Immunocytochemical Expression of BAX and BAK Proteins in Cervical Smears of Women Positive for HPV Types: A Study of 120 Cases

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Abstract

Objective: Human Papillomavirus (HPV) infection is clearly associated with cervical cancer development. However, only a very small percentage of HPV-infected women will eventually develop cancer and the factors determining that progression have not yet been sufficiently clarified. It is known that HPV oncoproteins E6 and E7 interact with various squamous cell molecules towards promoting cell immortalization and carcinogenesis. Among these molecules are the proapoptotic proteins Bax and Bak, two key regulators of the intrinsic apoptotic pathway. The aim of this study is to test for possible statistically significant differences in the Bax and Bak expression in the Pap smears of HPV-positive and HPV-negative women and thus examine their potential value as prognostic markers.

Methods: One hundred and twenty women were subtyped for HPV using microarrays hybridization and then Bax and Bak expression was assessed using immunocytochemistry staining on cytocentrifuged ThinPrep samples.

Results: Statistical analysis determined that there was no statistically significant difference between the expression of Bax and Bak in the HPV-positive and HPV-negative women as this expression was detected by immunocytochemical assessment of ThinPrep samples.

Conclusion: Although in several published studies there is evidence of HPV oncoproteins affecting the expression of Bax and Bak on squamous cells, our study indicates that this effect is not apparent by immunocytochemical protein staining.

Keywords: HPV; Bax; Bak; cytology; cervical smears

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Introduction

Cancer of the uterine cervix is the second most frequent women’s cancer worldwide, with an incidence of 18.7/100000 women [1], as well as the second leading cause of cancer related deaths among women [2]. It accounts for 5% of the global tumor burden [2] and its mortality rate between 1980 and 2005 was between 1.44 and 2.48 deaths per 100000 women [1]. In Greece in particular, the overall incidence is estimated to be approximately 10.4-10.6/100000, although a slight increase is anticipated to occur in the following years due to the increased influx of immigrants from developing countries [3].

Human Papillomavirus (HPV) is to date the only microorganism whose presence and the lesions it causes in squamous cervical cells have been clearly correlated with precancerous cervical lesions. HPV DNA has been detected in 99.7% of squamous cervical cells and 94-100% of adenocarcinomas and adenosquamous carcinomas [4]. The HPV types that are most commonly detected in them are 16, 18, 45, 31, 33, 52, 58 and 35 [5-7]. HPV 16 and HPV 18 are responsible for approximately 70% of cervical cancers [2]. For HPV 16 and HPV 18 in particular there is also strong evidence of its being a causative agent in cancer of the vulva, vagina, penis, anus, oral cavity and oropharynx [8]. However, HPV infection is a necessary but not adequate condition for cervical cancer development. Eighty per cent of HPV infections are transient [4], whereas only 5% of them progress to cervical intraepithelial neoplasia, and only 10-20% of cervical intraepithelial neoplasias progress to cancer [9]. Therefore, the clarification of the mechanism by which HPV leads to carcinogenesis is of great clinical significance, as it would go a long way towards elucidating which other factors collaborate with HPV infection in order for normal squamous epithelium to undergo carcinogenesis, and possibly lead to discovering more efficient methods of treatment and prevention.

High-risk HPV types like HPV 16 and HPV 18 encode oncoproteins E6 and E7 which are considered the primary factors responsible for blocking cell cycle exit during cell differentiation, eventually leading to immortalization of the cell [10]. E6 induces ubiquitination and subsequent degradation of the tumor suppressor gene p53 whereas E7 inactivates the tumor suppressor gene pRb causing increased DNA synthesis and cell division [11]. There is also evidence indicating that E6 and E7 decrease the expression of proapoptotic proteins Bax and Bak whilst increasing the expression of antiapoptotic protein Bcl-2, apparently independently of p53 ubiquitination [11-14].

Bax and Bak are key regulators of the intrinsic (mitochondrial) apoptotic pathway and their expression is of great significance in physiological and pathological processes such as homeostasis and cancer [15]. They are members of the pro-apoptotic subgroup of the Bcl-2 family of proteins, which constitutes a pivotal checkpoint in the apoptotic process [16]. More specifically, when cells are deprived of survival signals anti-apoptotic proteins such as Bcl-2 and Bcl-xL are lost from the mitochondrial membrane and replaced by pro-apoptotic proteins such as Bax, Bak and Bim [17]. These proteins increase the permeability of the outer mitochondrial membrane by forming pores [18], which are called mitochondrial-induced apoptosis channels (MAC). Through those pores proteins such as cytochrome c leak into the cytoplasm and activate the caspase cascade. It has been proven that Bax is a structural component of MAC while Bak too has been shown to co-immunoprecipitate with Bax during early apoptosis, and there is also data suggesting that it can replace Bax as a MAC component in Bax-deficient cells.
Therefore for a cell to become apoptosis-resistant both Bax and Bak need to be inactivated [15]. This makes them ideal targets for many viral oncoproteins, such as BHRF1 protein of the Epstein-Barr virus, the SV40 TAg protein and the adenovirus E1B19K protein [19]. However, the extent of the influence of Bax and Bak expression in squamous cervical cells carcinogenesis hasn’t been clearly determined yet, although certain studies indicate that the Bax-dependent proapoptotic pathway is a significant target of the E6 oncoprotein [20,21]. Other studies have demonstrated that the HPV16 and HPV 18 E6 is capable of binding Bak and inhibiting Bak-induced apoptosis [13,19].

The purpose of this study is to examine whether there is statistically significant difference in the immunocytochemical expression of Bax and Bak in Pap smears of women positive for HPV infection, as opposed to women negative for HPV infection (control group), and, by extension, evaluate the possible usefulness of Bax and Bak immunocytochemical staining on cell samples as potential prognostic markers which could be implemented in the screening routine for cervical cancer in the future. A secondary smaller statistical analysis was conducted regarding the expression of Bax and Bak in Pap smears of women infected by HPV types 16 and 18 in particular, since they are the types whose effect on the functionality of Bax and Bak has been studied most extensively. The hypothesis of the study was “there is statistically significant difference between the staining of the Bax and Bak proteins in squamous epithelial cells of women infected with the HPV virus compared to women who are not infected, as it is expressed by the combination of the percentage of stained cells and the intensity of the staining.”

Materials and Methods

The women for this study were randomly selected; some of them had reported history of HPV infection but no HPV subtyping had been performed, whereas others had no prior history of HPV infection. After obtaining their informed consent, the women participating in this study had a conventional Pap smear and a Thin-Prep (PreservCyt Solution 20ml, Cytex, Marlborough, Massachusetts, USA) sample for HPV subtyping taken with a flexible brush. Microarrays hybridization was used for HPV types subtyping. After HPV subtyping, 2ml of the ThinPrep solution were taken from each case. Each sample was centrifuged in a cytocentrifuge (Shandon Cytospin) at 1200rpm for 5 minutes. The resulting slides were air-dried in room temperature for 30 minutes and then stored in deep freeze (-8° to -15°C).

The immunocytochemical staining of the slides was carried out according to the ENVISION protocol 3-1 UNMASKING with MW for cellular smears (Dako, Glostrup, Denmark). For the detection of Bax and Bak proteins we used anti-human polyclonal rabbit antibodies (Rb Anti-Bax Pab, 7ml, ready-to-use, diluted 1:1 and Rb Anti-Bak Pab, 7ml, ready-to-use, Spring Bioscience, USA), detection system EnVision/HRP Rabbit/Mouse, 100ml, ready-to-use (Dako, Glostrup, Denmark) with peroxidase and secondary antibodies against rabbit and mouse immunoglobulins molecules, and chromogen Liquid DAB + Chromogen, 50x Concentrate with Substrate Chromogen System, diluted 1:50 (Dako, Glostrup, Denmark). Mayer hematoxylin was used for counterstain.

The evaluation of the expression of the Bax and Bak protein was carried out by viewing at least 1000 cells per slide (50 40x power
fields) in order to be considered accurate. According to criteria cited in previous papers [22,23], evaluation of expression depends on two parameters: stain intensity and percentage of positive staining cells. Percentage of cells is graded as follows: 0: no reactive cells, 1: 1-25%, 2: 26-50%, 3: 51-75%, 4: 76-100%. Stain intensity is graded as follows: 0: no staining, 1: weak staining, 2: moderate staining and 3: intense staining (see Figure 1 for Bax and Figure 2, Figure 3 for Bak). The two values are multiplied and the result is the score for each field. If the field manifests heterogeneity, each separate area of the field is independently graded and the results are added together, e.g. if in a field 15% of the cells is intensely stained (1x3=3), 30% is moderately stained (2x2=4) and 55% is weakly stained (3x1=3), the field score is 3+4+3=10. This method was preferred because it was considered by the authors as more precise and more liable to yield statistically significant results than the semiquantitative evaluation of stain intensity proposed by other authors [24,25]. It should be noted that all previously mentioned studies were conducted on paraffin-embedded tissue samples whereas in our study we used cytocentrifuged ThinPrep samples of cervical smears. To our knowledge, this is the first time this method is used on cell smears instead of tissue samples.

Statistical analysis of the results was performed by use of the SPSS Statistic Packs 17.0 program (SPSS Inc., Chicago, Illinois, USA). Statistically significant difference was calculated by means of the t-test, whereas the normality of score distribution was determined by skewness and kurtosis and the Q-Q plots in groups with more than 50 cases, and with the Kolmogorov-Smirnov test in groups with less than 50 cases. In all cases, score distribution was normal. In all tests, p < 0.05 was taken as the significance limit.

Figure 1 Examples of negative, weak, moderate and strong Bax antibody staining intensity (Bax stain, x200). Negative staining, score 0 (thin arrow). Weak staining, score 1 (dashed arrow): hypochromatic, finely granular staining. Moderate staining, score 2 (wide arrow): denser, more hyperchromatic staining. Strong staining, score 3 (arrowhead): Intensely hyperchromatic staining occupying the entire cell. The cell nucleus is barely visible.

Figure 2 Examples of negative and strong Bak antibody staining intensity (Bak stain, x400). Negative staining, score 0 (thin arrow). Strong staining, score 3 (arrowhead). The staining features are the same as those of the Bax stain.
Results

HPV Subtyping

Samples of 120 patients were evaluated. Mean age was 33.4 (range, 16-62) years. Of the 120 cases, 70 were negative for the examined HPV types and 50 were infected by 1 or more of the examined types. These numbers did not reduce the validity of statistical analysis in any way. More particularly, 33 cases were infected by 1 type, 11 by 2 types, 2 by 3 types, 3 by 4 types and 1 by 5 HPV types. Of the detected types, the one more frequently encountered was type 42 in 12 cases, followed by types 44/55, 51, 59, 16, 31, etc. The frequency of the various types is displayed on Figure 4.

Bax and Bak score statistical analysis

The histograms of the Bax and Bak scores in all 120 cases, and the histograms of the Bax and Bak scores in women positive for HPV and women negative for HPV (control group) as well as the average, minimum and maximum scores are listed in Figure 5 and Figure 6. The question statistically analyzed was whether there was statistically significant difference between the Bax and Bak scores of a. women negative for HPV types (control group, n=70) and b. women positive for HPV types (n=50). The overall average Bax and Bak scores were 6.60 and 2.62 respectively. The average Bax and Bak scores in the...
control group were 6.56 and 2.68 respectively, whereas the average Bax and Bak scores in the HPV-positive group were 6.65 and 2.55 respectively. Statistical analysis by t-test showed that there is no statistically significant difference in the Bax and Bak scores between any of the aforementioned compared groups (p=0.810 for Bax and p=0.648 for Bak).

Figure 5 Panel A. Bax staining score histogram for all cases Panel B. Bax staining score histogram for HPV-positive cases Panel C. Bax staining score histogram for HPV-negative cases (control group). The mean, minimum and maximum score and standard deviation are displayed. In all histograms the distribution curve is normal.

In our secondary analysis, the question statistically analyzed was whether there was statistically significant difference between the Bax and Bak scores of a. women positive for HPV types 16 and 18 and b. women positive for HPV types other than 16 and 18. For validity purposes the scores of the subgroup positive for HPV 16 and HPV 18 were also compared to the scores of the control group. Statistical analysis by t-test showed there is no statistically significant difference in the expression of Bax and Bak between the ‘HPV 16/HPV 18 positive’ subgroup and the ‘other HPV types’ subgroup or between the ‘HPV 16/HPV 18 positive’ subgroup and the control group (p=0.815 for Bax and p=0.937 for Bak in the comparison between the two subgroups and p=0.739 for Bax and p=0.866 for Bak in the comparison between the ‘HPV 16/HPV 18 positive’ subgroup and the control group).

Figure 6 Panel A. Bak staining score histogram for all cases Panel B. Bak staining score histogram for HPV-positive cases Panel C. Bak staining score histogram for HPV-negative cases (control group). The mean, minimum and maximum score and standard deviation are displayed. In all histograms the
distribution curve is normal.

Because the study did not yield statistically significant results, a post hoc power calculation of all t-tests performed in this study was conducted, in order to determine the study’s statistical power. According to the calculation the power of this study was below 10% in the performed t-tests. The significance of this finding will be further analyzed in the Discussion section.

It should also be mentioned that in the two aforementioned studies where Bax and Bak were evaluated by both intensity and percentage of stained cells, the authors graded tumor cells only, taken from tissue sections of malignant neoplasms [22,23]. In our case, as this method was used as part of a routine screening test, most of the cells in the cell samples were normal, even in the HPV-positive women, since HPV infection does not always lead to visible cell alterations. Even in those cases where there were abnormal cells, their morphological assessment on the antibody-stained slides was problematic because most of the time the antibody staining obscured the cells’ morphological details. Therefore, the results obtained refer to a representative sample of 1000 cells per slide, regardless of their morphological features, which we considered is in keeping with our approach of testing this method’s value as a screening test, adjunctive to the Papanicolaou test.

**Discussion**

Over the years quite a few studies have been conducted regarding the influence of HPV oncoproteins E6 and E7 in squamous cell carcinogenesis. Apart from their well-known ability to bind and inactivate the tumor suppressor genes p53 and pRb, there is evidence of them binding to a number of other proteins as well, including the proapoptotic proteins Bax and Bak, and several papers have been published regarding their effect on these proteins. The presence of such evidence naturally led to an investigation of a possible association between HPV oncoproteins and Bax and Bak expression in human squamous cells, both normal and cancerous.

As mentioned above, strong causal association between HPV infection, especially by the HPV 16 type [26], and head and neck squamous carcinoma has been established [27-29]. On the other hand, a study by Delehedde et al. [30] in 1999 showed evidence of a markedly increased expression of the Bax and Bak protein in tissue sections of squamous cell carcinomas of the skin, which was later corroborated by other tissue section studies where it was shown that Bax expression was maintained in oral and skin epithelial dysplasias [31,32].

Surprisingly, only a few studies have focused especially on the expression of Bax and Bak in carcinomas of the uterine cervix and their precancerous lesions rather than squamous cell carcinomas in general. As early as 1998, a study by Kokawa et al. [33] showed that Bax expression, while strong in cervical adenocarcinomas, was rather weak in squamous carcinomas. However, a large study of primary cervical carcinomas demonstrated diffuse cytoplasmic Bax staining in 83% of the examined cases, although no correlation with other prognostic factors was proved [34]. In another study by Cheah and Looi [35] there was upregulation of Bax in cervical carcinoma as opposed to normal cervical tissue but not in high-grade squamous intraepithelial dysplasia. Other studies compared Bax and Bak expressions amongst tissue samples with precancerous lesions and cervical carcinomas. In a study by Cheung et al. in 2001 [36], there was a statistically significant reduction in Bak expression in carcinoma as opposed to high-grade intraepithelial dysplasia, but no corresponding reduction in Bax expression. It should be noted that in all the aforementioned studies Bax and Bak expression was measured by immunohistochemistry on tissue sections, whereas in our study we utilized immunocytochemistry staining of cytocentrifuged ThinPrep samples of cervical smears.

Another issue of interest is the impact of HPV oncoproteins on Bax and Bak expression in squamous cells following irradiation or
chemotherapy. In most studies investigating this topic impaired apoptosis following irradiation or chemotherapy treatment was observed in HPV-positive cells, which coincided with reduced steady-state levels of both Bak and Bax [37-41]. More particularly a study by Struijk et al. in 2007 demonstrated that Bak expression was lower only in HPV 5 and HPV 38-containing squamous cells, whereas the expression of Bax was lower in HPV 5 and HPV 8-containing cells but not in HPV 16 and HPV 38-containing ones [40]. In other studies, however, although resistance to chemotherapy-induced apoptosis is indeed observed in HPV 16 positive cell lines, Bax expression is unaffected, suggesting that there are other mechanisms by which high-risk HPV types interfere with apoptosis mechanisms [42]. Clearly the oncoproteins of the various HPV types have varying effects on the apoptosis functionality of the squamous cells, but nevertheless it is undeniable that they play a critical role in apoptosis regulation and cell immortalization. It seems, however, that although impaired, apoptosis is not completely suppressed by HPV oncoproteins. A study by Sultana et al. showed that after chemotherapy Bax expression was observed more frequently in the subgroup of patients who responded to the treatment [43], whereas Struijk et al. demonstrated evidence that after exposure to UVB irradiation the expressions of both Bax and Bak increased to levels comparable to those of control cells [40].

While molecular studies have revealed that HPV E6 is directly involved in the degradation of several apoptotic proteins, including Bax and Bak [21,44], that fact alone does not suffice to explain the mechanism by which HPV infection causes carcinogenesis in squamous cells or the evident discrepancies between HPV-infected women’s potential for developing cervical cancer. This variation is probably due to a number of factors, both viral and host ones, such as variations in the HPV genome, E6 and E7 expression, p53 pleomorphism and others. Even within HPV 16 alone five phylogenetic variants were identified which seem to demonstrate varying levels of viral activities such as p53 degradation and Bax down-regulation [45] so it is more than probable that such variations also exist among the different HPV types and their respective variants, making it all the more difficult to predict the biological behavior of HPV-infected squamous cells. It is therefore of importance to identify possible prognostic factors in HPV-infected women, and since there is substantial evidence that HPV oncoproteins directly influence the expression of Bax and Bak proteins it is only logical that these proteins be tested as potential prognostic markers.

Although it has been already established by molecular and immunohistochemical studies that Bax and Bak expression is affected in HPV-infected women, it has not yet been investigated whether this altered expression would be evident in immunocytochemical staining of cells of a routine Pap smear, which is currently the easiest and most frequently performed cervical cancer screening test. The aim of our study was to investigate that particular possibility, in the hope of identifying a marker of potential prognostic significance that could be easily detected with a relatively simple examination. In each patient the same ThinPrep sample was utilized both for HPV subtyping and Bax and Bak immunocytochemical staining for the purpose of consistency, while a conventional Pap smear taken concurrently served for cytological evaluation of the patients. According to our results, there seemed to be no statistically significant difference in the expression of Bax or Bak in women positive for HPV compared with the control group. However, according to the post hoc power calculation the power of this study was low, which means there is a high probability of a Type II error, namely a false assumption that there is no statistically significant difference in the Bax and Bak scores between HPV infected women and the control group. According to Onwuegbuzie and Leech, t-test studies with nonsignificant statistical differences found and low post hoc power need to be replicated independently, and preferably on larger sample sizes in order to determine the reliability of the nonsignificant statistical
difference [46]. Moreover, further studies will also be required to determine whether a statistically significant difference might become apparent with other more complex methods such as immunoblotting analysis and mRNA expression.

Furthermore, the oncoproteins of HPV 16 and HPV 18 in particular have been known by previous studies to target various apoptotic molecules, including Bax and Bak [13], a fact which has not been established for other HPV types, even high-risk ones. Therefore, despite the relatively small number of women infected by HPV 16 and HPV 18 in our study we performed an additional statistical analysis to detect possible statistical differences in the expression of Bax and Bak in squamous cervical cells of women infected by those two types compared to other HPV types. In our results there were no statistically significant differences, however due to the small number of cases and low statistical power of the study it is the authors’ opinion that larger studies will be required in order to confirm this result.

References


