the most oncogenic and virulent types, and altogether are responsible for 70% of uterine cervix cancer (5, 6). Sexual intercourse is the main route of transmission of HPV infection; the frequency of HPV infection in different populations and countries varies widely (7). High risk HPVs, especially HPV 16 and 18, probably impose their early carcinogenesis effect by impairing the cell cycle through modulation of different genes' expression, including E6/E7 proteins (8). These early proteins, E6 and E7, bind and inactivate a tumour-suppressor gene product, p53, and the retinoblastoma tumour-suppressor protein, respectively. In cervical lesions caused by HPVs, the increased proliferation of suprabasal epithelial cells is attributed to the expression of the viral oncogenes, E6 and E7. E7 associates with Rb protein and other members of the pocket protein family, and disrupts the association

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Received: 18.10.2013 Accepted: 01.07.2014 • DOI: 10.5152/balkanmedj.2014.13175
Available at www.balkanmedicaljournal.org

between pRb and the E2F family of transcription factors, irrespective of the presence of external growth factors. E7 also associates with other proteins involved in cell proliferation, including histone deacetylases (9), and the cyclin-dependent kinase inhibitors p21 and p27 (10). The Viral E6 protein completes the E7 function and, in the high risk HPV types, the two proteins are expressed simultaneously from a single polycistronic mRNA species (11). Association of E6 with p53, in the case of high risk HPV types, mediates p53 ubiquitination and degradation via E6-AP, a ubiquitin ligase, which results in the inhibition of the transcriptional regulatory activities of the p53 protein. Furthermore, association of E6 with Bak (12) and Bax (13) also emphasises the general role of E6 as an anti-apoptotic protein. Consequently, E6 plays a central role in the development of cervical cancers, by compromising the effectiveness of the cellular DNA damage response and allowing the accumulation of secondary mutations. The ligand domain of the E6 protein of the high risk HPV types also plays a role in cell proliferation independent of E7 (14). E6 can mediate suprabasal cell proliferation (15, 16) and disrupt normal cell adhesions which may contribute to the development of metastatic tumours.

In productive lesions, mitotic activity is found only in the lower epithelial layers, and depends on the lesion grade and the infecting HPV type; these extend towards the epithelial surface to varying degrees (17). In the case of high risk HPV types associated with cervical cancer, the relative thickness of these layers increases with the grade of neoplasia, while the extent of epithelial differentiation decreases. In such cases, high risk HPVs may be responsible for overexpression of the p53 tumour suppressor and Bcl2 anti-apoptotic factor (18, 19). Paradoxical overexpression of p53 could be interpreted by inactivation and stabilisation of the p53 protein against the Mouse double minute 2 homolog (MDM2) degradation effect mediated by the E6 protein of high risk HPVs. Conversely, Bcl2 may be negative in almost half of the cases, which could be related to direct obstruction of p53 through E6 oncoprotein (19). On the other hand, overexpression of p53 in the majority of cases and Bcl2 and Bax expression in half of the cases of cervical SCC have been demonstrated (20-23). Previous studies have shown that p53 and Bcl2 are effective in the progression of *in situ* lesions towards invasive SCC (24); it is possible that the predominance of the Bcl2 anti-apoptotic factor effect over the Bax apoptotic factor may be responsible for development of the carcinogenesis process[4,24]. Furthermore, recent studies have shown that the alteration of additional factors with a crucial role in cell cycle progression, telomerase maintenance, apoptosis and chromosomal stability are also equally important in malignant transformations (25).

Regarding the possible close relationship between high risk HPVs and the expression of apoptosis regulators, for the first time we have conducted a study to compare the expression of p53 and Bcl2 family (Bcl2 and Bax) products in squamous cell carcinoma of the cervix in high risk HPV positive and negative cases.

MATERIAL AND METHODS

In this cross-sectional study, 60 paraffin-embedded blocks of cervical tissue specimens with histopathological diagnosis of invasive SCC and pre-established HPV 16/18 status by polymerase chain reaction (PCR) (positive in 30 cases and negative in the rest) were retrieved from Mashhad educational hospitals (Pathology departments of Ghaem, Imam reza, and Omid). The study was approved by the local ethics committee of Mashhad University of Medical Sciences. After revision of the corresponding slides, 12 cases were excluded from the study due to massive haemorrhage and/or necrosis in the specimen or exclusively consist of foci of *in situ* carcinoma. An additional 6 cases were also excluded because of insufficient tissue for immunohistochemistry (IHC).

Immunohistochemistry studies: 4µ thick sections were deparaffinised using heat (80ºC) and Xylol. Afterwards, hydration was achieved using 100%, 96% and 70% ethanol and distilled water (30 sec for each) and washed with Phosphate Buffered Saline (PBS Buffer) (1x) for 30 seconds. Internal peroxidase was inactivated using 3% H_2O_2 for one hour in a dim and humid media and washed again in PBS buffer for 5 min.

For IHC staining, p53 and Bcl2 slides were incubated in tris-EDTA buffer 0.05 mM/L (PH=7.6) and Bax slides in citrate buffer 10 mM/L (PH=6) in 100ºC wet bath for 40 min and 25 min for nuclear antigens (p53) and cytoplasmic antigens (Bax, Bcl2), respectively. The slides were washed with PBS buffer, marked by DaKo markers, augmentation solution was added for 20 min, and finally antibodies (Monoclonal Mouse Anti-Human p53 Protein Clone DO-7- DaKo Company; Monoclonal Mouse Anti Human Bcl2 oncoprotein- DaKo Company; Rabbit Anti Human Bax - DaKo Company) (DaKo Company; Denmark) before being incubated overnight at 4ºC with 1/200 dilutions. After rinsing slides with PBS buffer for 10min, secondary Antibodies (Envision) were added and washed again with PBS buffer half an hour later. Finally, diluted chromogen (chromogen x50 +3,3'-Diaminobenzidine) was added and the samples were washed with PBS buffer 5min later. Haematoxylin staining was done and the dehydration process using 70%, 96% and 100% ethanol was performed. The slides were exposed to Xylol for 15 min and then fixed.

Evaluation of Slides: IHC stained slides were evaluated for H-score (Histo Score), multiplicative and Additive Quick score by two pathologists using the criteria mentioned in Table 1. The H-score was calculated as the sum of the percentage of stained cells multiplied by a number (0-3) reflecting the intensity staining (0= none, 1= weak, 2= moderate, 3= strong). In cases

TABLE 1. H score with additive and multiplicative Quick score systems

Scoring system	Stained malignant cells % (A)*	Intensity (B)	Range
H score[1]		0 (negative) +1 (mild) +2 (moderate) +3 (severe)	0-300
Additive[2] and Multiplicative[3] Quick score	1 (0-4%) 2 (5-19%) 3 (20-39%) 4 (40-59%) 5 (60-79%) 6 (80-100%)	0 (negative) 1 (weak) 2 (moderate) 3 (strong)	1-9 (additive Q score) 0-18 (multiplicative Q score)

*In order to compare to the previous qualitative studies, cut off point >5% (A>1) were considered positive
[1]H score =((+1 cells %)x1) + ((+2 cells %)x2) + ((+3 cells %)x3)
[2]Additive Quick score =A+B
[3]Multiplicative Quick score =AxB

TABLE 2. Semi-quantitative evaluation of p53 marker staining

	H score			Additive Quick score			Multiplicative Quick score		
HPV 16/18 status	Mean (SD)	Range	p value	Mean (SD)	Range	p value	Mean (SD)	Range	p value
Negative	50.7 (68.9)	0-190	0.013	3.5 (2.9)	1-8	0.007	3.5 (4.5)	0-15	0.001
Positive	106.4 (69.3)	0-230		5.7 (2.1)	1-9		8.2 (4.4)	0-18	

HPV: human papillomavirus; SD: standard deviation

TABLE 3. Semi-quantitative evaluation of Bcl2 marker staining

	H score			Additive Quick score			Multiplicative Quick score		
HPV 16/18 status	Mean (SD)	Range	p value	Mean (SD)	Range	p value	Mean (SD)	Range	p value
Negative	4.5 (20.7)	0-95	0.006	1.3 (1.3)	1-7	0.008	3.0 (1.3)	0-6	0.006
Positive	68.4 (94.4)	0-230		3.6 (3.4)	1-10		4.0 (5.3)	0-12	

HPV: human papillomavirus; SD: standard deviation

of controversy, the mean results were recorded. P53 nuclear staining and Bcl2, Bax cytoplasmic staining were considered positive; Bax in normal Colon, Bcl2 in reactive lymph node and p53 in adenocarcinoma of Colon were used as positive controls.

Statistical analysis

One-sample Kolmogorov-Smirnov test was used for testing normality, and then the independent t-test was used for comparing the means. To evaluate the correlation between the variables, the Spearman correlation coefficient was used, while for comparing result percentages, the Fisher's exact test and Pearson chi square test were used. All calculations were performed using Statistical Package for the Social Sciences (SPSS) version 18 (SPSS Inc., Chicago, IL, USA), and a p value below 0.05 ($p<p0.05$) was considered significant.

RESULTS

In total, 42 cases of uterine cervix SCC were included in this study, in which high risk HPV was positive in 21 cases and negative in 21 cases. The patients ranged in age between 40 and 76 years with an average age of 59.52±9.36 years for mean±standard deviation (SD) in the HPV positive group and 61.23±9.47 years in the HPV negative group. There were no significant differences between the two groups analysed by independent t-test (p=0.56).

Expression of p53, Bcl2 and Bax markers in HPV 16/18 infected group was 85.7%, 38.1% and 100%, respectively, and expression of these markers in the non-infected group was 52.4%, 4.8% and 90.5%, respectively. Fisher's exact test showed a significant difference between the two groups for the expression of p53 (p=0.009) and Bcl2 (p=0.02), but there were no significant differences between the two groups for Bax expression (p=0.488).

Semi-quantitative evaluation of IHC stained p53, Bcl2 and Bax markers showed a significantly higher expression of p53, Bcl2 and Bax markers in the HPV 16/18 infected group compared to uninfected ones (Table 2, 3, 4). The mean H score for p53, Bcl2 and Bax markers in the HPV 16/18 infected group were 106.4, 68.4 and 234.0, respectively, and the mean H score of these markers in the non-infected group were 50.7,

TABLE 4. Semi-quantitative evaluation of Bax marker staining

	H score			Additive Quick score			Multiplicative Quick score		
HPV 16/18 status	Mean (SD)	Range	p value	Mean (SD)	Range	p value	Mean (SD)	Range	p value
Negative	218.8 (55.5)	30-290	0.414	7.9 (1.0)	4-9	0.045	12.3 (3.6)	3-18	0.036
Positive	234.0 (41.0)	150-299		8.4 (5)	8-9		14.6 (3.0)	12-18	

HPV: human papillomavirus; SD: standard deviation

4.5 and 218.8, respectively. The mean Additive Quick score for p53, Bcl2 and Bax markers in the HPV 16/18 infected group were 5.7, 3.6 and 8.4, respectively, and these scores in the non-infected group were in 3.5, 1.3 and 7.9, respectively. The Multiplicative Quick score for p53, Bcl2 and Bax markers in HPV 16/18 infected group were 8.2, 4.0 and 14.6, respectively, and these scores in the non-infected group were in 3.5, 3.0 and 12.3, respectively. There was a significant difference between the two groups analysed by independent t-test in the results of the Multiplicative Quick score for he Expression of p53 (p=0.001), Bcl2 (p=0.006) and Bax (p=0.036). Correlation coefficient between H score and Quick score results in p53, Bcl2 and Bax markers were 0.709, 0.998 and 0.657, respectively (p=0.0001).

DISCUSSION

Apoptosis can be measured by different methods. IHC is a reliable, valuable and simple method for the detection of apoptosis. The results of this study demonstrated that expression of the apoptotic markers p53, Bcl2 and Bax in HPV 16/18 infected squamous cell carcinoma of the cervix (case group) were significantly higher than the non-infected group. It has been reported that some viruses associate with the genesis of various benign disorders and some malignant tumours such as cervical cancer (26-29). Although uterine cervix malignancy is reported to be the fifth most common cancer among Iranian females (30), in many other countries, cervical squamous cell carcinoma is the second most prevalent malignancy (31, 32). For a long time, HPV infections have been considered one of the most important aetiological factors in the development of uterine cervix SCC. In some studies, other factors, such as p53 polymorphism (apoptosis markers), have been introduced as predisposing risk factors (33). The expression of p53 in the HPV 16/18 infected group of our study was 85.7% and this was 52.4% in the non-infected group. According to the qualitative evaluation of p53 by H score, additive and multiplicative Quick score, the expression of p53 was higher significantly in the high risk HPV (16/18) group compared to the other SCCs in this study. These findings are comparable with those of Grace et al. (92.6% in HPV^+ and 38.1% in HPV-) (18). The findings of Feng et al. (19), Giarnieri et al. (24) and Win et al. (21) were comparable with our HPV positive group results, which could be related to the higher prevalence of high risk HPV in their population. Regarding the well-known effect of high risk HPV E6 protein in p53 ubiquitination and inactivation, it seems that the inactivated and stabilised p53 preserves its IHC antibody target epitope.

Evaluation of Bcl2 markers by IHC showed a significantly higher score in the high risk HPV group of SCCs rather than the other one for all three scoring systems. In this study, the expression of Bcl2 was 38.1% in the HPV 16/18 infected group and 4.8% in the non-infected group. Compared to the study by Grace et al. (18), as in our study, the percentage of Bcl2 expression was higher in the high risk HPV group; our results were different from theirs with regard to percentage of Bcl2 expression in HPV negative SCCs (38.1% in the study of Grace et al. (18) versus 4.8% in our study), the low risk HPV group (0%), the HPV 18 positive group (100%) and the HPV 16 positive group (94%) versus 38.1% in HPV 16/18 positive SCCs in our study. Our results were also different from Baskaran et al. (4), Giarnieri et al. (24), Tjalma et al. (20) studies, in which Bcl2 were positive in 65%, 65% and 63%, respectively, versus 21.45% in our study. Nevertheless, our finding was more compatible with result of Feng et al. (19) (45%), Protrka et al. (34) (36.3%) and a more widespread study in Norway by Van de Putte et al. (35) (27%). This paradoxical negativity of Bcl2 anti-apoptotic marker in more than half a cases of cervical SCCs could be interpreted by direct obstruction of p53 by the E6 oncoprotein.

Semi-quantitative evaluation of the Bax pro-apoptotic marker in HPV 16/18 positive and negative SCCs showed controversial results. According to H score, there was no significant difference between the two groups (p value=0.414); according to the additive and multiplicative Quick scores, there was a significant difference (p value=0.045 and 0.036, respectively). Logically, based on the role of the HPV E6 protein in p53 inactivation and Bax degradation, and the direct role of p53 in exciting Bax transcription, compared to our results, a decrease in Bax expression in HPV infection is expected; in spite of this, regarding the recent findings of Oh et al. (36), that high risk HPV E5 stimulates ubiquitin-proteasome-mediated degradation of Bax pro-apoptotic protein, and by assuming the preservation of the IHC target epitope of non-functional ubiquitinated Bax, our result is rationalised. On the other hand, overexpression of Bax in the high risk HPV negative group of SCCs is interpretable by higher expression of functional

p53 following genotoxic stress stimulation. Nevertheless, due to less known carcinogenesis pathway in this group, the possibility of pro-apoptotic protein degradation or ubiquitination should also be considered.

The overall expression of Bax in SCCs in our study was comparable to the study of Van de Putte et al. (35) (88%) and western blot based assay by Mozzetti et al. (3) (83%), but differ from the study of Baskaran et al. (4) (45%). Because of the different results in comparison with high risk HPV positive and negative groups of SCC by H score and Quick score, evaluation of a larger population is needed for a straightforward conclusion. Since Bax is a candidate of target therapy, it is probable that not all of the IHC based Bax positive cases of cervical SCCs would be responsive, and the presence of functional Bax pro-apoptotic protein should be confirmed by another method, including western blot (37).

The Correlation coefficient between H score and additive/multiplicative Quick score for p53, Bcl2 and Bax markers was 0.709, 0.998 and 0.657, respectively, which demonstrates a significant statistical correlation (p value=0.001). This confirms the intra-observer and inter-observer reproducibility of these two methods, and the possibility of using the simpler Quick scoring system instead of time consuming method of H score.

Final speech: The expression of all three markers in our study (p53, Bcl2 and Bax) was significantly higher in the HPV 16/18 positive group of SCCs than in the negative one, which suggests different p53, Bcl2 and Bax-related apoptotic pathways in the early stages of carcinogenesis. Also, a statistically meaningful correlation coefficient between H score, and Additive and Multiplicative Quick score suggests that the time consuming H score system could be replaced by the simpler Quick score system.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Mashhad University of Medical Sciences.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - H.A.,M.H.S.; Design - H.A., M.H.S.; Supervision - H.A., M.H.S.; Resource - H.A., M.H.S., N.S.; Materials - H.A., M.H.S., A.A.; Data Collection&/or Processing - H.A., M.H.S., A.A.; Analysis&/or Interpretation - H.A., M.H.S., A.A.; Literature Search - H.A., M.H.S., H.R.G.; Writing - H.A., M.H.S., H.R.G.; Critical Reviews - H.A., M.H.S.

Acknowledgements: The authors would like to thank research vice chancellor of Mashhad University of Medical Sciences and also Mrs.Behnaz Barazandeh for performing IHC.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was financially supported by a grant from Mashhad University of Medical Sciences (No. 87558).

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