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Original Research Article

Molecular markers of the progression of conjunctival neoplastic epithelial lesions and its correlation with P16 and HPV expression

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ABSTRACT

Epithelial lesions of the conjunctiva include both benign and transitional malignant and malignant lesions.[1,2] Therefore, it is important to identify molecular markers of malignant progression of these lesions.

The aim of our study was to study molecular markers determining the risk of progression of epithelial neoplastic processes of the conjunctiva. The following markers were detected by standard immunohistochemical methods: Ki67, PHH3, Bcl2, P16, HPV16/18, P53, P63 and CK7.

A squamous-epithelial index was also derived by evaluating standard hematoxylin-eosin (H&E)-stained fluorescence and the P63/CK7 ratio.

The results of the study showed that during the progression of intraepithelial neoplasia of the conjunctiva, the content of squamous epithelium and, accordingly, the squamous-gland index increases significantly.

In addition, according to proliferative and apoptotic characteristics, conjunctival intraepithelial lesions can be divided into two groups, namely, low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia.

1. Introduction

Epithelial lesions of the conjunctiva include both benign and transitional malignant and malignant lesions.[3,4] They are tumors that appear in the conjunctiva, which is the transparent mucous membrane that covers the eyeball from the corneal edge (limbus) to the conjunctival fornix. These tumors require early diagnosis in order for them to be treated appropriately. Squamous cell carcinoma of the conjunctiva is the end-stage of a spectrum of disease referred to as ocular surface squamous neoplasia (OSSN).[2,5] Some conjunctival tumours are directly related to excessive exposure to the sun. Others, such as conjunctival squamous neoplasia (CoIN), have been found to be related to infection by human papillomavirus and HIV.[6–8] Some melanocytic tumours (pigmented) have been linked to smoking. The limbal epithelial cells appear to be the progenitors of this disease.[9,10] The incidence around the world is about 0.1 people per 100,000 population per year. Recent studies indicates that the mean age of OSSN patients is around 40 years and 70% are males.[4,11]

Since malignant lesions can recur over time, either in the same place, nearby or in a different location, once treated, they should be regularly monitored. The molecular markers determining the risk of progression of epithelial neoplastic processes of the conjunctiva is still poorly understood and needs further evaluation.

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2. Materials and Methods

Study included formalin-fixed and paraffin-embedded (FFPE) tissue sections of 10 normal conjunctivitis, 12 actinic keratosis, 25 pterigeas, 14 CoIN1, 12 CoIN2, 8 CoIN3 and 7 squamous cell carcinomas, altogether 88 cases. FFPE tissue blocks were retrieved from the teaching, research and diagnostic laboratory of Tbilisi State Medical University. H&E stained sections were revised and diagnosed by two independent pathologists (SH.K, G.B.). Squamous-glandular index was evaluated in H&E-stained specimens as the number of glands in 10 HPF. Tissue sections were stained by standard immunohistochemical procedure, using antibodies against: Ki67, p16, High-Risk HPV (16/18), p53, p63 and CK7. The evaluation of marker expression has been performed by two independent pathologists (SH.K., G.B.) in two major compartments of the lesion, including the basal cell layer and superficial cell layer. The percentage of marker positive cells has been recorded and analyzed with the following statistical methods: correlations were assessed using Spearman's rank test and comparisons between groups were evaluated using Mann-Whitney and Kruskal-Walli's test. The sensitivity and specificity of the test was assessed using 95% confidence interval. P value <0.05 was considered as statistically significant. All statistical tests were performed using SPSS statistical software V20.00.

3. Results and Discussion

The evaluation of proliferation markers in conjunctival epithelial lesions showed the following results: Ki67 labeling index in basal layer of normal conjunctiva was 5.4±2.1 and in superficial layer it was 0±0, in actinic keratosis Ki67 labeling index was 19.7±3.6 in basal layer and 3.8±1.2 in superficial layer. In pterygium, the Ki67 labeling index was 8.1±2.4 in the basal layer and 2.2±0.9 in the superficial layer. The CoIN1 Ki67 labeling index was 19.6±6.2 in the basal layer and 0±0 in the superficial layer. In CoIN2 Ki67 the labeling index was 21.3±7.8 in the basal layer and 2.5±1 in the superficial layer. In CoIN3 Ki67 labeling index was 25.3±4.2 in basal layer and 23.7±5.2 in superficial layer in CSCC Ki67 labeling index was 40.6±4.6 in basal layer and 36.7±7.3 in superficial layer.

The p53 mutations which were detected as complete loss of p53 or strong overexpression of p53 protein was not seen in normal conjunctiva; in actinic keratosis p53 mutations were detected in 24±4.3 cells in basal layer and 16±2.6 in superficial layer; in pterigea p53 mutations were detected in 12±3.8 cells in basal layer, in CoIN1 it was detected in 11±3.6 in basal layer and in CoIN2 it was detected in 15±2.2 in basal layer; p53 mutations were not detected in superficial layer of pterygium, CoIN1 and CoIN2; p53 mutations were detected in 27±4.3 in basal layer and 13.7±3.3 cells in superficial layer in CoIN3; p53 mutations were detected in 36±5.4 in basal layer and 16.9±5.2 in superficial layer in CSCC.

The study of epithelial squamous and glandular epithelial markers, such as p63 and CK7 respectively showed the following results: the distribution of p63 in normal conjunctiva was 45±5.2 in basal cell layer and 50.5±5.5 in superficial layer; p63 distribution in actinic keratosis was 76±4.9 in basal layer and 82.5±5.6 in superficial layer; p63 distribution in pterygium was 52±2.4 in basal layer and 79.3±9.1 in superficial layer; in CoIN p63 distribution was 65±3.7 in basal layer and 67.2±8.2 in superficial layer; in CoIN3 the p63 distribution was 80±6.1 in basal layer and 86.8±10.2 in superficial layer; in CSCC the p63 distribution was 90±4.3 in basal layer and 95.2±12.3 in superficial layer.

The distribution of CK7 showed the following results: in normal conjunctiva 10±2.9 in basal layer and 48±10.3 in superficial layer; in actinic keratosis 2.7±1.7 in basal layer and 4.3±1.3 in superficial layer; in pterygium 9±1.8 in basal layer and 42±9.9 in superficial layer; in CoIN1 7.9±3.4 in basal layer and 34±8.3 in superficial layer; in CoIN2 7±3.1 in basal layer and 25±6.7 in superficial layer; in CoIN3 2.1±0.9 in basal layer and 2.3±1.2 in superficial layer; in CSCC 1.7±0.2 in basal layer and 1.9±0.7 in superficial layer.

The study of P63/CK7 index showed the following results: in normal conjunctiva 4.5±1.8 in basal layer and 1±0.5 in superficial layer; in actinic keratosis 28.1±3 in basal layer and 19±4.3 in superficial layer; in pterygium 5.7±1.3 in basal layer and 1.9±0.9 in superficial layer; in CoIN1 8.2±1 in basal layer and 2±1 in superficial layer; in CoIN2 10.6±1.5 in basal layer and 3±1 in superficial layer; in CoIN3 2.1±0.9 in basal layer and 2.3±1.2 in superficial layer; in CSCC 52.9±21.5 in basal layer and 50.1±17.6 in superficial layer.

The analysis of the results of squamous index, glandular index and squamous-glandular index have shown that the squamous index is lowest in normal conjunctiva and it is significantly increased in line with the increase of atypia in squamous epithelial lesions. The highest proportion of squamous index has been found in CSCC. Whilst, the opposite trend has been seen with regards to glandular index. Hence, squamous-glandular index is also increased in line with the increase of atypia, with the dramatic increase in CSCC. In actinic keratosis the changes of squamous index, glandular index and squamous glandular index are similar to CoIN3.

The analysis of the distribution of proliferation markers Ki67 in conjunctival epithelial lesions showed that the lowest proliferation index is seen in pterygium, followed by actinic keratosis, CoIN1 and CoIN2. The maximal proliferation index was detected in CSCC.

p53 mutations, detected as the complete absence of p53 or the strong expression of p53 protein, it was not seen in normal conjunctiva. Lowest rates of p53 mutations were

Table 1: The distribution of P63, CK7 and P63/CK7 index in conjunctival epithelial lesions

	P63		CK7		P63/CK7 Index	
	Basal	**Superficial**	**Basal**	**Superficial**	**Basal**	**Superficial**
Normal Conjunctiva	45 ± 5.2	50.5 ± 5.5	10 1 2.9	48 ±10.3	4.5	1.0
Actinic Keratosis	76 ± 4.9	82 ± 5.6	2.7 ± 1.7	43 ± 1.3	28.1	19.0
Pterigea	52 ± 2.4	79.3 ± 9.1	9 ± 1.8	42 ± 9.9	5.7	1.9
CoIN1	65 ± 3.7	67.2 ± 8.2	7.9 ± 3.4	34 ± 8.3	8.7	2.0
CoIN2	74 ± 4.8	76.1 ± 7.1	7 ± 3.1	25 ± 6.7	10.6	3.0
CoIN3	80 ± 6.1	86.8 ± 10.2	2.1 ± 0.9	2.3 ± 1.2	38	37.7
CSCC	90 ± 4.3	952± 12.3	1.7 ± 0.2	1.9 ± 0.7	52.9	50.1

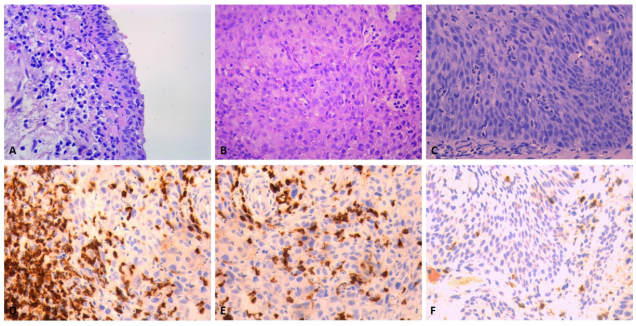

Fig. 1: A): Pterygium, H&E, x100; **B):** CoIN2, H&E, x200; **C):** CoIN3, H&E, x200; **D):** CSCC, IHC P16 expression, x400; **E):** IHC, P16 expression in COIN2 x400; **F):** IHC, P16 expression in COIN1 x400

detected in the basal layer of actinic pterygium, CoIN1 and CoIN2, whilst the superficial layer did not show any sign of p53 mutations. P53 mutations were detectable in both layers of actinic keratosis, CoIN3 and CSCC.

The analysis of squamous epithelial marker P63 and glandular epithelial marker CK7 showed that the distribution of P63 is nearly equal to the distribution of CK7 in normal conjunctiva. Whilst the expression of P63 is significantly increased in conjunctival epithelial lesions and CK7 is significantly decreased. The CSCC epithelium is almost virtually represented by squamous epithelium marked by P63.

The analysis of squamous-glandular index, based on the ratio of P63 and CK7, showed that the highest P63/CK7 ratio is detected in actinic keratosis, CoIN3 and CSCC. In addition, this index is significantly higher in basal layers of normal conjunctiva, actinic keratosis, pterygium, CoIN1 and CoIN2, whilst it is almost equal in basal and superficial layers of CoIN3 and CSCC.

The study of markers, such as p16 and High-Risk HPV respectively showed the following results:

The distribution of p16 in normal conjunctiva as well as in Actinic Keratosis in Basal and Superficial layers was 0 also. In CoIN1 p63 distribution in Basal layers was 23±1.5 in superficial layer 0. In CoIN2 p63 distribution in Basal layers was 29±2.3 in superficial layer 12.3±1.2. In CoIN3 p63 distribution in Basal layers was 35±1.5 in superficial layer 21.5±2.2. In CSCC p63 distribution in Basal layers was 49±2.2 in the superficial layer there was no significant expression.

The distribution of High-Risk HPV in normal conjunctiva as well as in Actinic Keratosis in Basal and Superficial layers was 0 also. In CoIN1High-Risk HPV distribution in Basal layers was 16.3±1.7 in superficial layer 0. In CoIN3 High-Risk HPV distribution in Basal layers was 24.0±2.1 in superficial layer 12.7±1 in the superficial layer there was no significant expression.

To the best of our knowledge, we are first who analyzed the squamous-glandular index in conjunctival epithelial lesions, by the evaluation of standard H&E specimens and as the ratio of P63/CK7 by immunohistochemistry. We have found that this ratio is significantly increased with the progression of conjunctival intraepithelial neoplasia and reaches its maximum in squamous cell carcinoma. Therefore, we suggest that squamous-glandular index may be used as an objective measure of CoIN progression. Moreover, one of the previous studies of P63 did not show any relationship of P63 expression with the progression of CoIN, although it was significantly higher compared to normal conjunctiva.[7] With regards to proliferation and apoptosis markers, they are not also very well studied in conjunctival intraepithelial lesions. The study from Ohara et al., showed that Ki67 labeling index is significantly increased during the progression of CoIN[8] and this finding is in line with our results.

4. Conclusions

Squamous-glandular index, based on the evaluation of H&E -stained specimens as well as P63/CK7 ratio, represents the objective measure of the progression of conjunctival epithelial lesions. During this process the glandular epithelium is gradually, almost virtually, replaced by squamous epithelium. Based on proliferation, apoptotic and epithelial characteristics, CoIN2 is more similar to CoIN1, whilst there is a dramatic difference between CoIN1/2 and CoIN3. Therefore, we suggest that CoIN1

Table 2: The distribution of HPV and P16 in conjunctivale pithelial lesions

	P16		High-Risk HPV	
	Basal	**Superficial**	**Basal**	**Superficial**
Normal Conjunctiva	0	0	0	0
Actinic Keratosis	0	0	0	0
COIN1	23 ± 1.5	0	16.3 ± 1.7	0
COIN2	29 ± 2.3	12.3 ± 1.2		
COIN3	35 ± 1.5	21.5 ± 2.2	24 ± 2.1	12.7 ± 1
CSCC	49 ± 2.2	Superficially no expression	15 ± 1.3	Superficially no expression

and 2 should be considered as low-grade dysplasia, whilst CoIN3 should be considered as high-grade dysplasia. Based on our study results, pterygium represents the benign entity. However, the presence of p53 mutations in pterigea indicates its potential malignant progression potential. Actinic keratosis, represents the intermediate entity between low grade dysplasia and high-grade dysplasia of the conjunctival epithelium, which can also be progressed in high grade dysplasia.

5. Source of Funding

None.

6. Conflict of Interest

None.

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