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ADAM3A copy number gains occur in a subset of conjunctival squamous cell carcinoma and its high grade precursors

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Abstract

Conjunctival squamous cell carcinoma (cSCC) and its precursors are among the most frequent ocular surface neoplasms worldwide. Copy gain of 8p11.22 and *ADAM3A* overexpression have been recently identified in invasive cSCC. We sought to study copy number gains using fluorescent in situ hybridization (FISH) in cSCC and the spectrum of precursor lesions. A total of 54 cases conjunctival squamous intraepithelial neoplasia (CIN), carcinoma in situ (CIS), or cSCC were studied using FISH with an *ADAM3A* (8p11 locus) probe and a chromosome 8 (Chr 8) centromere reference probe. Eighty one percent (44/54) of the cases presented in men and 19% (10/54) in women. The age at presentation ranged from 12 to 94 years (mean 65.5 years). Severe CIN was diagnosed in 45% (24/54) of the cases, followed by CIS in 31% (17/54), moderate CIN in 15% (8/54), invasive cSCC in 7% (4/54), and mild CIN in 2% (1/54). Nine (of 54)(17%) cases harbored *ADAM3A* or Chr 8 gains, with one of these cases demonstrating high level amplification. All *ADAM3A* alterations were restricted to high-grade lesions, including 2/17 (12%) cCIS, 1/4 (24%) cSCC, 5/24 (20%) severe CIN and 1/8 (12%) moderate CIN. Monosomy 8 was detected in 2 (4%) cases. No *ADAM3A* alterations were detected in non-neoplastic controls. Gains of *ADAM3A*/chromosome 8 occr in a subset of cSCC and its precursors. Alterations were present in high-grade lesions, sparing non-neoplastic conjunctiva and absent in tested controls. Thus, the specificity of this alteration as a biomarker for ocular SCC deserves further study.

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Conflicts of interest: None.

Keywords

ADAM3A; conjunctiva; ocular surface carcinoma; squamous cell carcinoma; conjunctival squamous intraepithelial neoplasia

Introduction

Conjunctival squamous cell carcinoma (cSCC) and its precursors, conjunctival squamous intraepithelial neoplasia (CIN), are among the most frequent ocular surface neoplasms worldwide. These lesions are the most common conjunctival malignancies in the USA. Recognized risk factors include UV-B irradiation (sun exposure), immunosuppression, smoking, hepatitis B and C, previous injury and xeroderma pigmentosum (1–4). The term “ocular surface squamous neoplasia” encompasses the whole spectrum of these lesions, ranging from dysplasia to overt squamous cell carcinoma (5).

Despite its relative frequency at this anatomic site, the biologic mechanisms responsible for squamous lesions remain understudied. A variety of alterations have been postulated to result from UV irradiation, including p53 positivity (6) and *TP53* mutations. *TP53* mutations were identified in 11/22 (52%) cases of conjunctival squamous cells carcinoma in one study, although they were also identified in a minority of the controls (7). *TERT* promoter mutations were also reported in 43% samples of conjunctival squamous lesions, including CIN and SCC (8). Conjunctival squamous carcinoma also has differential gene expression patterns when compared to conjunctiva proper (9).

ADAM3A gene (A Disintegrin And Metalloprotease Domain 3a) is part of a family (ADAMs) that encodes proteins with both potential adhesion and protease domains and was initially described in spermatogenic cells (10). Amplification of this gene has been found in neoplasms like nasal NK/T cell lymphoma (11). Additionally, copy gain of 8p11.22 and *ADAM3A* overexpression has been also recently identified in invasive cSCC (12). The aims of this study were to confirm *ADAM3A* alterations in cSCC using FISH, to assess these alterations in earlier stages of conjunctival squamous neoplasia including CIN and conjunctival carcinoma in situ (cCIS), as well as their potential as a diagnostic biomarker.

Materials and Methods

Patients

A cohort of 54 cases was studied through a ten-year retrospective review of the clinical and pathology records at JHH/Wilmer (2006–2016). Histologic confirmation was performed in all cases by three pathologists (CGE, FJR, MAV). Grading of all lesions was performed using criteria outlined in the 4th Edition of the *WHO Classification of Tumours of the Eye* (13). Three pterygia without atypia or dysplasia were tested as controls. Appropriate institutional review board approval was obtained for the study and informed consent or waiver of informed consent per IRB guidelines was followed.

Fluorescence in situ hybridization (FISH)

FISH studies were performed on formalin-fixed paraffin embedded tissues using established methods. In brief, slides were placed in a 90°C oven for 15 minutes. Slides were then deparaffinized with xylene (2 times, 15 minutes each) at room temperature (RT), dehydrated in 100% ethanol for 5 minutes at RT, and placed in 10mM Citric Acid (pH 6.0) and microwaved for 10 minutes. Following this, the slides were immersed in 2x standard saline citrate (SSC) for 5 minutes at 37°C followed by digestion in 0.2 % pepsin working solution (1.2 grams pepsin/600 mL 0.9% NaCl pH 2.5) at 37°C for 12 minutes. Immediately after digestion, the slides were dehydrated using an ethanol series (70, 85, 100%) 2 minutes each at RT. Working solution of *ADAM3A* (Empire Genomics, Buffalo, NY, USA) and CON 8(Chromosome 8 centromere probe, Empire Genomics) was made by mixing 2 μL of concentrated *ADAM3A* probe and 1 μL of concentrated CON 8 probe with 7 μL of in Situ Hybridization Buffer (Empire Genomics). The working solution was applied to the target areas, coverslipped, co-denatured with a ThermoBrite™ at 83°C for 5 minutes, and hybridized overnight in a humidified oven at 37 °C. Following hybridization, slides were soaked in RT 2xSSC/0.1% NP-40 to remove coverslips, placed in 2xSSC/0.1% NP-40 at 74°C for 1 minute and then placed into RT 2xSSC/0.1% NP-40 for 2 min. The slides were stained with 4'−6,-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and coverslipped. Slides were evaluated by two observers using an Olympus fluorescence scope with appropriate filters. At least 50 non-overlapping nuclei were evaluated per case. Gains were defined with a target/control probe ratio >1.2, with at least 15% cells containing >3 ADAM3A but 1–2 centromere 8 signals. High level amplification was defined as a ratio >2 and at least 1% of cells with innumerable (>20) target probe signals. Monosomy was defined by loss of the centromere probe in greater than 60% of nuclei in the presence of a normal target/control probe ratio.

Results

Grading of squamous conjunctival lesions

Eighty one percent (44/54) of the cases presented in men and 19% (10/54) in women. The age at presentation ranged from 12 to 94 years (mean 65.5 years). Grading of squamous lesions was done using established criteria (13). Lesions with mild dysplasia were rare and characterized by alterations limited to the lower third of the epithelium. In moderate dysplasia the middle third was also involved, while lesions with severe dysplasia were characterized by involvement of the upper third of the epithelium but with preservation of some differentiation at the surface. cCIS was characterized by squamous abnormalities involving the complete layer, often with overt increases in thickness.

Severe CIN was diagnosed in 45% (24/54) of the cases, followed by CIS in 31% (17/54), moderate CIN in 15% (8/54), invasive cSCC in 7% (4/54), and mild CIN in 2% (1/54). Clinicopathologic features are summarized and illustrated in figures 1–2 and tables 1 and 2.

ADAM3A copy gains in squamous conjunctival squamous intraepithelial lesions

Nine of 54 (17%) cases harbored *ADAM3A* or Chr 8 gains (figure 3). Monosomy 8 was detected in 2 (4%) cases. All cytogenetic alterations were restricted to high-grade lesions,

including 2/17 (12%) cCIS, 1/4 (24%) cSCC and 5/24 (20%) severe CIN and 1/8 (12%) moderate CIN. *ADAM3A* gains specifically (n=5) were identified in lesions with at least severe dysplasia (5/45) and were absent in lesions with mild/moderate dysplasia (0/9). *ADAM3A* gains where usually low level with ratios of target to control probe ranging from 1.28 to 1.51. A single case demonstrated high level amplification, with greater than 20 signals (innumerable) *ADAM3A* gains involving a small subset of tumor cells (1–5%) (figure 3D). No *ADAM3A* alterations were detected in non-neoplastic controls, including adjacent conjunctiva of all abnormal cases or pterygia. Molecular cytogenetic results are summarized in figure 4.

Discussion

cSCC and its precursors are important causes of morbidity in specific populations, particularly the immunosuppressed and those with significant sun exposure. These lesions are difficult to treat, and therefore understanding their biological basis is imperative for the development of novel therapies. In addition, pathologic grading of conjunctival intraepithelial squamous lesions is challenging in specific cases, and may require specialized expertise. Identification of relevant biomarkers that more objectively recognize high grade lesions, may therefore have significant practical utility for diagnosis.

Prior genetic studies of ocular squamous lesions focused on individual genes, e.g. *TP53*, HPV status and association with xeroderma pigmentosum. However, high resolution techniques are increasing our understanding of cSCC and other cancer types. A prior study of global gene expression reported upregulation of matrix metalloproteinases MMP-9 and MMP-11, as well as the gene encoding the calcium binding protein S100A2 in cSCC (9). In another study of conjunctival squamous cell carcinomas using array comparative genomic hybridization, Asnaghi et al. identified amplification of the 8p11.22 locus as the most common cytogenetic abnormality, present in 75% of the cases (9). This region is of interest since it contains several ADAM members, including the *ADAM1B*, *ADAM3A*, and *ADAM5P* genes. In addition, a greater than 100 fold increase in *ADAM3A* mRNA was identified by quantitative PCR in cSCC with 8p11.22 gain compared to normal bulbar conjunctiva.

How *ADAM3A* may mediate tumorigenesis in conjunctival squamous lesions is unclear. Members of the ADAM group of proteins appear to play a variety of roles, including cell migration, adhesion, proteolysis and signaling in various pathways, which may be protumorigenic in the right context (14). This gene has also been found to be amplified in NK/T lymphomas (11), but intriguingly, homozygous loss of *ADAM3A* has also been described in pediatric gliomas (15). It is also important to note that *ADAM3A* is classified as a pseudogene (16). Although pseudogenes have been traditionally interpreted as being non-functional, more recent data actually assigns novel biologic roles to pseudogenes, particularly transcriptional and post-transcriptional regulation, which is relevant to cancer (17). Some studies have also highlighted the role of germline copy number alterations in *ADAM3A* in the predisposition to autoimmune disorders, including Addison disease (18) and systemic lupus erythematosus (19). In our study we did not detect copy number

alterations in normal adjacent regions of conjunctiva in positive cases, which suggests that the alterations in *ADAM3A* in CIN is somatic.

ADAM3A does not seem only to be gained in conjunctival squamous lesions, but also overexpressed, and therefore a hitherto unknown function remains to be discovered. It is important that *ADAM3A* as a pseudogene codifies a lncRNA, many of which have been implicated in a variety of regulatory functions.

In our study, the frequency of gains of *ADAM3A*/Chromosome 8 was relatively lower (17% of all CIN and SCC lesions) than in the study of Asnaghi et al., (75%) which may be secondary in part to the different techniques employed. FISH is a useful technique that preserves tissue architecture. However, aCGH may identify smaller chromosome alterations not detectable by FISH. Different genetics or factors related to geographic location, may also be relevant, since the prior cytogenetic study used tumors developing in Saudi patients.

In summary, gains of *ADAM3A* locus/Chr 8 may be detected by FISH in a subset of cSCC and its precursor lesions. Gains predominated in lesions with at least severe dysplasia. All alterations were limited to the neoplastic regions, sparing the non-neoplastic conjunctiva and absent in tested controls. The specificity of this alteration as a biomarker for ocular cSCC may be of interest and deserves further study in the future.

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Highlights

- Gains of *ADAM3A* locus/Chr 8 may be detected by FISH in a subset of cSCC and its precursor lesions.
- ADAM3A gains predominate in lesions with at least severe dysplasia.
- All ADAM3A/Chr8 alterations were limited to the neoplastic regions, sparing the non-neoplastic conjunctiva.

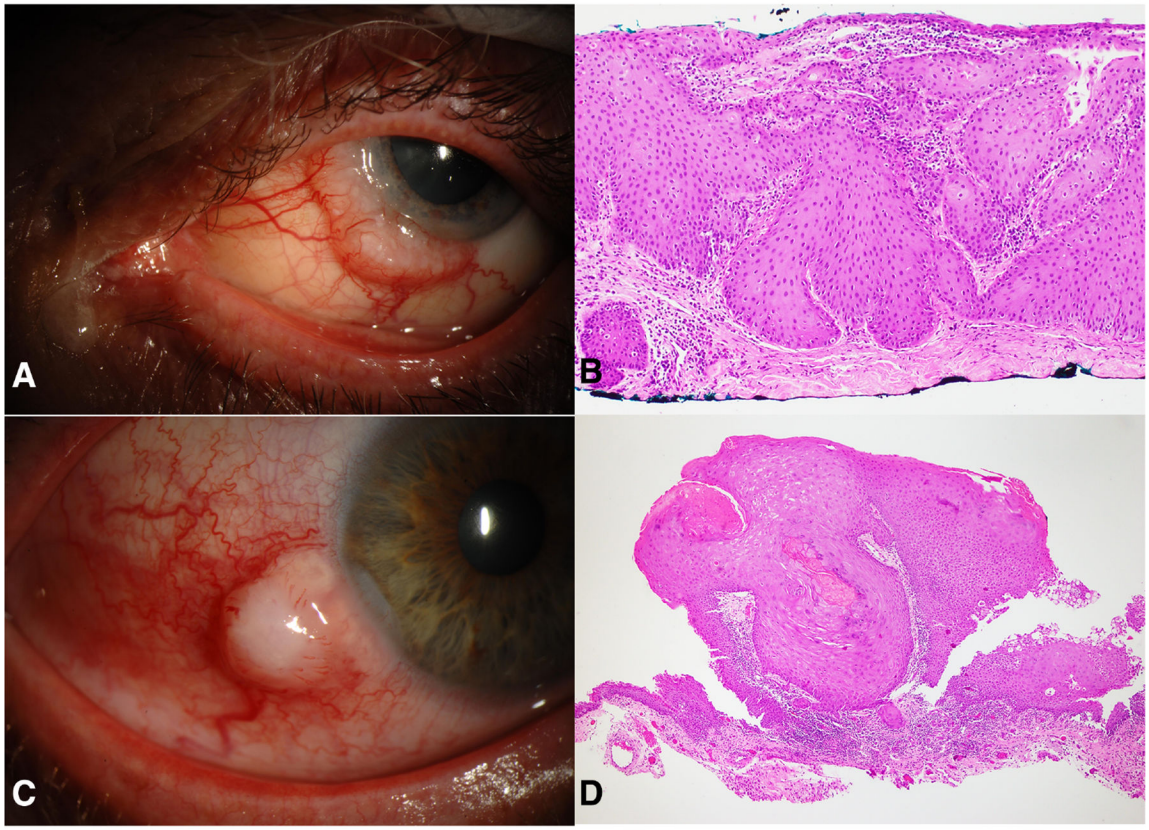

Figure 1. Clinical features of conjunctival squamous lesions.

Case 54: 68 year old male who presented for evaluation of a conjunctival lesion in the left eye. Slit-lamp biomicroscopy revealed an elevated, gelatinous mass adjacent to the limbus, spanning six clock hours from 4 to 10 o'clock, with feeder vessels. The patient elected to undergo resection of the lesion, which was conducted with a no-touch technique using subconjunctival mitomycin, double freeze-thaw cryotherapy, and 99.7% alcohol to the limbus, followed by three months of topical mitomycin therapy **(A)**. Histologic sections demonstrated severe squamous atypia and architectural features of squamous cell carcinoma (**B**). Case 51. 59-year-old white male who presented with a gelatinous limbal lesion with prominent feeding vessels in the right eye. Patient underwent excision with no touch technique, double freeze thaw cryotherapy and amniotic membrane graft. Noted to have a deep positive margin on pathology so underwent second surgery to remove additional tissue in the area of the base of lesion 2 weeks later which was subsequently negative on pathology (**C**). Histologic sections demonstrated an invasive squamous cell carcinoma (**D**).

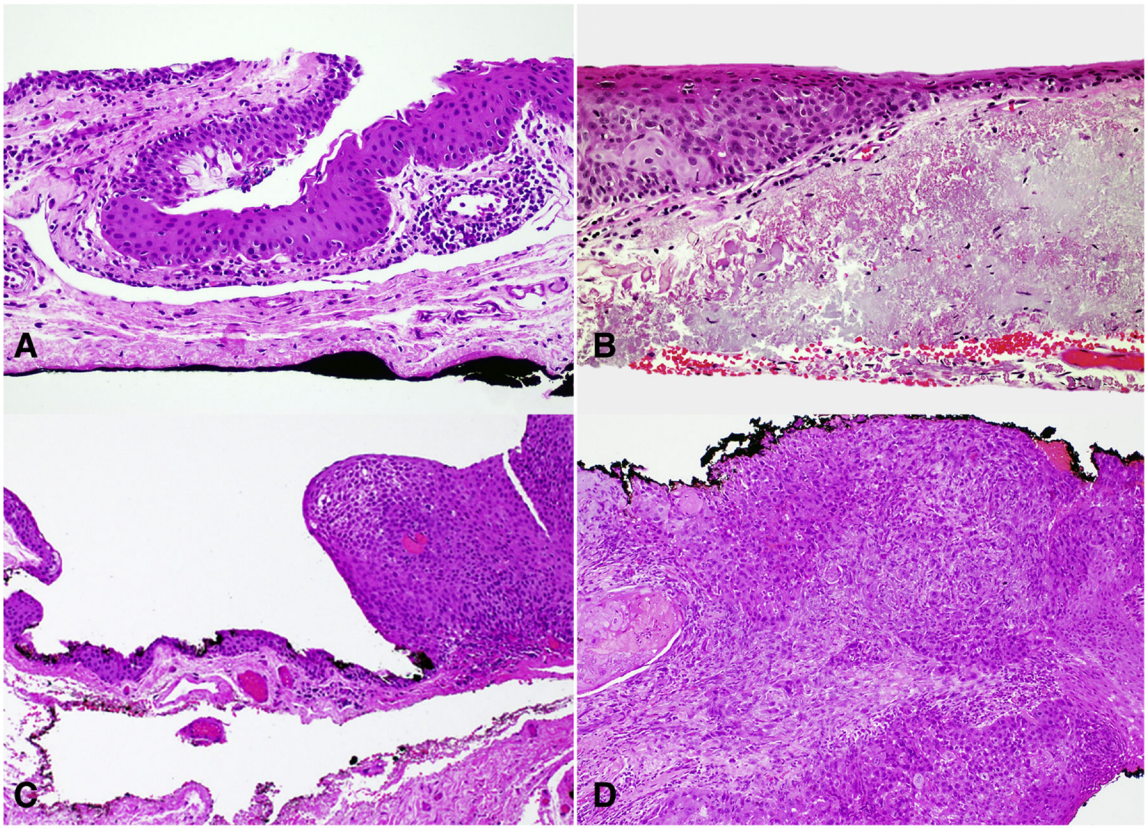

Figure 2. Pathologic features of conjunctival squamous cell carcinoma and intraepithelial lesions.
Conjunctival lesions with mild (A) and severe (B) dysplasia, as well as squamous cell carcinoma in situ (C) or invasive carcinoma (D) are illustrated.

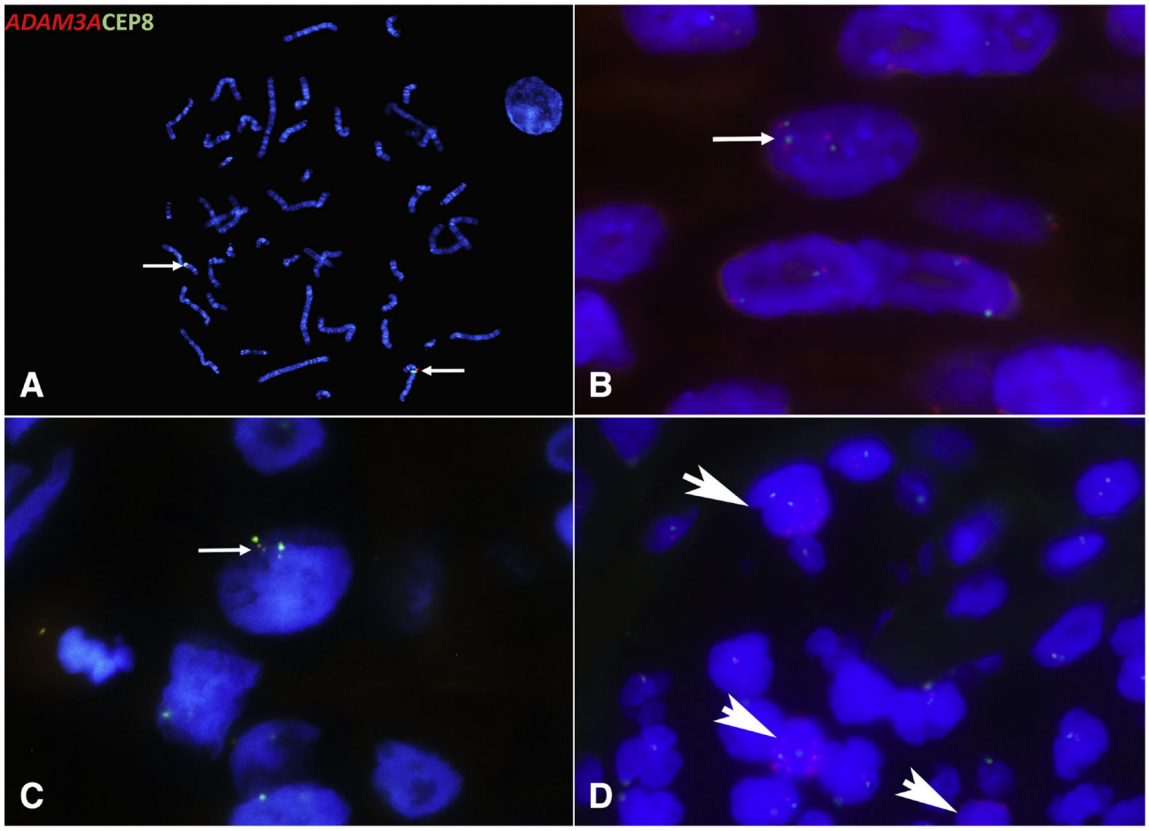

Figure 3. *ADAM3A* copy gains in squamous conjunctival lesions.

A FISH probe specific for the *ADAM3A* locus in chromosome 8 was validated in normal human metaphases **(A)**. Alterations identified included whole chromosome 8 gain **(B)** and gains of the *ADAM3A* locus **(C)**. A single case of conjunctival intraepithelial neoplasia with severe dysplasia demonstrated high level amplification in a small subset of tumor cells, characterized by innumerable signals of the target probe and normal signals for the control probe (arrows)**(D)**.

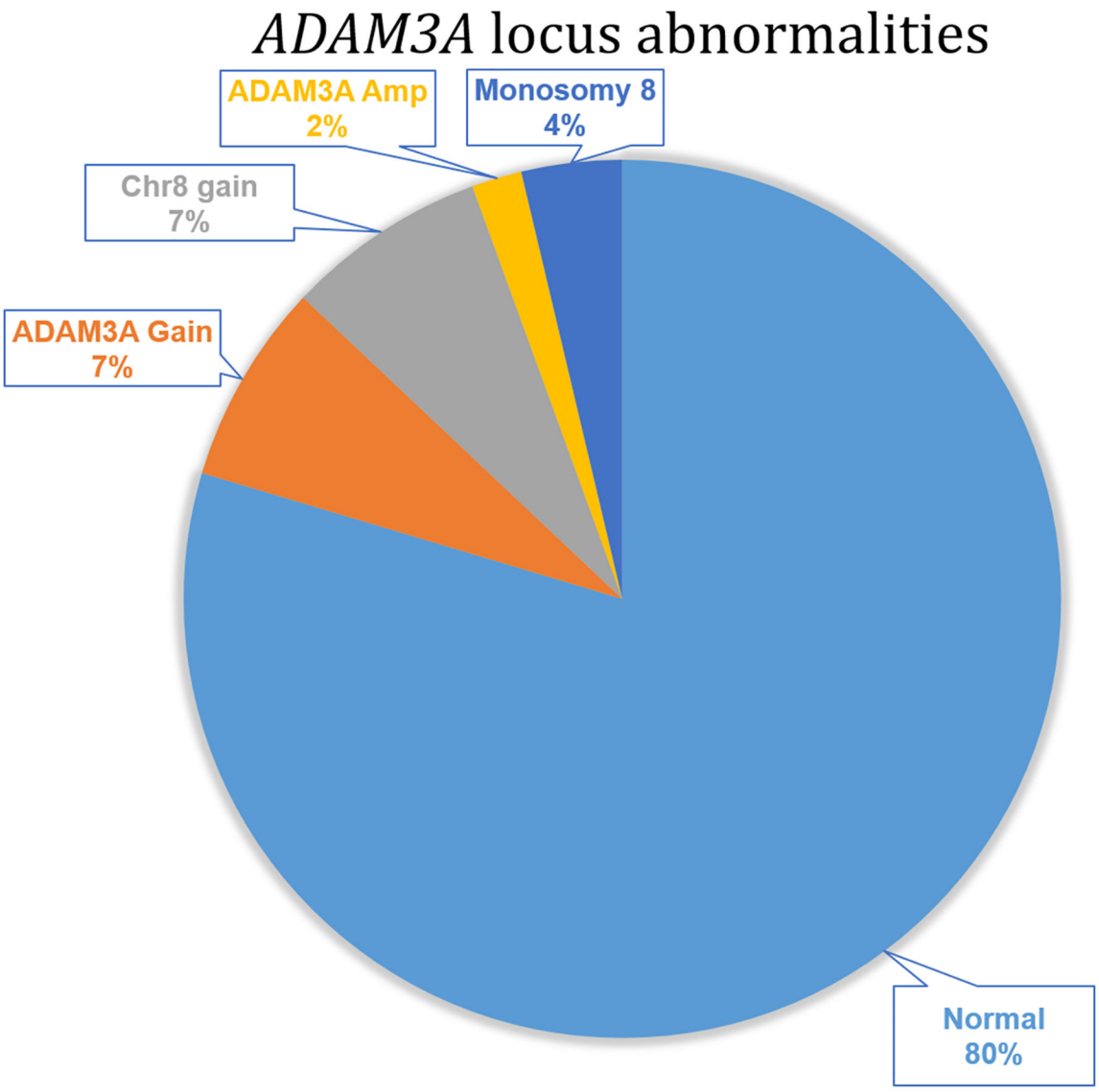

Figure 4. Distribution of *ADAM3A* copy gain abnormalities in squamous conjunctival lesions.