Original Article

Immunohistochemical localization of human papilloma virus in conjunctival neoplasias: A retrospective study

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Background: The extent of association of human papilloma virus (HPV) in human conjunctival neoplasias has been debated in studies originating from different parts of the world, but no substantial evidence has been generated on Indian subjects. This prompted us to carry out a retrospective study on conjunctival neoplasias diagnosed over the past 12 years.

Materials and Methods: Histopathological and immunohistochemical analysis of 65 specimens of ocular neoplasias and 30 normal controls diagnosed between 1991 and 2002 at a tertiary eye care hospital, was undertaken. Formalin-fixed, paraffin-embedded tissues were reviewed for confirming histopathological diagnosis, presence of koilocytosis and changes related to actinic keratosis. Immunohistochemical analysis was done using HPV-specific monoclonal antibodies. Clinicopathological correlation and the association of HPV antigen with the histopathological features were performed.

Results: Out of the 65 cases analyzed, 35 were papillomas and 30 were ocular surface squamous neoplasias (OSSN). The mean age was 48 years with a male preponderance. Histologically, koilocytosis was observed in 17.1% of papillomas and 36.6% of OSSN. Actinic keratosis was present in 33% of OSSN. Immunohistochemically 17.1% conjunctival papillomas stained positive for HPV antigen, all cases of OSSN were negative for HPV. There was no correlation between koilocytosis or actinic keratosis and the detection of HPV antigen.

Conclusions: The association between HPV and conjunctival neoplasias is variable in different geographical areas and also depends on the methods of detection used. This study warrants the need for applying more advanced techniques at a molecular level to determine the possible etiology of HPV in conjunctival neoplasias among Asian-Indians.

Key words: Conjunctiva, human papilloma virus, immunohistochemistry, monoclonal antibodies, ocular surface squamous neoplasias, papilloma

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Many DNA viruses including human papilloma virus (HPV) have been found to play significant role in the induction of human malignancies.[1] Human papilloma viruses are receiving attention for their role in the pathogenesis of cancer, especially of the cervix, anogenital area and larynx but it has also been detected in squamous tumors of the eye both benign and malignant.[2-4]

Conjunctival squamous neoplasias are classically referred to be actinic in origin. The reports relating to the association of HPV with conjunctival neoplasias are variable. This is attributed both to diversity of populations studied and / or absence of a "gold standard" test for HPV detection.[5] Although HPV is considered one of the probable etiologic agents in proliferative ocular surface and lacrimal sac lesions worldwide, only scant to no published data is available from India. The present retrospective study was undertaken to investigate the association between HPV and conjunctival neoplasias in the Indian population.

Materials and Methods

Biopsies obtained from 35 cases of conjunctival papilloma (including palpebral conjunctiva and it's junction with the eyelid epidermis) and 30 cases of ocular surface squamous neoplasia (OSSN) diagnosed in the pathology section over a 12-year period (1991 to 2002), were included. The formalin-fixed, paraffin-embedded blocks and hematoxylin-eosin stained sections of these 65 cases were retrieved from the histopathology records. In addition 30 age and sex-matched controls of normal conjunctiva were also obtained from the eye bank. The mean age of the controls was 48 years.

The hematoxylin-eosin stained sections were reviewed to confirm the diagnosis, document koilocytes and actinic keratosis (seen as elastotic degeneration of the subepithelial tissues). Verhoeff's elastic stain confirmed the elastotic degeneration. Cases of OSSN were further graded into mild, moderate and severe dysplasias based on the thickness of the epithelial dysplastic changes.[6]

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Unstained sections of 5 μm thickness were cut on polylysine-coated slides from the paraffin blocks and subjected to immunohistochemical analysis using the Avidin-biotin indirect method.[7] In brief, antigen retrieval was done with citrate buffer, endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol for 30 min, followed by overnight incubation at 4ºC with monoclonal antibodies (DAKO, CA, USA) against HPV (1:50 dilution). This was followed by sequential incubations with biotinylated-linked secondary antibody and peroxidase-labeled streptavidin according to manufacturer's protocol (Dakocytomation LSAB+ System-HRP kit) and final incubation with 3,3'-diaminobenzidene (DAB) peroxidase substrate to give brown stain and counterstained with hematoxylin.

Positive and negative controls were run simultaneously in each batch (n =10). Positive controls were obtained from a case of penile condylomata and phosphate buffer saline instead of primary antibody was used in the negative controls.

Results

The mean age of the study subjects of squamous papilloma was 41 years (three to 88 years) with a male preponderance (62.8%). Duration of the symptoms varied between one month to 20 years. The conjunctival papillomas showed presence of goblet cells. Koilocytosis was observed in only 6/35 cases (17.1%) [Table 1]. The koilocytes are superficial or intermediate, mature squamous cells characterized by perinuclear vacuolation, dense staining peripheral cytoplasm and a nucleus with an undulating nuclear membrane and a rope-like chromatin [Fig. 1]. Immunohistochemically, only six cases of conjunctival papilloma showed positivity for HPV antigen. The monoclonal antibody used reacts with a major polypeptide of the capsid protein VPI which is broadly expressed among the different HPV types 6,11,16,18,31,33,4 2,51,52,56 and 58. The normal controls (30) were all negative for HPV antigen. Positive immunostaining was largely confined to the nuclei of infected cells [Fig. 2]. The positivity was seen as brown-black staining of >50% of the cells in the superficial layers. Occasionally, the cyptoplasm was observed to be immunoreactive. No correlation was found between koilocytosis and positivity for HPV.

The mean age of patients with OSSN was 55 years (19 to 80 years) with marked male preponderance of 73.3%. Eight of the cases (27%) had recurrent lesions and two cases (7%) were bilateral. Duration of symptoms varied between 10 days to five years. Histopathologically there were nine cases of mild dysplasia, 10 cases of moderate and 11 cases of severe dysplasia. Elastotic degeneration in the form of fragmented elastic fibers which stained as dark wavy lines on Verhoeff's elastic stain were observed in 10 (33%) cases. Koilocytes were observed in 11 cases (37%). Immunohistochemically, all the cases were negative for HPV antigen [Table 1].

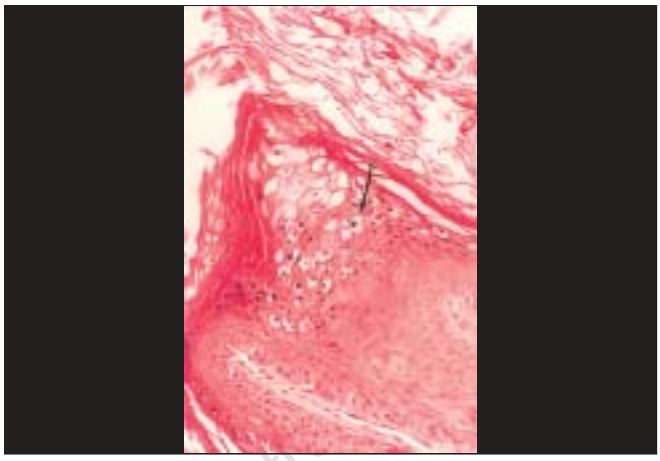

Figure 1: Squamous papilloma, arrow points to koilocyte (H/E, x200)

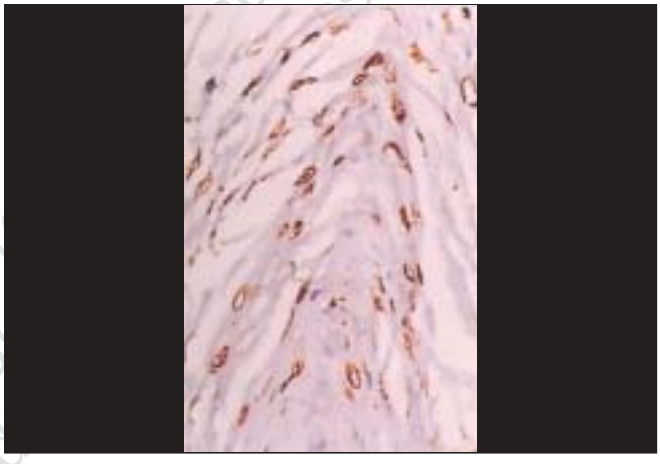

Figure 2: Positive nuclear and cytoplasmic staining for human papilloma virus antigen in a case of squamous papilloma (Avidin-biotin, x400)

Discussion

The etiology of conjunctival papillomas is not well established, but HPV is thought to be one of the several important factors. Human papilloma virus positivity varying from 44 to 92% has been reported using various detection methods.[8-10] We conducted a retrospective immunohistochemical analysis on paraffin sections and found HPV positivity of 17.1% in squamous papillomas.

Conjunctival papillomas are known to occur commonly in children and adults (20 to 39 years) with male preponderance (60%).[4,11] Koilocytosis is a common feature in lesions where HPV association is well documented. Most studies report koilocytosis in the range of 40-60%.[1,3,4,10-12] Koilocytosis was observed in 17.1% of our cases of squamous papillomas. However, we did

Table 1: Histopathological and Immunohistochemical results

Histopathology diagnosis	No. of cases	Koilocytosis	Actinic keratosis	HPV antigen positivity by IHC
Papillomas	35	6	0	6
Ocular surface squamous neoplasias	30	11	10	0
Normal controls	30	0	0	0
Total	95	17	10	6

HPV- human papilloma virus; IHC- immunohistochemistry

not observe any association between koilocytosis and HPV positivity. In the study by Mc Donnell *et al.* also, no correlation was documented between positive staining for papilloma virus and the presence of koilocytosis.[3] Sjo *et al.* have also concluded that the presence of koilocytosis was of a low diagnostic value for HPV infection in conjunctival papillomas.[10] However, Nakamura *et al.* have stated that histological assessment of koilocytosis, immunohistochemical staining and polymerase chain reaction (PCR) were more reliable than *in situ* hybridization for detection of HPV in benign lesions.[2] Thus there is a wide intergroup variation and this has consequently focused the interest on methods of detection of HPV.

Ocular surface squamous neoplasia is a serious ocular disease and because of its high prevalence and the potential to cause disability, it has a high impact on public health.[13] Epidemiological and histopathological findings strongly suggest that ultraviolet radiation is a major risk factor in the development of dysplastic and neoplastic changes in the conjunctiva and cornea.[6] In our analysis of OSSN lesions, the mean age was 55 years with 73.3% males. Solar elastosis and koilocytosis were observed in 33% of the cases. Immunohistochemistry failed to detect HPV DNA in any of the OSSN cases or the normal controls. Tulvatana *et al* observed solar elastosis in 53.3% of OSSN and 3.3% of controls, but HPV DNA was not detected in any of their specimens using PCR and dot hybridization.[9] McDonnell and coworkers observed elastotic changes in 25 of 35 (71.4%) of their neoplastic specimens and 8.2% of dysplasias showed HPV antigen by immunoperoxidase. They felt that ultraviolet exposure alone cannot explain the development of conjunctival neoplasia in their series.[3] Two studies from Tanzania revealed 100% positivity using *in situ* hybridization and PCR.[14,15] Conjunctival squamous neoplasias, classically, are regarded to be actinic in origin or a combination of factors. Multiple interfering factors such as HPV, ultraviolet light, chronic inflammations and surface microtrauma may be responsible in combination.[1,13]

Human papilloma virus DNA in dysplastic lesions has been identified in percentages varying between 0 to 100.[2,4,5,131]Many other studies have failed to detect HPV antigen even by using more sensitive diagnostic techniques like *in situ* hybridization, immunohistochemistry and PCR.[5,9,11,16,17] In our study 0% positivity for HPV antigen was found in OSSN using immunohistochemistry. Two other studies have used immunohistochemistry for detection and found a positivity of 50% and 8.2% respectively in OSSN.[2,3] In an attempt to standardize immunohistochemical method for the detection of HPV antigen, we used specific monoclonal antibodies in contrast to the more commonly used polyclonal antibodies.

The HPV antigen detection depends on several factors including the population studied and the method of detection employed as a diagnostic tool. Although immunohistochemistry has its own diagnostic specificity, PCR has an edge over it, as it can detect even a single viral DNA which may have been missed during immunohistochemical analysis. As such we are not aware of any published study conducted in the Indian population to detect the association between HPV and conjunctival papillomas.

The role of HPV in ocular lesions in the Indian population thus remains unanswered and warrants further investigations. Most studies available on HPV in OSSN are from the west and very few are from southeast Asia including Japan and China.[2,5] The results of this preliminary study should encourage further research using more sensitive and sophisticated techniques to determine the association of HPV with conjunctival neoplasias in the Indian population.

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