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Whole Exome Profiling and Mutational Analysis of Ocular Surface Squamous Neoplasia

Nallely Ramos-Betancourt, MD^{1,*}, Matthew G. Field, MD, PhD^{2,*}, Jesus H. Davila-Alquisiras, MD¹, Carol L. Karp, MD², Luis F. Hernández-Zimbrón, PhD^{3,4}, Roberto García-Vázquez, MD¹, Kristian A. Vazquez-Romo, MD¹, Gaofeng Wang, PhD^{2,5}, Jans Fromow-Guerra, MD, MSc³, Everardo Hernandez-Quintela, MD, MSc, FACS^{1,3}, Anat Galor, MD, MSPH^{2,6}

¹Department of Cornea and Refractive Surgery, Asociación para Evitar la Ceguera, IAP, Mexico City, Mexico.

²Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida

³Research Department, Asociación para Evitar la Ceguera en México, IAP, Mexico City, Mexico

⁴Biochemistry Department, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City, Mexico

⁵John P. Hussman Institute for Human Genomics, University of Miami, Miller School of Medicine, Miami, Florida

⁶Department of Ophthalmology, Miami Veteran Affairs Medical Center, Miami, Florida

Abstract

Purpose: To determine genetic mutational profiles in patients with Ocular Surface Squamous Neoplasia (OSSN) using whole exome sequencing.

Methods: Prospective, case-series study. Patient recruitment was conducted in a single tertiary referral center from April to September 2017. Specimens were obtained by incisional biopsies of tumors from ten eyes with histopathologic confirmation of OSSN. DNA whole exome sequencing and mutation analysis were performed.

Results: Ten patients with clinically-diagnosed OSSN underwent DNA whole exome sequencing analysis. Deleterious mutations in 305 genes known to drive tumor development and progression were found. These mutations centered around two main pathways: DNA repair/cell cycle and development/growth. All ten samples had at least one mutation in a DNA repair/cell cycle gene and all but one sample had one in a development/growth gene. The most common mutation was

Corresponding author: Nallely Ramos-Betancourt, MD. Address: Vicente García Torres 46, Barrio San Lucas, Coyoacán, Mexico City, Mexico, 04030. Phone: (+52) 5510841400 Ext. 1290. n.ramosbetancourt@apec.com.mx.

*These authors contributed equally to this work.

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⁶.Conflict of Interest Statement

The authors have no conflicts to disclose.

found in *TP53* and *HGF* (both present in 50% of cases) and mutually exclusive mutations were found in *BRCA1* and *BRCA2* (50% of cases). Mutations in *APC*, *MSH6*, *PDGFRA*, and *PTCH1* were found in 40% of cases. Global mutation analysis identified ultraviolet induced radiation as the only mutational signature present in the dataset.

Conclusions: Mutations found in samples from patients with OSSN are mainly induced by ultraviolet radiation and occur within two main pathways related to DNA repair/cell cycle and development/growth. There are many clinically available drugs and several others being evaluated in clinical trials that target the genes found mutated in this study, offering new therapeutic options for OSSN.

Keywords

Ocular Surface Squamous Neoplasia; whole exome sequencing; mutational analysis

1. Background

Ocular surface squamous neoplasia (OSSN) is the most common ocular surface malignancy, encompassing squamous epithelial disorders of the cornea and conjunctiva ranging from mild dysplasia to invasive squamous cell carcinoma.^{1,2} Major risk factors implicated in OSSN pathogenesis include ultraviolet (UV) radiation from sunlight exposure, smoking, immunosuppression, genetic susceptibility, ocular surface injury, chemical exposure, and vitamin A deficiency.³ Human papillomavirus (HPV) has also been implicated in the pathogenesis of OSSN but is considered more of a cofactor in the development in already susceptible hosts.^{4,5,6}

In recent years, significant improvements have been made in the diagnosis and treatment of OSSN. Specifically, High Resolution Optical Coherence Tomography (HR-OCT) has aided in the clinical diagnosis and enabled improved detection of even the smallest lesions.⁷ Additionally, treatment of OSSN has shifted from surgery to medical management with interferon alpha 2b (IFN α -2b), 5 fluorouracil (5-FU), or mitomycin C (MMC), as these drugs have shown comparable efficacy to surgery with fewer side effects and low recurrence rates.⁸ Even with improved diagnosis and treatment, there are a number of refractory cases and recurrences can occur in up to 10-15% of cases.

A wide variety of pathophysiological mechanisms are thought to be involved in the development of OSSN, such as DNA damage, failure of DNA repair mechanisms and reduced immunity.⁹ Ultraviolet B (UV-B) radiation from sun exposure causes local and systemic photo-immunosuppression, DNA damage, and formation of pyrimidine dimers. These pyrimidine dimers have already been shown to cause CC/TT base pair dimer transformations of the *TP53* tumor-suppressor gene in OSSN, which allows cells past the G1-S cell cycle checkpoint; all together delaying DNA repair and leading to an increase of somatic mutations and oncogenesis.⁹⁻¹¹ Only one published study exists using whole exome sequencing technology on OSSN, studying a cohort of seven individuals. In that study, the most frequent mutations in genes known to promote oncogenesis were hepatocyte growth factor (*HGF*) and CREB binding protein (*CREBBP*).¹² Accordingly, this study was designed to conduct whole exome sequencing on a larger cohort of patients in a population of

Hispanic individuals from Mexico, with vastly different genetic and environmental risk factors than that of the previous exome study, in order to further our understanding of the genetics and molecular biology of OSSN.

2. Methods

2.1 Study design:

Prospective case series

2.2 Study population:

This study adhered to the tenets of the Declaration of Helsinki and Good Laboratory and Clinical Practices and received Institutional Review Board approval. Patients were prospectively recruited from the Cornea and Refractive Surgery Department from April to September 2017. Signed informed consent was obtained from each individual after explanation of the nature of the study. Patients included were 18 years or older with clinical signs of OSSN (nodular conjunctival lesion, opalescent cornea, leukoplakia, papillomatous or gelatinous appearance, presence of feeder vessels), and typical features of OSSN by HR-OCT (thickened, hyper-reflective epithelium with an abrupt transition line between normal and abnormal epithelial tissue, RTVue, Optovue, Freemont, CA).⁷ Patients with a history of previous surgical or topical treatment for OSSN were excluded.

2.3 Clinical data:

Clinical information collected included age at presentation, sex, race/ethnicity, past ocular and medical history, self-reported history of extensive UV exposure, tumor location, tumor characteristics, tumor area, and epithelial thickness (microns) by HR-OCT. The tumor size and location provided the basis for American Joint Committee on Cancer (AJCC) clinical stage of the tumor.¹³

Incisional biopsies were performed on all subjects. For each specimen, one sample was sent for histopathological examination and another sample was flash frozen at -80°C for later DNA extraction. HPV-related status was determined by the presence of koilocytes on histopathological examination. After histopathological confirmation and grading of OSSN, topical Interferon- α -2b (IFN α -2b) at a 1millionU/ml concentration was prescribed 4 times a day for patient treatment until clinical and HR-OCT resolution was achieved. Patients were examined every month until clinical resolution. Non-responders were defined as those tumors that did not resolve after 3 months of treatment.

2.4. Molecular analysis:

Total DNA was extracted from frozen tumor specimens using the Quick-DNA Miniprep Plus Kit (ZYMO Research Corporation, Orange, CA, USA) according to manufacturer's protocol and sent immediately to Bascom Palmer Eye Institute for further analysis. Whole exome sequencing analysis of OSSN specimens was conducted in the sequencing core facility at the University of Miami as previously described.¹² Sequenced samples had a mean exome coverage target of 62x. Sequences were aligned to the human reference genome GRCh37/hg19 using NovoAlign (Novocraft Technologies Sdn Bhd). As matched normal samples

were not collected as part of the study design, we created a panel of normal samples by calling mutations on exomed blood specimens (n=117) from patients of varying ethnic and racial backgrounds in the TCGA dataset and from patients seen at Bascom Palmer Eye Institute. None of these patients had known or detected germline genetic disorders. Variant calling with MuTect2 was done using the panel of normals and a high-coverage blood sample sequenced in the same manner as the cases in this study to filter out technical artifacts and likely silent germline polymorphisms.¹⁴ To further minimize artifacts introduced by the specimen archiving process, we filtered out all genetic variants present in less than 10% of sequencing reads and mutations with fewer than 3 alternate reads. To filter out likely passenger and germline mutations, we excluded mutations in non-coding regions, mutations present in greater than 0.5% of the population, and those predicted to be non-damaging using ANNOVAR (Children's Hospital of Philadelphia, Pennsylvania). Mutations were first evaluated following the above filtering steps and then further filtered and re-evaluated using stricter filtering based on a recently published Comprehensive Characterization of Driver Genes and Mutations dataset, which comprehensively analyzed the TCGA cancer datasets to identify driver oncogenic mutations.¹⁵ Though all mutations described in this study were predicted to detrimentally affect the function of known oncogenic genes, the existence of rare germline variants cannot be excluded. Protein-protein interaction networks of mutated genes were created using STRING (Search Tool for Retrieval of Interacting Genes/Proteins).¹⁶ Mutation signatures were analyzed using predicted deleterious SNPs (Single Nucleotide Polymorphisms) from coding and splicing regions with pmsignature in R.¹⁷

2.5 Statistical analysis:

Additional statistical analyses were conducted using SPSS Version 25 (IBM Corporation, Armonk, NY, USA). Specifically, Fischer exact test was used to assess statistical differences between several groups (mutation profile by tumor response, histologic grade, clinical phenotypes, sex, age and self-reported history of extensive UV exposure). P-values <0.05 were considered to be of statistical significance.

3. Results

3.1 Study population:

All ten patients identified as Mexican mestizo and had a mean (SD) age of 69 (± 12.38) years; 6 of 10 (60%) were male. Full demographics and clinical features of the study population are presented in Table 1. HR-OCT typical features of OSSN were present in all ten cases, specifically an abrupt transition between healthy and neoplastic epithelium, with increased thickness and hyper-reflectivity (Figure 1), which disappeared upon resolution of the tumor. The mean epithelium thickness was 227.6 μ m, (SD 89.1, 126 to 377 μ m).

On histopathologic examination, 3 cases were graded as mild dysplasia, 3 as moderate dysplasia, and 4 as carcinoma in situ. Patients were treated with topical IFN α -2b for 4 to 8 months (mean 6.4 months, SD 1.43) (Figure 2). Two patients (20%) were non-responsive to IFN α -2b treatment after three months of therapy. Of these two cases, 1 underwent successful surgical treatment (excisional biopsy with no touch technique and cryotherapy),

and the other was lost to follow-up (after 3 months). Mean time of follow-up was 8.5 months (SD 3.14, 3 to 13 months); no recurrence was seen in any cases treated with topical chemotherapy or surgery.

3.2 DNA mutations:

Over 8000 mutations were found amongst the 10 samples, consisting of 305 mutations predicted to be deleterious in genes known to drive tumor development and progression.¹⁵ (Supplementary Table 1) Closer analysis of these 305 mutations demonstrated that they centered around two main pathways: DNA repair/cell cycle and development/growth (Figure 3A). All samples had at least one mutation in a DNA repair/cell cycle gene and all but one sample (patient 6) also had one in a development/growth gene. Within each of these pathways, extensive protein-protein interaction networks exist between the mutated genes (Figure 3B), suggesting that dysregulation of these pathways is essential for OSSN tumorigenesis.

The most common mutation was found in *TP53* and *HGF* (both present in 50% of cases). Mutations in the DNA mismatch repair genes *MSH2* (one case) and *MSH6* (four cases) were mutually exclusive with each other and found in 50% of cases. Additionally, *BRCA1* (three cases) and *BRCA2* (two cases) were also mutually exclusive with each other. Development/growth genes *APC*, *PDGFRA*, and *PTCH1* were each found in four samples. Other major driver mutations found in at least two cases within these samples included *NOTCH1*/*NOTCH2* (three cases), *ERBB2*/*ERBB3*/*ERBB4* (three cases), *AXIN1*/*AXIN2* (three cases), *CTNNB1* (two cases), *TCF7L2* (two cases), *ATM* (two cases), *ATR* (two cases), and *ATRX* (two cases). Other mutations identified were *POLQ*, *CHEK2*, *SMC1A*, *ABL1*, *PMS1*, *ERCC2*, *MTOR*, *MSH3*, *RB1* and *CDKN2A*. The only mutational signature found within the dataset was that of UV-induced mutagenesis (C>T mutation signature) (Figure 3C).

3.3 Clinical correlation of DNA mutations:

When comparing mutations by histologic grade, mutation in *NOTCH2* was present in 75% (3/4) of patients with carcinoma in situ (CIS) compared to none (0/6) in those who were only partial thickness (the CIN group) (P=.03). Also, a *NOTCH2* mutation was present in 3 out of 4 tumors with leukoplakia, compared to none (0/6) when this clinical feature was absent (P=.03). After comparing the presence of mutations based on tumor response, sex, age, HPV status and self-reported history of extensive UV exposure, no statistical differences were found for any mutation.

4. Discussion

In this study on OSSN, we found both established and novel mutations in several genes known to promote tumorigenesis and tumor progression. These mutations clustered into two different pathways: DNA repair/cell cycle and development/growth regulators. For the former, all samples had 1 mutation in a DNA Repair/Cell Cycle gene with the most common mutation found in the DNA repair gene *TP53* (50% of samples). Gene mutations in *TP53* have previously been described in OSSN and are well known to promote the development and progression of cancer.^{9,18} In a study of 21 OSSN specimens, 52.4% (n=

11) had mutations in exons 5-9 of TP53, seven mutations were C>T transitions.¹⁹ This finding is not surprising as UVB solar radiation is a known promoter of both TP53 mutations and OSSN, and all the TP53 mutations in this study had the characteristic UV-induced C>T mutation.¹⁸ In fact, the only mutational signature present in the tumor samples and the one that embodied the vast majority of mutations in all samples analyzed was UV induced C>T mutation. This finding in combination with the self-reported history of extensive sunlight exposure in all patients further supports that UV light is a major risk factor in OSSN pathogenesis, and highlights the importance in counseling patients about this risk.

The other major DNA repair/cell cycle mutations found in this study, including mismatch repair genes (*MSH2*, *MSH3*, *MSH6*), *BRCA1/BRCA2*, and *ATM*, *ATR*, and *ATX* have not previously been reported in OSSN. Mutations in these genes are known to propagate malfunctions within the DNA repair machinery, leading to accumulation of progressively more mutations.²⁰ Interestingly, *MSH2* mutations have been reported in precancerous skin lesions and keratoacanthoma,²¹ and *BRCA1*, *BRCA2*, and *ATR* mutations have been implicated in skin melanoma pathogenesis.^{22,23} Drug development and clinical trials targeting DNA repair machinery is at the forefront of clinical and basic science research in cancer and many exciting developments have been made and are currently being explored as therapeutic options which may be tested in OSSN in the future.²⁰

All but one sample (9 out of 10) carried a mutation in a Development/Growth pathway gene. Specifically, the most common mutations consisted of HGF (5 cases), APC (4 cases), PTCH1 (4 cases) and PDGFRA (4 cases). These mutations are present within well-known development pathways, including WNT (CTNNB1, APC, AXIN1, AXIN2), Notch (Notch1/2) and hedgehog (PTCH1) and mutations in all of these genes are well known promoters of tumorigenesis. *CTNNB1*, *APC*, *AXIN1*, and *AXIN2* are all heavily implicated in colon, colorectal and hepatocellular carcinoma (HCC).^{24,25} *NOTCH* mutations drive human T-cell lymphoblastic leukemia/lymphoma, B-cell chronic lymphocytic leukemia, and cutaneous and lung squamous cell carcinomas (SCCs).²⁶ Interestingly, in this study, mutations in *NOTCH2* were only found in cases with carcinoma in situ, suggesting that *NOTCH2* either develops later in tumor development or promotes a more aggressive phenotype. Though these tumors still responded to conventional therapy, due to the limited number of cases and correlation with a more aggressive phenotype, *NOTCH2* mutations in OSSN should be assessed in a larger dataset and correlated with clinical outcome and response to therapy. Finally, *PTCH* mutations have been observed in Syndrome-Associated Childhood Medulloblastoma and Gorlin syndrome (naevoid basal cell carcinoma).²⁷

When comparing our results to our prior OSSN study in a Miami population¹², the only shared mutation present in multiple cases was in the HGF gene. HGF binds to its receptor c-MET to mediate downstream cellular signaling and has been shown to regulate tumor growth and metastasis via multiple modalities, including proliferation, angiogenesis, stimulating motility to form micrometastases, and branching morphogenesis.²⁸ There are many drugs currently available and in development that target this pathway, including c-MET tyrosine kinase inhibitors²⁹ and monoclonal antibodies against HGF that may also be useful in OSSN.³⁰ This dissimilarity in mutational profiles between studies might be

because the population is quite distinct in terms of race/ethnicity (100% Hispanic vs 30%).¹² On the other hand, patients were older (mean age 69 years $\pm$ 12.38, vs 57.29 $\pm$ 15.41) and tumors were bigger (40.6mm² $\pm$ 38.87 vs 19.0mm² $\pm$ 17.7) in the present study.

We also recognize that a major limitation of the current study is the small number of samples and that further shared mutations between each cohort as well as novel ones may be uncovered when analyzing a larger number of samples. Additionally, the lack of sequenced germline samples impaired the ability to identify whether all mutations identified were somatic, requiring strict filtering for mutations predicted to be detrimental in known putative cancer genes and in < 0.5% of the population, though the existence of rare germline variants cannot be completely excluded. The presence of only two non-responders to IFN α -2b makes identification and interpretation of genetic alterations promoting therapy resistance difficult. A larger dataset with increased numbers of non-responders is required for such analyses.

5. Conclusions

Despite these limitations, this study centrally implicates UV sunlight exposure in driving OSSN pathogenesis through mutation of genes in DNA repair/cell cycle and development/growth pathways. Many of these genes have actionable drug targets and thus set the foundations for testing new therapeutic options in OSSN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Limited data is available on genetic alterations promoting OSSN
- DNA whole exome sequencing and mutation analysis were performed
- Mutations found were related to DNA repair/cell cycle and development/growth cycle
- All samples had a ultraviolet radiation-induced mutational signature

Figure 1. Typical features of OSSN by HR-OCT. Abrupt transition line between normal and abnormal epithelium, with hyper-reflectivity and epithelial thickening is seen in the right eye of patient 2.

Figure 2.

Slit lamp examination before and after treatment with IFN α -2B. A. Diffuse and gelatinous conjunctival tumor, with leukoplakia and corneal involvement before treatment (pathology confirmed carcinoma in situ) in the right eye of patient 2. B. After successful treatment with topical interferon eyedrops for 6 months.

Figure 3.

Co-mutation plot, protein-protein interactome, and mutational signature analysis of ocular surface squamous neoplasia (OSSN) tissue samples. A. Co-mutation plot demonstrating the distribution of mutations in OSSN samples with enrichment for mutations in genes involved in DNA repair/cell cycle and development/growth. B. Protein-protein interaction networks demonstrating the connectivity of the genes mutated within the DNA repair/cell cycle (top) and development/growth (bottom) pathways. C. Mutation signature analysis demonstrating the UV-light induced (C>T) mutation signature in the OSSN samples. BG, background signal.

Table 1.

Demographics and Clinical Features of Study Population

Patient ID	Age, years	Sex	UV exposure	Tumor location	Tumor Characteristics	Tumor Area	Pathologic grade	AJCC Stage	HPV-related	Response to IFN
1	83	M	Yes	Superior OD	Gelatinous, Corneal Opacity	130 Mm ²	CIS	T3	Yes	No
2	61	M	Yes	Temporal OD	Gelatinous, leukoplakia, Corneal Opacity	34 mm ²	CIS	T3	No	Yes
3	80	M	No	Temporal OD	Gelatinous, Corneal Opacity	20 mm ²	CIN1	T3	Yes	Yes
4	56	F	Yes	Nasal OD	Gelatinous, Corneal Opacity	11 mm ²	CIN2	T3	No	Yes
5	69	M	Yes	Temporal OS	Gelatinous, Corneal Opacity	62 mm ²	CIN1	T3	No	Yes
6	63	F	No	Temporal OD	Gelatinous, Corneal Opacity	10 mm ²	CIN2	T3	No	Yes
7	55	F	No	Temporal OD	Gelatinous, Corneal Opacity	78 mm ²	CIN1	T3	No	Yes
8	90	M	Yes	Temporal OD	Gelatinous, leukoplakia, Corneal Opacity	28 mm ²	CIN2	T3	Yes	No, lost to follow-up
9	58	M	Yes	Temporal OD	Gelatinous, leukoplakia, Corneal Opacity	25 mm ²	CIS	T3	Yes	Yes
10	75	F	No	Nasal OS	Gelatinous, leukoplakia, Corneal Opacity	8 mm ²	CIS	T3	Yes	Yes

Abbreviations: M=male; F=Female; UV, Ultraviolet light exposure defined as self-reported history of extensive UV exposure; AJCC, American Joint Committee on Cancer; HPV, Human Papilloma Virus; IFN, Interferon; OD, right eye; OS, left eye; CIS, Carcinoma in situ; CIN, Conjunctival Intraepithelial Neoplasia