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Altered gene expression in conjunctival squamous cell carcinoma

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Conjunctival squamous cell carcinoma is a malignancy of the ocular surface. The molecular drivers responsible for the development and progression of this disease are not well understood. We therefore compared the transcriptional profiles of eight snap-frozen conjunctival squamous cell carcinomas and one *in situ* lesion with normal conjunctival specimens in order to identify diagnostic markers or therapeutic targets. RNA was analyzed using oligonucleotide microarrays, and a wide range of transcripts with altered expression identified, including many dysregulated in carcinomas arising at other sites. Among the upregulated genes, we observed more than 30-fold induction of the matrix metalloproteinases, MMP-9 and MMP-11, as well as a prominent increase in the mRNA level of a calcium-binding protein important for the intracellular calcium signaling, S100A2, which was induced over 20-fold in the tumor cohort. Clusterin was the most downregulated gene, with an approximately 180-fold reduction in the mRNA expression. These alterations were all confirmed by qPCR in the samples used for initial microarray analysis. In addition, immunohistochemical analysis confirmed the overexpression of MMP-11 and S100A2, as well as reductions in clusterin, in several independent *in situ* carcinomas of conjunctiva. These data identify a number of alterations, including upregulation of MMP-9, MMP-11, and S100A2, as well as downregulation of clusterin, associated with epithelial tumorigenesis in the ocular surface.
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Conjunctival squamous cell carcinoma generally arises from the bulbar conjunctiva and limbus. It is the most advanced type of ocular surface squamous neoplasia, which also includes conjunctival intraepithelial neoplasia, and conjunctival carcinoma *in situ*.[1] Squamous carcinomas are the commonest non-pigmented malignancies of the conjunctiva, with a yearly incidence that ranges from 0.02 to 3.5 per 100 000.[2] One of the main risk factors is exposure to the high ultraviolet (UV-B) light, thus the incidence of conjunctival squamous cell carcinoma has a geographic variability and is highest at shorter distances from the equator. Another important risk factor is immunosuppression, often due to human immunodeficiency virus (HIV) infection.[3,4] The role of human papilloma virus (HPV) in the pathogenesis of conjunctival squamous cell carcinoma remains controversial.[5,6]

The tumors are locally invasive, with intraocular and orbital involvement reported in 2–15% and 12–16% of cases, respectively.[7,8] It rarely spreads to distant organs, with occasional metastases to regional lymph nodes. Primary therapy is surgical removal. Cryotherapy, radiation, and chemotherapy have been used after excision to reduce recurrence rates. Topical mitomycin C,[9] 5-fluorouracil,[10] and interferon alpha-2b[11,12] have been successfully used for recurrent lesions.

From a biological standpoint, little is known about the molecular drivers responsible for the formation and progression of conjunctival squamous cell carcinoma. Inherited mutations in DNA repair

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Table 1 Clinical and pathological characteristics of the ocular surface squamous neoplasia specimens

Case	*Sex*	*Vascularized*	*Histopathology*	*Invasion outside conjunctiva*	*Subsequent recurrence*	*Immunosuppression*
1	M	Yes	Conjunctival carcinoma *in situ*	Cornea	No	Unknown
2	M	Yes	Conjunctival squamous cell carcinoma	Cornea orbit	No	No
3	M	Yes	Conjunctival squamous cell carcinoma	Cornea	No	No
5	F	Yes	Conjunctival squamous cell carcinoma	Cornea orbit	No	No
7	M	Unknown	Conjunctival squamous cell carcinoma	Unknown	No	Unknown
10	M	Unknown	Conjunctival squamous cell carcinoma with focal adnexal differentiation	Orbit	No	No
12	M	Yes	Conjunctival squamous cell carcinoma	Cornea	Yes	No
13	M	Yes	Poorly differentiated recurrent conjunctival squamous cell carcinoma	Cornea sclera orbit	No	Unknown
14	M	Yes	Recurrent conjunctival squamous cell carcinoma	Sclera orbit	Yes	No

Abbreviations: F, Female; M, male. The numerical progression of the case number corresponds to the cases analyzed in our previous report.[15]

enzymes can increase the incidence of these tumors, and p53 alterations have also been reported.[13,14] We previously documented DNA copy number alterations in a cohort of 10 snap-frozen conjunctival squamous cell carcinoma tumor specimens and two *in situ* carcinomas by array-based comparative genomic hybridization assay, which allowed us to determine the most frequent DNA gains and losses.[15] We now compare transcriptional profiles of nine of these tumor specimens from a single center in Saudi Arabia to normal conjunctiva in order to identify dysregulated mRNAs representing molecular targets potentially useful for diagnosis and/or therapy.

Materials and methods

Clinical Specimens

Eight conjunctival squamous cell carcinomas and one conjunctival carcinoma *in situ* cases were identified through review of pathology and tumor bank records at King Khaled Eye Specialist Hospital, Riyadh, Saudi Arabia. Only tumors with tissue snap-frozen at the time of surgery were used in this study. Three ophthalmic pathologists (Drs Alkatan, Edward, and Maktabi) reviewed the histopathological slides in all cases to confirm the presence of carcinoma. With Institutional Review Board approval, relevant clinical data were abstracted from the clinical record and linked to the frozen research specimens using anonymized sample identification numbers. The clinical characteristics of these cases are summarized in Table 1. Two normal conjunctiva and one limbal specimen resected from autopsy eyes at the Johns Hopkins Hospital were used as controls for gene expression profiling. We also examined formalin-fixed paraffin-embedded surgical specimens resected at Johns Hopkins Wilmer Eye Institute with a diagnosis of conjunctival intraepithelial neoplasia with severe dysplasia or conjunctival squamous cell carcinoma using immunohistochemistry.

RNA Extraction

Immediately after resection, the specimens were transported to the laboratory on ice, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Total RNA was isolated from frozen tumor tissues using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. DNase enzyme digestion was performed as per the manufacturer's instructions to exclude genomic DNA contamination. RNA concentration and quality were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Microarray Analysis

Microarray assay was performed using SurePrint Agilent human GE 4× 44 K version 2 one-color expression array (Agilent Technologies, Santa Clara, CA, USA). RNA quality was assessed by bioanalyzer (2100 Bioanalyzer, Agilent) and 400 ng of RNA was used for cDNA synthesis. Array hybridizations were carried out at the Microarray Core Facility of the Sidney Kimmel Cancer Center in Johns Hopkins University. Microarray raw data were analyzed using GeneSpring 12.6 software (Agilent). *P*-values in Tables 2 and 3 were calculated using Moderated t-test. *P*-values lower than 0.05 were considered statistically significant. Ingenuity Pathway Analysis (IPA) software (Qiagen) was used to determine the main signaling pathways that are regulated by the genes overexpressed or downregulated in the gene expression profile analysis performed in the conjunctival squamous cell carcinoma samples, compared with normal conjunctiva. *P*-values in Table 4 were calculated using Fisher's Exact test.

Quantitative Real-Time PCR

The mRNA levels of MMP-9, MMP-11, and S100A2 were also analyzed by quantitative real-time PCR (qPCR). Two hundred nanograms of RNA were used

Table 2 Genes upregulated in ocular surface squamous neoplasia compared with normal conjunctiva; fold change (FC) > 10

P-value	FC (abs)	Gene symbol	Chromosome	Gene name
0.00490036	48.10617	CD24	chrY	CD24 molecule
0.00120449	37.94295	MMP-9	chr20	Matrix metallopeptidase 9
2.48E-05	34.33098	TMEM132A	chr11	Transmembrane protein 132A
0.00193533	33.137703	COL1A1	chr17	Collagen, type I, alpha 1
2.32E-04	32.71715	MMP-11	chr22	Matrix metallopeptidase 11 (stromelysin 3)
6.60E-06	25.115358	CENPF	chr1	Centromere protein F, 350/400kDa (mitosin)
1.16E-05	25.001253	SCD	chr10	Stearoyl-CoA desaturase (delta-9-desaturase)
2.66E-04	22.138256	S100A2	chr1	S100 calcium-binding protein A2
0.00146693	21.938208	HK2	chr2	Hexokinase 2
0.00267661	18.583275	KRT17	chr17	Keratin 17
2.85E-06	18.415516	CKS2	chr9	CDC28 protein kinase regulatory subunit 2
2.43E-05	18.185926	UBE2C	chr20	Ubiquitin-conjugating enzyme E2C
0.00293024	17.505316	CYTH4	chr22	Cytohesin 4
0.00261672	15.982199	LGALS7	chr19	Lectin, galactoside-binding, soluble, 7
3.48E-06	15.73342	RECQL4	chr8	RecQ protein-like 4
5.47E-04	13.731016	AK4	chr1	Adenylate kinase 4
0.0016305	13.466698	KRT42P	chr17	Keratin 42 pseudogene
0.00433193	12.890687	STAT1	chr2	Signal transducer and activator of transcription 1
0.00145267	11.87778	TK1	chr17	Thymidine kinase 1, soluble
6.66E-05	11.28387	CDCA8	chr1	Cell division cycle associated 8
2.05E-04	11.265495	C17orf96	chr17	Chromosome 17 open reading frame 96
0.00195755	11.210659	TNFRSF12A	chr16	Tumor necrosis factor receptor superfamily, 12A
0.00447209	11.119723	CDH3	chr16	Cadherin 3, type 1, P-cadherin (placental)
4.00E-06	11.028542	FANCI	chr15	Fanconi anemia, complementation group I
6.02E-05	10.597402	SMC2	chr9	Structural maintenance of chromosomes 2
0.00508388	10.348655	KLHL6	chr3	Kelch-like 6 (Drosophila)
1.87E-06	10.16844	CENPW	chr6	Centromere protein W
1.19E-04	10.124681	KIF23	chr15	Kinesin family member 23
0.00435048	10.109373	CD52	chr1	CD52 molecule
1.90E-04	10.10572	C1QTNF6	chr22	C1q and tumor necrosis factor related protein 6 (C1QTNF6)

to synthesize cDNA using Reverse Transcription Reagents (Applied Byosystems, Foster City, CA, USA). qPCR for the selected genes was carried out as previously described.[16] All qPCR reactions were performed in triplicate with MyiQ2 two-color real-time PCR detection system (Bio-Rad, Hercules, CA, USA), using SYBR Green (Applied Biosystems) as fluorescent dye, and normalized to β-actin mRNA levels. Data are presented as the mean ± standard deviation. Primers for MMP-11 were designed using Primer3 software, while those specific for MMP-9, S100A2, and Clusterin were previously described.[17–19] The sequences for the primers are the following: S100A2: 5′-CTGGCTGTGCTGGTCACTAC-3′ (forward); 5′-TGGGCAGCTCCTTGTGCAGA-3′ (reverse); MMP-9: 5′-GCACGACGTCTTCCAGTACC-3′ (forward), 5′-CAG GATGTCATAGGTCACGTAGC-3′ (reverse); MMP-11: 5′-CTCGTGGGTCCTGACTTCTT-3′ (forward), 5′-GCAG TTGTCATGGTGGTTGT-3′ (reverse); Clusterin: 5′-GAG CAGCTGAACGAGCAGTTT-3′ (forward), 5′-CTTCGCC TTGCGTGAGGT-3′ (reverse); β-Actin: 5′-CCCAGCACA ATGAAGATCAA-3′ (forward), 5′-CGATCCACACGGA GTACTTG-3′ (reverse).

Immunohistochemistry

Immunohistochemical staining was performed on 4-μm-thick sections using standard techniques. Briefly, the slides were deparaffinized and antigen retrieval was performed by incubating the slides in hot sodium citrate, pH 6.0, for 30 min. Slides were incubated for 10 min in 3% hydrogen peroxide, washed in distilled H_2O, and incubated for 30 min with 0.4% Triton X100/TBS pH 7.2. Non-specific binding was blocked by treating the slides with 4% normal goat serum in 0.1% Triton X100/TBS for 1 h. Slides were then incubated overnight in the following rabbit anti-human primary antibodies: MMP-9 (Cell Signaling Technology, #13667, Danvers, MA, USA), MMP-11 (Abcam, #ab52904, Cambridge, MA, USA), S100A2 (Abcam, #ab109494), and mouse anti-human Clusterin antibody (Pharmingen, clone E5, #552886, San Jose, CA, USA). Secondary antibodies were purchased from Vector Laboratories, Burlingame, CA, USA (anti-rabbit: #PK-6101; anti-mouse: #PK6102) and diluted 1:200 in 2% normal goat serum/0.1% Triton X100/TBS. Formalin-fixed paraffin-embedded MCF7 and PANC1 cell pellets were used as positive control for MMP-11, while normal conjunctiva specimens were used as positive control for Clusterin staining. Incubations with no primary antibody as well as assessment of non-reactive stromal cells was used as negative control. Diaminobenzidine was used as chromogen and slides were counterstained with hematoxylin before mounting. Immunoreactivity was scored as follows: 0, no expression; 1+, weak expression; 2+, moderate expression; and 3+, strong expression in over 50% of cells.

Table 3 Genes downregulated in ocular surface squamous neoplasia compared with normal conjunctiva; fold change (FC)>11

P-*value*	*FC*	*Gene symbol*	*Chromosome*	*Gene name*
2.00E-06	178.1298	CLU	chr8	Clusterin
1.87E-04	72.28282	DKFZP547L112	chr15	Uncharacterized protein DKFZp547L112
7.29E-06	45.74096	KGFLP1	chr9	Fibroblast growth factor 7 pseudogene
4.59E-07	35.09851	AQP5	chr12	Aquaporin 5
0.001342	29.82204	TCAP	chr17	Titin-cap (telethonin)
4.44E-06	29.38312	CPAMD8	chr19	C3 and PZP-like, α-2-macroglobulin domain containing 8
2.70E-04	25.80205	CAPN5	chr11	Calpain 5
2.25E-04	25.23275	MOP-1	chr4	MOP-1
0.004186	24.81032	HOPX	chr4	HOP homeobox
1.12E-04	22.25546	LOC100507547	chr6	Uncharacterized LOC100507547
0.002928	22.09582	GDF15	chr19	Growth differentiation factor 15
9.52E-05	21.93042	SPARCL1	chr4	SPARC-like 1 (hevin)
5.25E-04	20.52862	SHOX	chrY	Short stature homeobox
1.80E-06	18.92113	PRB3	chr12	Proline-rich protein BstNI subfamily 3
0.001213	18.75985	ALDH3A1	chr17	Aldehyde dehydrogenase 3 family, member A1
5.73E-04	18.56191	EFEMP1	chr2	EGF-containing fibulin-like extracellular matrix protein 1
1.27E-04	17.88825	ZNF497	chr19	Zinc finger protein 497
4.19E-04	16.98899	SCARA3	chr8	Scavenger receptor class A, member 3
3.95E-04	14.59297	CES1	chr16	Carboxylesterase 1
3.31E-04	14.19237	CFHR3	chr1	Complement factor H-related 3
4.47E-04	13.14154	COL11A2	chr6	Collagen, type XI, alpha 2
9.27E-04	12.98495	SOD3	chr4	Superoxide dismutase 3
1.48E-04	12.66532	NR2F2	chr15	Nuclear receptor subfamily 2, group F, member 2
8.24E-06	12.66295	CYBRD1	chr2	Cytochrome b reductase 1
2.84E-05	12.44124	FLJ43860	chr8	FLJ43860 protein
3.54E-04	12.43122	KLF9	chr9	Kruppel-like factor 9
5.69E-05	12.3595	ADD3	chr10	Adducin 3 (gamma)
7.90E-07	12.33297	LOC389834	chr21	Ankyrin repeat domain 57 pseudogene
1.08E-04	12.3031	COX7A1	chr19	Cytochrome c oxidase subunit VIIa polypeptide 1 (muscle)
0.001247	11.99697	C11orf96	chr11	Chromosome 11 open reading frame 96 (C11orf96)
0.005758	11.75529	HAPLN2	chr1	Hyaluronan and proteoglycan link protein 2 (HAPLN2)
4.05E-04	11.51033	S1PR1	chr1	Sphingosine-1-phosphate receptor 1 (S1PR1)
4.70E-04	11.43265	CFH	chr1	Complement factor H (CFH)
0.003684	11.3695	FAM48A	chr13	Family with sequence similarity 48, member A (FAM48A)
1.01E-04	11.21465	PFDN6	chr6	Prefoldin subunit 6
2.59E-05	11.21126	NPB	chr17	Neuropeptide B (NPB)

Results

Clinical Characteristics

Cases from our prior study[15] with sufficient frozen tissue available for RNA extraction and gene expression profiling were used. These included one primary conjunctival carcinoma *in situ*, six primary, and two recurrent conjunctival squamous cell carcinomas resected at King Khaled Eye Specialist Hospital between 2005 and 2010. In some of the recurrent cases, cryotherapy had been used in addition to surgery, but no prior chemotherapy exposure was documented. Four of the patients were between 45 and 65 years of age, with the remainder over 65 years old; all patients, except one, were males. None of the patients were known to be immunosuppressed or have a history of HPV infection. Clinical features are described in Table 1 using the same case numbers as our prior publication[15] to facilitate comparisons with those previously reported DNA alterations.

Gene Expression Profile Data

Microarray analysis was used to identify expression changes in the conjunctival squamous cell carcinoma and conjunctival carcinoma *in situ* cases as compared with normal conjunctiva. A large number of transcripts were upregulated in the tumors, and those with an increase of 10-fold or more are listed in Table 2. Among the most upregulated genes associated with other types of epithelial malignancy, we observed induction of the matrix metalloproteinases, *MMP-9* and *MMP-11*, which were increased by 38- and 33-fold, respectively, in the tumor samples as compared with normal controls. We also found overexpression of additional genes known to be elevated in other types of squamous cell carcinoma, such as collagen type I α1 (*COL1A1*), centromere protein F (*CENPF*), hexokinase 2 (*HK2*), and *STAT1*, which were increased by 33-, 25-, 22-, and 12-fold in the tumor cohort, respectively. A 22-fold induction of S100A2 was also noted. This calcium-binding protein important for the regulation of intracellular calcium signaling is known to be expressed in both mucosal carcinomas and in normal conjunctiva.[20]

Numerous reductions in expression were also identified in the carcinomas, and the 36 transcripts with a 11-fold or greater decrease as compared with normal conjunctiva are listed in Table 3. The most downregulated gene was *clusterin* (*CLU*), whose

Table 4 Top canonical pathways and upstream regulators modulated by the genes overexpressed or downregulated in conjunctival squamous cell carcinoma; *P*-values were calculated using Fisher's Exact test

Name	P-*value*
Top upregulated pathways	
RAN signaling	4.56E-04
Hereditary breast cancer signaling	5.04E-03
Cell cycle control of chromosomal replication	5.47E-03
lnosine-5′-phosphate biosynthesis II	6.41E-03
Purine nucleotides *de novo* biosynthesis II	9.67E-03
Top downregulated pathways	
Epithelial adherens junction signaling	1.09E-03
Ceramide signaling	1.93E-02
Cardiac-adrenergic signaling	2.00E-02
Production of nitric oxide and reactive oxygen species in macrophages	3.19E-02
Glutamine biosynthesis I	3.28E-02

expression was reduced 178-fold in the tumor samples. In addition to these more highly suppressed transcripts, the gene XPA, related to the DNA damage repair syndrome Xeroderma Pigmentosum, was downregulated 3.6-fold in the tumor cohort. This is consistent with our prior genomic study, which identified DNA deletions in conjunctival squamous cell carcinoma tumor specimens at loci encoding genes altered in Xeroderma Pigmentosum.[15]

Confirmation of Selected Upregulated and Downregulated Genes

Microarray results for selected loci were validated by quantitative PCR (qPCR) and immunohistochemistry. As shown in Figure 1a, MMP-9 gene expression was induced between 20- and 800-fold in the tumor cohort as compared with normal conjunctiva and limbus when assessed using qPCR in mRNA from the original snap-frozen cohort. Upregulation of MMP-11 was also confirmed by qPCR, with increases of up to 120-fold in the tumors (Figure 1b). Interestingly, the two tumors, which had less pronounced increases in MMP-11, were histopathologically distinctive. Case #1 was an *in situ* lesion, and the frozen specimen may have contained more admixed normal tissues than the invasive carcinomas. Case #10 showed features of adnexal differentiation, and had the lowest numbers of chromosomal alterations in our prior array-based comparative genomic hybridization analysis.[15] The final upregulated locus we sought to confirm using qPCR was S100A2, in which this second technique revealed mRNA levels from 5 to 50 times than in normal conjunctiva and limbus (Figure 1c).

The most prominently reduced mRNA in the tumors analyzed by microarray was encoded by *clusterin*. This change was also confirmed by qPCR, although it was not as prominent as in the initial expression profiles, with a decrease in *CLU* mRNA expression of 4- to 40-fold as compared with normal conjunctival and limbal samples (Figure 1d). Interestingly, the conjunctival carcinoma *in situ* (Case #1) again had an expression level closer to that of the normal tissue than the invasive tumors.

In order to visualize the expression of proteins within neoplastic cells, and in particular the relationship between intraepithelial or *in situ* lesions and normal conjunctiva, we used immunohistochemistry. Four cases of severely dysplastic conjunctival intraepithelial neoplasia or conjunctival carcinoma *in situ* resected at Johns Hopkins Hospital were examined. For S100A2, strong expression was noted in the basal layer of normal conjunctiva where proliferating progenitors are located, as well as diffusely in conjunctival intraepithelial neoplasia and conjunctival carcinoma *in situ* (−) cells (Figure 2a). MMP-11 protein expression was also strong in dysplastic cells, with weaker and patchy staining in adjacent non-neoplastic conjunctiva (Figure 2b). Finally, in normal conjunctiva, clusterin protein expression was moderate and diffuse, while in the conjunctival intraepithelial neoplasia and conjunctival carcinoma *in situ* specimens, no immunoreactivity was noted (Figure 2c). For all three of these antibodies, expression in neoplastic cells and differences with normal conjunctiva were the same in all four cases examined.

Pathway Analysis

We used Ingenuity Pathway Analysis (IPA) to determine the top five signaling pathways that are regulated by the genes that we found to be overexpressed in the conjunctival squamous cell carcinoma cases compared with normal conjunctiva tissues. As summarized in Table 4, these pathways include RAN signaling, hereditary breast cancer signaling, chromosomal replication, inosine-5’-phosphate biosynthesis, and purine nucleotides *de novo* biosynthesis. Significantly elevated upstream regulators of these pathways included GLI1, TGFβ1, ERBB2, HSF1, and SPDEF, which might be potentially responsible for driving tumorigenesis in conjunctival squamous cell carcinoma. We also determined the top five signaling pathways modulated by the genes that we found to be downregulated in the conjunctival squamous cell carcinoma cases compared with normal conjunctiva tissues. These pathways include epithelial adherens junction signaling, ceramide signaling, cardiac-adrenergic signaling, nitric oxide production, and glutamine biosynthesis (Table 4). The upstream regulators of these pathways included SOX9, LAMTOR5, XBP1, NR1H3, and SOX4.

Discussion

Conjunctival squamous neoplasms are non-pigmented malignant tumors of the ocular surface. They are more frequent in countries located near the equator, with increased exposure to UV-B light, which is considered one of the main etiologic factors for the pathogenesis of the disease.[3] Only limited information is known about

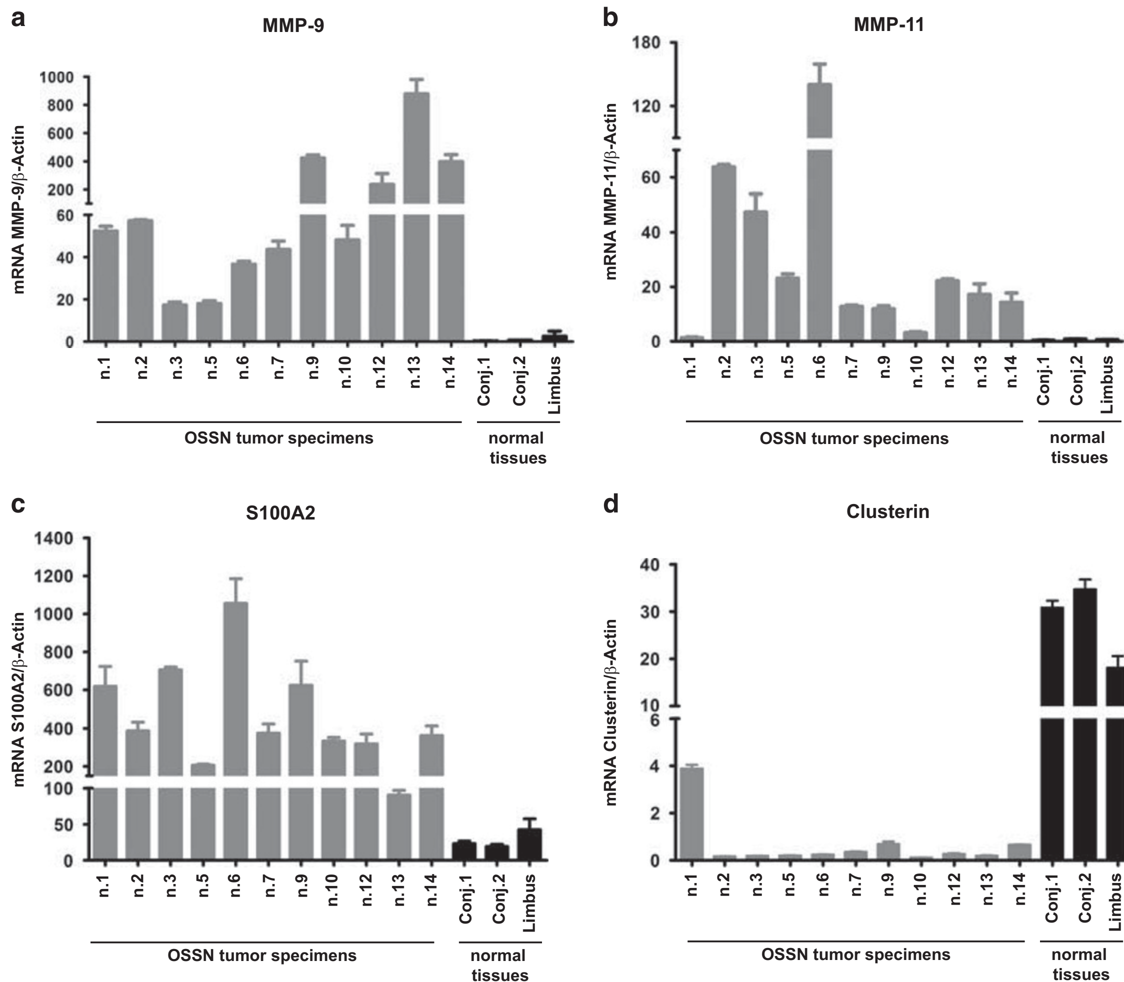

Figure 1 mRNA levels of MMP-9, MMP-11, S100A2, and Clusterin in ocular surface squamous neoplasia tumors *vs* normal conjunctiva and limbus. (**a**) mRNA levels of MMP-9 were induced more than 20-fold in the tumors as compared with normal conjunctiva and limbus, as determined by qPCR in 11 specimens of ocular surface squamous neoplasia. Cases #6 and #9 were not included in the microarray analysis and their clinical and pathological characteristics were previously described;[15] (**b**) mRNA levels of MMP-11 were increased from 15- to 120-fold in the tumors as compared with normal conjunctiva and limbus, as determined by qPCR in 11 specimens of ocular surface squamous neoplasia; (**c**) mRNA levels of S100A2 were increased from 5- to 50-fold in tumor samples as compared with normal conjunctiva and limbus, as determined by qPCR in 11 specimens; (**d**) mRNA levels of clusterin were reduced from 4- to 40-fold in ocular surface squamous neoplasia tumors as compared with normal conjunctiva and limbus, as determined by qPCR in 11 specimens.

the molecular alterations that drive tumorigenesis. The main goal of our study was to characterize the transcriptional profile of conjunctival squamous cell carcinoma and compare it with normal conjunctiva in order to identify new diagnostic markers and therapeutic targets. We identified a number of dysregulated genes, many of which have been previously implicated in the pathobiology of other types of cancer.

Among the most upregulated genes were the matrix metalloproteinases, MMP-9 and MMP-11, whose mean expression was increased in tumors by 38- and 33-fold, respectively, compared with normal conjunctiva. Reports by Ng *et al.*[21] and Di Girolamo *et al.*[22] implicating MMPs in the pathogenesis of conjunctival squamous cell carcinoma have specifically investigated MMPs and their inhibitors by immunological methods and are in support of our findings. On the basis of their analysis of MMPs, which included MMP-9 and -11, Di Girolamo *et al.* proposed an imbalanced enzyme/inhibitor ratio which could promote invasive activity. MMPs belong to a family of closely related Zinc-finger endopeptidases, involved in the degradation of specific components of the extracellular matrix and basement membrane, promoting tumor invasion and metastasis.[23] Their crucial role in the metastatic progression of the primary tumor has led to the discovery of MMP inhibitors for use as anticancer treatments.

The induction of MMP expression in other types of squamous cell carcinoma has been reported in previous studies, including cutaneous squamous cell carcinoma, where MMP-2 and MMP-9 expression was found to correlate with depth of lesion, inflammation, and microvessel density.[24] In another

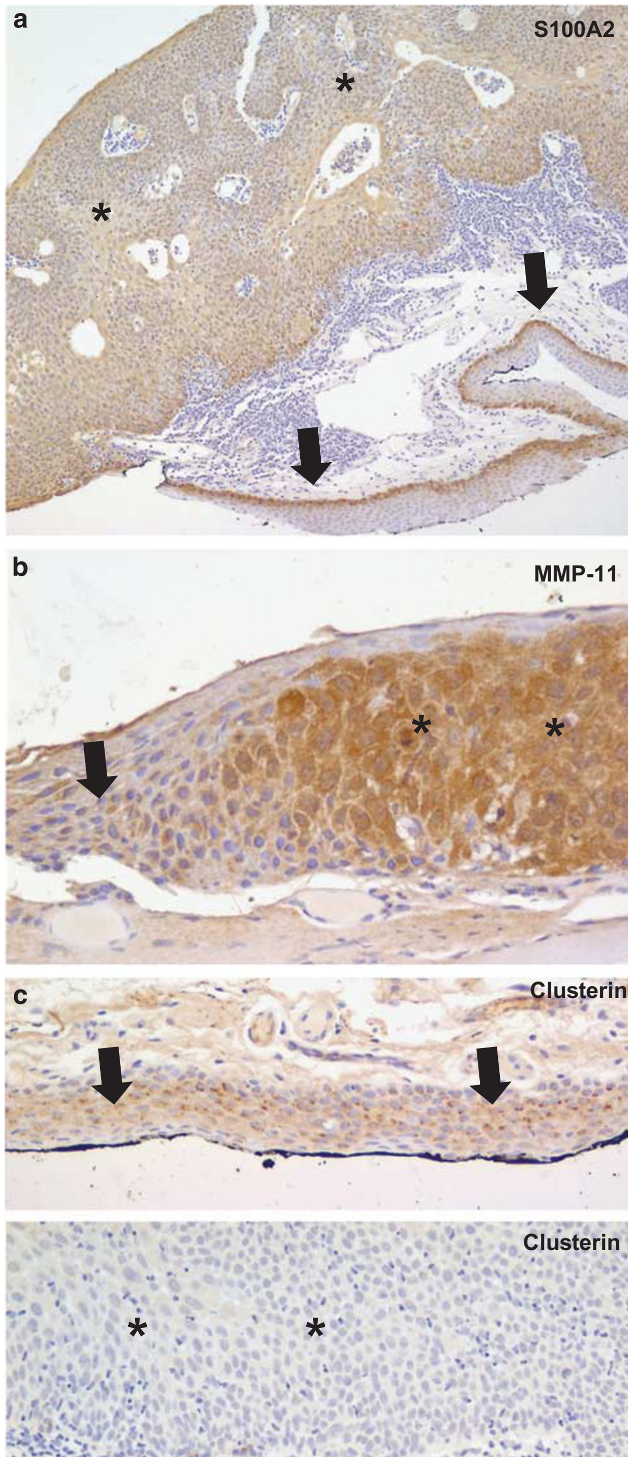

Figure 2 Immunohistochemical analysis of conjunctival intraepithelial neoplasia and carcinoma *in situ*. (**a**) S100A2 is expressed in the epithelial base of normal conjunctiva present in the bottom of the panel (arrows), and more diffusely in the conjunctival carcinoma *in situ* (asterisks; original magnification 100 ×). (**b**) The dysplastic portion of the conjunctival epithelium (asterisks) is strongly positive for MMP-11, while weaker immunoreactivity is noted in the less atypical cells adjacent to the lesion (arrow; original magnification 400 ×). (**c**) Clusterin shows diffuse moderate expression in the normal conjunctival epithelium in the upper panel (arrows), while no expression was present in an adjacent conjunctival carcinoma *in situ* shown in the lower panel (asterisks; original magnification 200 ×).

report, high expression of MMP-9 was observed in the tumor stroma of squamous cell carcinoma of the skin, providing evidence for the involvement of MMP-9 in tumor invasiveness of keratinocyte-derived tumors.[25] Interestingly, in head and neck squamous cell carcinomas, MMP-9 immunoreactivity has been observed in 82% of the cases, and is associated with shortened relapse-free survival, with high MMP-9 expression linked to more aggressive relapses.[26] Stromelysin-3 (ST3/MMP-11) is also associated with human tumor progression. Elevated expression of MMP-11 has been observed in carcinomas with poor prognosis, whereas in benign tumors, MMP-11 is mildly detectable or not expressed at all.[27] Interestingly, MMP-11 protein has been demonstrated using immunohistochemistry in 70% of oral squamous cell carcinoma, where it is associated with lymph node involvement, but it is not a significant prognostic indicator.[28]

Additional upregulated loci we identified previously associated with related cancers include collagen type I α1 (COL1A1), which is known to be induced in oral squamous cell carcinoma, where it has an important role in distinguishing oral squamous cell carcinoma from controls.[29] Our transcriptional profile analysis also showed upregulation of centromere protein F (CENPF) and hexokinase 2 (HK2), which are known to be overexpressed in esophageal[30] and cervical squamous cell carcinoma. Finally, the expression of HK2 is associated with radiation resistance.[31]

An additional gene meriting further investigation is the calcium-binding protein S100A2, which was previously shown to be expressed in the normal epithelium of the ocular surface.[20] We found that this gene was upregulated more than 20-fold in our tumor cohort compared with normal conjunctiva, and confirmed expression of the S100A2 protein in both the basal proliferating layer of non-neoplastic epithelium and more diffusely in tumor cells. Our data are consistent with the previous report which used immunofluorescence to show high S100A2 expression in ocular surface carcinomas.[20] These investigators also documented a reduction in S100A2 levels as limbal stem cells reduced their proliferative capacity and differentiated.[20] Together with this prior study, our findings support the concept that S100A2 may have an important role in promoting proliferation and inhibiting differentiation of the normal conjunctiva and limbus, and that its overexpression is a common feature of neoplastic transformation in these tissues.

The most downregulated gene in our conjunctival squamous cell carcinoma cohort was *Clusterin* (*CLU*), which is also known as apolipoprotein J. This multifunctional protein is associated with clearance of cellular debris and apoptosis, as well as with lipid transport, membrane recycling, cell adhesion, tumor progression, and neurodegenerative disorders.[32] Clusterin is known to be expressed in the ocular surface epithelium, but it is significantly

reduced in the keratinized conjunctiva, suggesting its potential role in maintaining the ocular surface epithelium in a non-keratinizing state.[33,34] In addition, it has been found that Clusterin strongly binds MMP-9, inhibiting its enzymatic activity and MMP-9-mediated degradation of the tight junction structures formed between human epithelial cells, thereby preventing tumor invasion.[35] However, as we had only two cases of recurrent conjunctival squamous cell carcinoma in our tumor cohort, we cannot infer at the moment any statistical association between the expression of the most upregulated or downregulated genes and the clinical outcome or progression of the disease.

By performing pathway analysis, we observed that the epithelial adherens junction signaling was the most downregulated signaling pathway in the tumor cohort. Interestingly, the expression of Notch1 receptor, one of the genes related with this pathway, was reduced 2.7-fold in the conjunctival squamous cell carcinoma samples compared with normal conjunctiva tissues. It has been shown that Notch1 has an oncosuppressor role in the skin by inhibiting β-catenin signaling.[36] Therefore, the downregulation of this receptor that we observed in the conjunctival squamous cell carcinoma cases suggests that Notch1 might have a tumor suppressor function also in the SCC of the ocular surface, and its upregulation might represent a new potential therapeutic strategy in the treatment of conjunctival squamous cell carcinoma.

In summary, here, we present evidence for dysregulation of several genes associated with epithelial tumorigenesis in the ocular surface by gene expression analysis. We provide further validation of the gene expression data by qPCR and immunohistochemistry for four genes, MMP-9, MMP-11, S100A2, and Clusterin. Overall, gene dysregulations observed in our samples show multiple hallmarks of cancer including proliferation and invasion. Further investigations would be needed to understand the significance of individual aberrations as well as the pathways that they disrupt. Thus far, such a global gene expression study has not been reported for ocular surface squamous neoplasia and together with our previous array-based comparative genomic hybridization study, we report a comprehensive analysis of chromosomal and transcriptional aberrations in the same set of tumors. Our data form the basis for future investigations to delineate the molecular mechanisms underlying the development and progression of ocular surface squamous neoplasia.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Kiire CA, Srinivasan S, Karp CL. Ocular surface squamous neoplasia. Int Ophthalmol Clin 2010;50: 35–46.
2 Yang J, Foster CS. Squamous cell carcinoma of the conjunctiva. Int Ophthalmol Clin 1997;37:73–85.
3 Lee GA, Hirst LW. Ocular surface squamous neoplasia. Surv Ophthalmol 1995;39:429–450.
4 Shields CL, Ramasubramanian A, Mellen PL *et al.* Conjunctival squamous cell carcinoma arising in immunosuppressed patients (organ transplant, human immunodeficiency virus infection). Ophthalmology 2011;118: 2133–2137.
5 Ateenyi-Agaba C, Franceschi S, Wabwire-Mangen F *et al.* Human papillomavirus infection and squamous cell carcinoma of the conjunctiva. Br J Cancer 2010;102: 262–267.
6 Peralta R, Valdivia A, Estañol P *et al.* Low frequency of human papillomavirus infection in conjunctival squamous cell carcinoma of Mexican patients. Infect Agent Cancer 2011;6:24.
7 Tunc M, Char DH, Crawford B *et al.* Intraepithelial and invasive squamous cell carcinoma of the conjunctiva: analysis of 60 cases. Br J Ophthalmol 1999;83:98–103.
8 McKelvie PA, Daniell M, McNab A *et al.* Squamous cell carcinoma of the conjunctiva: a series of 26 cases. Br J Ophthalmol 2002;86:168–173.
9 Shields CL, Naseripour M, Shields JA. Topical mitomycin C for extensive, recurrent conjunctival-corneal squamous cell carcinoma. Am J Ophthalmol 2002;133: 601–606.
10 Midena E, Angeli CD, Valenti M *et al.* Treatment of conjunctival squamous cell carcinoma with topical 5-fluorouracil. Br J Ophthalmol 2000;84:268–272.
11 Boehm MD, Huang AJ. Treatment of recurrent corneal and conjunctival intraepithelial neoplasia with topical interferon alfa 2b. Ophthalmology 2004;111:1755–1761.
12 Schechter BA, Koreishi AF, Karp CL *et al.* Long-term follow-up of conjunctival and corneal intraepithelial neoplasia treated with topical interferon alfa-2b. Ophthalmology 2008;115:1291–1296 1296.e1.
13 Smith ML, Ford JM, Hollander MC *et al.* p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. Mol Cell Biol 2000;20:3705–3714.
14 Mahomed A, Chetty R. Human immunodeficiency virus infection, Bcl-2, p53 protein, and Ki-67 analysis in ocular surface squamous neoplasia. Arch Ophthalmol 2002;120:554–558.
15 Asnaghi L, Alkatan H, Mahale A *et al.* Identification of multiple DNA copy number alterations including frequent 8p11.22 amplification in conjunctival squamous cell carcinoma. Invest Ophthalmol Vis Sci 2014;55:8604–8613.

16 Asnaghi L, Ebrahimi KB, Schreck KC *et al.* Notch signaling promotes growth and invasion in uveal melanoma. Clin Cancer Res 2012;18:654–665.
17 Matsubara D, Niki T, Ishikawa S *et al.* Differential expression of S100A2 and S100A4 in lung adenocarcinomas: clinicopathological significance, relationship to p53 and identification of their target genes. Cancer Sci 2005;96:844–857.
18 Safranek J, Pesta M, Holubec L *et al.* Expression of MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in lung tissue of patients with non-small cell lung cancer (NSCLC) and benign pulmonary disease. Anticancer Res 2009;29:2513–2517.
19 Cochrane DR, Wang Z, Muramaki M *et al.* Differential regulation of clusterin and its isoforms by androgens in prostate cells. J Biol Chem 2007;282:2278–2287.
20 Li J, Riau AK, Setiawan M *et al.* S100A expression in normal corneal-limbal epithelial cells and ocular surface squamous cell carcinoma tissue. Mol Vis 2011;17: 2263–2271.
21 Ng J, Coroneo MT, Wakefield D *et al.* Ultraviolet radiation and the role of matrix metalloproteinases in the pathogenesis of ocular surface squamous neoplasia. Invest Ophthalmol Vis Sci 2008;49:5295–5306.
22 Di Girolamo N, Atik A, McCluskey PJ *et al.* Matrix metalloproteinases and their inhibitors in squamous cell carcinoma of the conjunctiva. Ocul Surf 2013;11: 193–205.
23 Curran S, Murray GI. Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. Eur J Cancer 2000;36:1621–1630.
24 O'Grady A, Dunne C, O'Kelly P *et al.* Differential expression of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 in non-melanoma skin cancer: implications for tumour progression. Histopathology 2007;51:793–804.
25 Poswar FO, Fraga CA, Farias LC *et al.* Immunohistochemical analysis of TIMP-3 and MMP-9 in actinic keratosis, squamous cell carcinoma of the skin, and basal cell carcinoma. Pathol Res Pract 2013;209:705–709.
26 Ruokolainen H, Pääkkö P, Turpeenniemi-Hujanen T. Expression of matrix metalloproteinase- 9 in head and neck squamous cell carcinoma: a potential marker for prognosis. Clin Cancer Res 2004;10:3110–3116.
27 Asch PH, Basset P, Roos M *et al.* Expression of stromelysin 3 in keratoarcanthoma and squamous cell carcinoma. Am J Dermatopathol 1999;21:146–150.
28 Soni S, Mathur M, Shukla NK *et al.* Stromelysin-3 expression is an early event in human oral tumorigenesis. Int J Cancer 2003;107:309–316.
29 Chen C, Méndez E, Houck J *et al.* Gene expression profiling identifies genes predictive of oral squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 2008;17:2152–2162.
30 Pimkhaokham A, Shimada Y, Fukuda Y *et al.* Nonrandom chromosomal imbalances in esophageal squamous cell carcinoma cell lines: possible involvement of the ATF3 and CENPF genes in the 1q32 amplicon. Jpn J Cancer Res 2000;91:1126–1133.
31 Huang X, Liu M, Sun H *et al.* HK2 is a radiation resistant and independent negative prognostic factor forpatients with locally advanced cervical squamous cell carcinoma. Int J Clin Exp Pathol 2015;8:4054–4063.
32 Koltai T. Clusterin: a key player in cancer chemoresistance and its inhibition. Onco Targets Ther 2014;7: 447–456.
33 Nishida K, Kawasaki S, Kinoshita S. Clusterin may be essential for maintaining ocular surface epithelium as a non-keratinizing epithelium. Adv Exp Med Biol 1998;438:629–635.
34 Nakamura T, Nishida K, Dota A *et al.* Changes in conjunctival clusterin expression in severe ocular surface disease. Invest Ophthalmol Vis Sci 2002;43:1702–1707.
35 Jeong S, Ledee DR, Gordon GM *et al.* Interaction of clusterin and matrix metalloproteinase-9 and its implication for epithelial homeostasis and inflammation. Am J Pathol 2012;180:2028–2039.
36 Nicolas M, Wolfer A, Raj K *et al.* Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 2003;33: 416–421.