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Risk factors for conjunctival squamous cell neoplasia: a matched case-control study

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Aims: To identify roles of human papillomavirus (HPV) infection and solar elastosis as the risk factors for conjunctival squamous cell neoplasia (CSCN).**Methods:** 30 consecutive pathological specimens, ranging from conjunctival intraepithelial neoplasia, carcinoma in situ, to invasive squamous cell carcinoma were retrieved from tissue archives. 30 controls were disease free conjunctiva from age and sex matched patients undergoing extracapsular cataract extraction. Two masked pathologists studied haematoxylin and eosin stains on paraffin embedded conjunctival tissues. Elastic stain for solar elastosis was blindly interpreted in comparison with negative and positive controls. HPV infection was studied by polymerase chain reaction and dot hybridisation.**Results:** The mean age of CSCN patients was 54.9 years. The male to female ratio was 1:1. Solar elastosis was seen in 53.3% of CSCN and in 3.3% of controls with an odds ratio of 16.0 (95% CI, 2.49 to 670.96; p value = 0.0003). HPV DNA were not detected in any of the specimens.**Conclusion:** Solar elastosis is much more frequently found in CSCN cases than in their matched controls and is a risk factor for CSCN. These data are insufficient to conclude that HPV infection is a risk factor for CSCN.

Conjunctival squamous cell neoplasia (CSCN) is the most common malignant tumour of the ocular surface.^{1,2} The disease is prevalent in tropical areas including Thailand.^{3,4} Early manifestations are small masses at or around the limbus mimicking pterygia, occurring in middle aged patients. The tumours then grow slowly, invading the nearby tissues including the eyeball, eyelids, and orbital tissues leading to severe visual loss, loss of the eye, and severe facial deformities.

Similar to the squamous cell carcinoma of the uterine cervix, the stagings of conjunctival squamous cell carcinoma are classified by the thickness of epithelial dysplastic changes and the tumour invasion into the substantia propria. The disease severity varies from conjunctival intraepithelial neoplasia (CIN), carcinoma in situ (CIS), to invasive squamous cell carcinoma.

So far the causes of the disease are not adequately understood. There have been a number of investigations on the relation between the tumour to several factors, including solar exposure^{5–7} and human papillomavirus (HPV).^{8–12}

Solar exposure has been observed to cause the epithelial malignancy. Newton *et al*⁵ related the prevalence of this malignancy to tropical location of the patient dwellings. Sun *et al*⁶ found an association between ultraviolet exposure and the prevalence of squamous cell neoplasia of the conjunctiva and the eyelids. However, these two studies were conducted as non-comparative studies. Lee *et al*⁷ found a relation of sun exposure to squamous cell dysplasia in a case-control study, but did not include the pathological study on the actinic damage of the tissue.

A number of studies have been successful in detecting HPV type 16 and 18 in CSCN.^{8–12} However, benign conjunctival lesions have been shown to contain the infection as well.^{13–16} Tuppurainen *et al*¹⁷ failed to demonstrate HPV DNA in conjunctival malignancy by using in situ hybridisation and polymerase chain reaction (PCR).

This study was designed to identify the association between sun exposure and human papillomavirus infection to CSCN.

MATERIALS AND METHODS

Thirty cases were retrieved from the most recent pathological tissue diagnosed to be conjunctival squamous cell neoplasia at various stages. The earliest case was from May 1995 and the latest one was from November 2000. Controls were disease-free conjunctiva from age and sex matched patients who underwent planned extracapsular cataract extraction. Age matching was done by stratifying age groups of cases into 5 year strata—50–54, 55–59, 60–64, etc. The controls were recruited from consecutive patients whose age at the time of operation fell into the similar stratum of the carcinoma cases. The recruitment process took place from July 2000 to January 2001. All control patients agreed to participate in the study and provided informed consent. The study protocol was reviewed and approved by the ethics committee of the Faculty of Medicine, Chulalongkorn University.

All tissues were preserved in 40% buffered formalin, routinely processed overnight, and embedded in paraffin wax. The tissues in paraffin blocks were sectioned by microtomes into thin slices of 4 µm thickness. Staining with haematoxylin and eosin was performed. Specimens of both the cases and the controls were coded and mixed in order to blind the interpreting pathologists.

Two masked pathologists interpreted haematoxylin and eosin stained slides. Carcinoma cases were classified into different stages. If the readings disagreed, sections were reviewed to make the diagnosis and staging under consensus.

Solar elastosis

Conjunctival sun exposure has been demonstrated by solar elastosis in the substantia propria.^{18–21} By applying the elastic stain (Merck, Germany) using Verhoeff's technique,²² the abnormal pseudoelastic material in the substantia propria appears as dark brown wavy stripes. External positive controls were three pterygium tissues. Internal controls could be demonstrated only in some cases where the arterial walls were identified. Internal elastic lamina, shown as concentric rings of dark wavy lines, was seen if the cross sections of the arteries were found. Negative controls were from normal appearing

conjunctival tissues of other irrelevant diagnosis. All slides were coded to mask the interpreters. The codes were revealed after the interpretation was complete, before statistical analysis.

HPV DNA detection

Fifteen sections of 10 µm from paraffin embedded tissue were obtained. The tissue was carefully handled to avoid viral DNA contamination to other specimens. Each microtome blade was singly used for a specimen. DNA extraction was prepared by using QIAamp DNA mini kit (Qiagen Inc, USA), based on the spin column technique.²³ The tissues were deparaffinised with Xylene, rinsed with ethyl alcohol, and completely dissolved by proteinase K at 56°C. DNA was precipitated, filtered through silica membrane column, and preserved at -20°C. Quantitative analysis of DNA was performed by ultraviolet spectrophotometer at 260 and 280 nm. Detection of HPV DNA was first carried out by PCR amplification of HPV L1 region, using consensus primer MY09 and MY11, in parallel with the human β globin primers, GH20 and PC04, and followed by dot hybridisation with HPV generic probes, GP01, and GP02, as previously described.^{24, 25} Interpretation of positive and negative viral DNA detection were compared with the positive (HeLa DNA) and the negative (human DNA and distilled water) controls, respectively.

Statistical analysis

McNemar's χ^2 test was used to analyse the odds ratio. The statistical analyses were performed under STATA 6.0 program (Stata Corporation, TX, USA).

RESULTS

Thirty cases of CSCN were collected from the files of the pathology department (15 males, 15 females, age range 21–84 years, mean age 54.9 years). There were 16 cases of invasive squamous cell carcinoma, seven cases of CIS, and seven cases of CIN. Mild dysplasia was found in one case and severe dysplasia in six others.

Positive staining of elastic tissue in the substantia propria, representing pathologically proved solar damage of the conjunctiva, was seen in 53.3% of neoplastic samples. There were only 3.3% of positive elastic stains in the control group. The odds ratio, calculated from the discordant pairs, was 16.0 (95% confidence interval, 2.49 to 670.96; $p = 0.0003$).

DNA was not quantifiable in 14 specimens (three cases, 11 controls). However, in this study, all samples were measured. Among those 14 specimens, nine (two cases, seven controls) were unable to amplify human β globulin. PCR inhibitors were not detected because the amplified products were obtained after adding HPV DNA into the tested samples. With positive DNA and human β globulin (28 cases and 23 controls), HPV was not found in any of the specimens.

DISCUSSION

Conjunctival squamous cell neoplasia is a serious problem with a high impact on public health owing to its relatively high prevalence and the potential to cause severe disability. We conducted a study to find the association of sun exposure and HPV infection and the neoplasm.

The study was designed in such a way to minimise biases. Sampling bias may occur if only selected cases were included. In this study we searched the archival tissue for the most recent cases. We found a variety of stages of diagnosis. However, these cases were from a hospital based population, which is an unrepresentative sample of the general population. In order to obtain a comparable group of samples with equal opportunity to reach medical attention, the controls were recruited from the same hospital.

With a further attempt to minimise any unknown confounders, control specimens were age and sex matched to

the cases. Matching increased the power of the comparison between groups by balancing the number of cases and controls at each level of the constitutional factors. Elastic staining and HPV DNA detection were compared with the positive and negative controls to enhance the validity of measurement. Two pathologists interpreted the results to increase the reliability of the study. We blinded the outcome assessors of the case-control status in order to prevent ascertainment bias.

Solar elastosis is a pathological manifestation of actinic damage to the tissue where collagen, ground substance, and fibrocytes alter to abnormal "elastotic" material. This finding was used as a pathognomonic sign in the pathological diagnosis of degenerative diseases of the conjunctiva such as pinguecula and pterygia.¹⁸ Solar elastosis can be demonstrated in routine haematoxylin and eosin stain as blue-grey, wavy, and irregular sizes of strands in the subconjunctival areas. This material changes its colour to dark brown after elastic staining. We found different proportions of positive solar elastosis between neoplastic cases and non-diseased conjunctival controls. The odds ratio showed that solar elastosis is related to malignant changes of the conjunctiva. Owing to the small sample size, the confidence interval of the odds ratio was rather large.

The dose-response relation is one of the criteria to establish a causal effect of exposure to the disease. We, however, did not measure the solar elastosis quantitatively because the retrieved specimens were previously sectioned to generate pathological reports, and later to detect HPV infection.

We extracted DNA from paraffin embedded specimens with the most appropriate technique as previously described by Chan *et al.*²³ Unfortunately, we could not find any positivity on HPV infection in the study. This might be because of low prevalence of the viral infection.

Multiple factors may contribute to the development of the disease. Further studies to explore all factors in a single situation would contribute to more useful information. Since HIV infection is a possible confounding factor,^{26–30} the investigation on HPV and HIV infections together with special characteristics on pathological figures will predict the diseases more definitely.

In conclusion, sun exposure, as demonstrated by pathologically proved solar elastosis of the subconjunctival tissue, is one of the risk factors to develop conjunctival squamous cell neoplasia. More studies need to be performed to explore the relation of this tumour to multiple factors.

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