Identification of Multiple DNA Copy Number Alterations Including Frequent 8p11.22 Amplification in Conjunctival Squamous Cell Carcinoma

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PURPOSE. Little is known about the molecular alterations that drive formation and growth of conjunctival squamous cell carcinoma (cSCC). We therefore sought to identify genetic changes that could be used as diagnostic markers or therapeutic targets.

METHODS. The DNA extracted from 10 snap-frozen cSCC tumor specimens and 2 in situ carcinomas was analyzed using array-based comparative genomic hybridization (aCGH), and further examined with NanoString and quantitative PCR.

RESULTS. The number of regions of DNA loss ranged from 1 to 23 per tumor, whereas gains and amplifications ranged from 1 to 15 per tumor. Most large regions of chromosomal gain and loss were confirmed by NanoString karyotype analysis. The most common alteration was amplification of 8p11.22 in 9 tumors (75%), and quantitative PCR analysis revealed 100-fold or greater overexpression of ADAM3A mRNA from 8p11.22 locus. In addition, recurring losses were observed at 1q13.2 and 22q11.23, both lost in 5 (42%) of the 12 tumors, and at 12p13.31, lost in 4 (33%) of the 12 samples. Of the eight loci associated with the DNA damage repair syndrome xeroderma pigmentosum, three showed loss of at least one allele in our aCGH analysis, including XPA (9q22.33, one tumor), XPE/DDB2 (11p11.2, one tumor) and XPG/ERCC5 (13q33.1, three tumors).

CONCLUSIONS. Conjunctival SCC contains a range of chromosomal alterations potentially important in tumor formation and growth. Amplification of 8p11.22 and overexpression of ADAM3A suggests a potential role for this protease. Our findings also suggest that defects in DNA repair loci are important in sporadic cSCC.

Keywords: conjunctiva squamous cell carcinoma (cSCC), array CGH, NanoString assay

A range of precancerous and cancerous epithelial lesions can arise in the cornea and conjunctiva, including actinic keratosis, conjunctival intraepithelial neoplasia (CIN), conjunctival carcinoma in situ (cCIS), and conjunctival squamous cell carcinoma (cSCC). Carcinomas arise most commonly in the interpalpebral area, and involve the bulbar conjunctiva and the limbus.1,2 Although these tumors have a low metastatic potential, they can be locally invasive.3 Most of the lesions grow slowly and are unilateral. The main reported risk factors for this group of ocular surface squamous neoplasms (OSSN) are high ultraviolet (UV-B) exposure, infection with high-risk subtypes of human papilloma virus (HPV), and suppression of the immune response due to infection with human immunodeficiency virus or medication.4,6

The role of HPV in the pathogenesis of cSCC is somewhat controversial. Early investigations detected HPV, but several recent studies have not identified the virus in CIN or cSCC lesions, calling its role in the initiation and growth of these tumors into question.7-9 The strong association between OSSN and exposure to sunlight, however, is clear, with a striking increase in their incidence in geographic areas that are close to the equator.10,11 In addition, patients with xeroderma pigmentosum, who are more susceptible to the effects of solar UV light due to mutation or loss of DNA repair factors, are especially prone to develop the disease.12 Despite this evidence supporting a role for mutations and other DNA alterations in OSSN pathogenesis, the genetic drivers responsible for their initiation and progression are largely unknown. We therefore sought to identify chromosomal abnormalities in a series of cSCC and in situ lesions resected at a single center in Saudi Arabia so as to find molecular alterations potentially useful for diagnosis and as targets for therapy.
Comprehensive Molecular Profiling of cSCC

Table 1. Clinical and Pathological Features of the Ocular Surface Squamous Neoplasia Specimens

<table>
<thead>
<tr>
<th>Case</th>
<th>Array CGH</th>
<th>NanoString</th>
<th>Age Group</th>
<th>Sex</th>
<th>Size Group</th>
<th>Vascularized</th>
<th>Histopathology</th>
<th>Invasion Outside Conjunctiva</th>
<th>Subsequent Recurrence</th>
<th>Immunosuppression</th>
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<td>Yes</td>
<td>Yes</td>
<td>3</td>
<td>M</td>
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<td>cCIS</td>
<td>Cornea</td>
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<td>Yes</td>
<td>4</td>
<td>M</td>
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<td>Cornea</td>
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<td>4</td>
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<td>Yes</td>
<td>4</td>
<td>M</td>
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<td>3</td>
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<td>Orbit</td>
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<td>Sclera</td>
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<td>No</td>
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</tbody>
</table>

Age groups: 3, 45–65 years; 4, >65 years. Size group: 4, 5–10 mm; 5, >10 mm. M, male; F, female; cCIS, conjunctival carcinoma in situ; cSCC, conjunctival squamous cell carcinoma.

Materials and Methods

Clinical Information

In situ and invasive conjunctival squamous cell carcinoma cases were identified through review of pathology and tumor bank records at King Khaled Eye Specialist Hospital (KKESH), Riyadh, Saudi Arabia. Only cases with tissue snap-frozen at the time of surgery were used in this study. The diagnostic slides in all cases were reviewed by ophthalmic pathologists (HA, DE, and AM) to confirm the presence of carcinoma. After Institutional Review Board approval, relevant clinical data were abstracted from the clinical record and linked to the frozen research cases using anonymized sample identification numbers. The clinical characteristics in these cases are summarized in Table 1.

Genomic DNA and Total RNA Extraction

Genomic DNA was extracted from 14 snap-frozen OSSN tumor tissues using DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) following manufacturer’s protocol, and the DNA concentration and quality were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA in 2 of the 14 samples was not sufficient to perform array comparative genomic hybridization array-based comparative genomic hybridization (aCGH) although one of these was used for NanoString analysis. Total RNA also was isolated from the tumor tissues and additionally from two normal bulbar conjunctiva specimens taken at autopsy using an RNeasy Mini Kit (Qiagen) according to manufacturer’s protocol and used for reverse-transcription and quantitative real-time PCR.

Array CGH

The Agilent SurePrint G3 Human 4x 180K Microarray (G4449A) was used in the study (Agilent Technologies, Santa Clara, CA, USA). This array contains 170,334 distinct biological probes chosen from human genome sequences. The integrity of genomic DNA was confirmed by low-voltage 0.6% agarose gel electrophoresis with a mean band size of approximately 50 Kb. Sample labeling was performed following Agilent’s recommendation for array CGH. Briefly, 1.5 μg genomic tumor DNA and normal reference DNA were digested with 50 units of Alu I and Rsa I (Promega, Madison, WI, USA) for 2 hours at 37°C. Samples were then purified by using QIAQuick PCR clean-up kit (Qiagen). Labeling reactions were carried out with restricted and purified DNA for 3 hours at 37°C using a BioPrime Array CGH Genomic Labeling Module (Invitrogen, Carlsbad, CA, USA) with 3 μmol Cy5-dUTP or Cy3-dUTP (Perkin Elmer, Waltham, MA, USA). Labeled samples were purified, concentrated on a Centricon YM-30 column (Millipore, Billerica, MA, USA), and then mixed with 10X blocking agent and 2X hybridization buffer (Agilent Technologies). Hybridization mixtures were first denatured at 95°C for 3 minutes and then immediately transferred to 37°C for 30 minutes. To remove any precipitates, the mixtures were centrifuged at 14,000g for 5 minutes. These mixtures were then hybridized to the microarrays for 40 hours at 65°C in a rotating oven (Robbins Scientific, Mountain View, CA, USA) at 10 rpm. Hybridized microarrays were washed and dried according to manufacturer’s protocols, and imaged with an Agilent G2565BA microarray scanner using default settings. Data were extracted using Feature Extraction Software v9.1 (Agilent Technologies) and analyzed using Agilent’s Genome Workbench software (version 7.0). Aberrant regions (gains and losses) were then identified using a build-in Hidden Markov

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Models algorithm with default settings. Dye swap experiments were performed for each sample and only aberrations present in both sets were considered for further analysis. The Log$_2$ [Tumor/Normal] for each probe in a chromosome region was determined and averaged for the region by the algorithm. Values between 0.75 and 1.0 were considered genomic gain, and ≥1.0 amplification, whereas those between −0.75 and −1.0 were classified as hemizygous loss, and ≤−1.0 as homozygous deletion.

**Quantification of Chromosomal Aberrations by NanoString Analysis**

For NanoString analysis, 600 ng purified genomic DNA was used from each sample and hybridized overnight in a thermocycler at 65°C with the human karyotype panel probes (NanoString Technologies, Inc., Seattle, WA, USA). Hybridization mixtures were then loaded into the nCounter Prep Station, and color-coded barcodes on the reporter probes were read and quantified by the nCounter Digital Analyzer (NanoString Technologies, Inc.). Copy number estimate for each probe was normalized using a normal DNA reference. Quality control, normalization, and data analysis were performed using nSolver software (NanoString Technologies, Inc.). After normalization, copy number estimate values between 2.5 and 3.0 were scored as genomic gains, greater than 3.0 as high-level amplifications, between 1.0 and 1.5 as hemizygous deletions, and lower than 1.0 as homozygous deletions. Technical variability was reported as being 15%; therefore, we used a conservative threshold of 25%.

**Real-Time Quantitative PCR**

Expression levels of ADAM3A and ADAM5P transcripts were determined using TaqMan one-step quantitative RT-PCR (Invitrogen), following manufacturer’s protocol. Primers specific for ADAM3A (ID: Hs01386688_m1), ADAM5P (ID: Hs99999901_s1), were purchased from Invitrogen. All reactions were performed in triplicate, using 50 ng RNA, in an IQ5 Multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA), using EXPRESS One-Step Superscript reverse-transcription and quantitative real-time PCR (qRT-PCR) Kit, universal mix (Invitrogen). The cycling program included the cDNA synthesis at 50°C for 15 minutes, followed by incubation at 95°C for 20 seconds and 40 cycles at 95°C for 3 seconds, and 60°C for 30 seconds, according to manufacturer’s protocol.

**RESULTS**

**Clinical Characteristics**

The cases with frozen tissue available included 1 primary and 1 recurrent cCIS, as well as 10 primary and 2 recurrent invasive cSCCs resected at KKEsH between 2005 and 2010. In some of the recurrent cases, cryotherapy had been used in addition to surgery, but no previous chemotherapy exposure was documented. Five of the patients were between 45 and 65 years of age, with the remainder older than 65; all but one patient were males. Most tumors were well vascularized, and most had invaded into the cornea, sclera, or orbit. None of the patients were known to be immunosuppressed or have a history of HPV infection. Clinical features are summarized in Table 1.

**Recurrent DNA Alterations Detected by Array CGH**

Array CGH analysis was successful in 12 cases, whereas in two tumors, DNA extracted was insufficient for this testing, as indicated in Table 1. All of the tumors had DNA copy number abnormalities, and a graphical representation of the regions of gain (red) and loss (green) among the entire cohort is shown in Figure 1. In Figure 2, representative data from selected single cases are shown. The changes illustrated include gains in chromosome 2 (p arm, case 8) and chromosome 3 (q arm, case 6). Representative losses in chromosome 2 (terminal part of the q arm, case 8), chromosome 3 (p arm, case 6), chromosome 11 (part of the p-q arms, case 12), and chromosome 13 (q arm, case 6) also are shown. Each tumor sample was analyzed twice using a dye swap protocol, and only alterations identified in both experiments were included in the Tables 2, 3, and Supplementary Tables S1 to S4.

The number of hemizygous or homozygous DNA losses ranged from 1 to 23 per tumor, whereas gains/amplifications ranged from 1 to 15 (Tables 2, 3). These ranged from small changes to entire chromosomal arms. The combined gains and losses varied from 3 to 34 per tumor (mean 15). Case 10, which only had two gains and one loss, was distinct microscopically, with adnexal features. The precise extent of the regions of DNA gain and loss, as well as the amplitude of the changes identified, is summarized in Supplementary Tables S1 and S2.

Large regions of DNA gain or amplification including all of chromosome arm 3q were identified in three cases, including two recurrent cCIS and one cSCC. A low-level DNA gain at 17p was also noted in one recurrent cCIS. The region 8p11.22, which was amplified in 9 (75%) of 12 cases. We also examined two frozen normal bulbar conjunctival specimens from adult autopsy patients, and did not identify any large regions of gain or loss. No amplification of 8p11.22 was noted in either of these healthy controls, but low-level DNA gains were found at this locus.

Large recurring regions of loss, encompassing one entire chromosome arm, included 3p, 4p, 13q, 14q, and 17p, with additional large regions lost in only single tumors (Supplementary Table S2). More focal regions of DNA loss, greater than 1 Mb in size were present in more than one case at 4q34.3–4q35.2, 5q11.1–5q14.3, 8p21.2–8p23.2, 9p13.2–9p24.2, 11p15.2–11p15.4, 12p13.2–12p13.32, and 18q12.3–18q22.3.

Recurrent large regions of DNA loss, including all of chromosome 13, all of the long arm of chromosome 17, and most of the long arm of chromosome 18, all of which were found to be deleted in 3 (25%) of the 12 samples, and 8p11.22 lost in 3 (25%) of the 12 samples. No amplification of 8p11.22 was noted in either of these healthy controls, but low-level DNA gains were found at this locus.
FIGURE 1. Graphic representation of all chromosomal changes. The regions of DNA gain (red) and loss (green) among the entire cohort are shown, with the magnitude of the change representing the number of cases with alterations in each region.
FIGURE 2. Chromosomal alterations in selected single cases. Each dot corresponds to a single probe, with deflections to the left of the “0” midline representing reduced hybridization ratio of tumor DNA to that probe, and deflections to the right representing an increased hybridization ratio of tumor DNA to that probe. These correspond to loss and gain of DNA at the respective chromosomal loci. Log₂ [Tumor/Normal] for each probe in a chromosome region was determined and values between 0.75 and 1.0 were considered genomic gain, ≥1.0 amplification, between −0.75 and −1.0 hemizygous loss, and ≤−1.0 homozygous deletion. The thin pairs of lines to the left or right of each alteration indicate that such loss or gain was identified in both of the paired analyses that were performed for each DNA sample according to the dye swap protocol.
Confirmation of Gains and Losses

We used the NanoString platform to confirm chromosomal alterations identified by array-based comparative genomic hybridization (aCGH; Fig. 3). A total of 338 probes were included in the NanoString karyotype panel, in addition to control probes targeting invariant genomic regions used for normalization. The much lower density of probes as compared to aCGH meant that only very large regions of gain or loss could be identified using this technique. Among larger regions of gain identified by array CGH at 8p11.22, thus we had to further analyze this locus using other methods.

Analysis of 8p11.22 Locus

The most frequent genomic alteration was observed on chromosome 3 (Fig. 4A), where the locus 8p11.22 was amplified in 9 (75%) of 12 tumors. Interestingly in the other three cases this locus appeared to be at least partially lost. The region contains a group of genes coding for oncogenic receptors, in tumor formation and cell migration. In particular, the locus includes the ADAM proteins, which are peptidases involved in the shedding and activation of oncogenic receptors, tumor formation and cell migration. They are peptidases involved in the shedding and activation of oncogenic receptors, tumor formation and cell migration.

**Table 3. Chromosomal Gains Detected in the Ocular Surface Squamous Neoplasia Samples**

<table>
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<tr>
<th>Case No.</th>
<th>Genomic Gains</th>
<th>No. Alterations</th>
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<tr>
<td>2</td>
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</tr>
<tr>
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<td>1p31.1; 12p13.31; 20q13.33; 8p11.22</td>
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<tr>
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<td>20</td>
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Amplifications (amplitude ≥ 1) are shown in bold.

**Table 3. Chromosomal Losses Detected in the Ocular Surface Squamous Neoplasia Samples**

<table>
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<td>11</td>
<td>12p13.1; 3q; 6p21.32</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>12p13.1</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>4q34.3–4q35.2; 4q22.1; 5q11.1–5q14.3; 6p22.3; 7p12.1–7p15.2; 9p21.3; 10q11.22; 10q22.2; 10q23.1–10q26.2; 11p13–11p15.4; 11q23.2–11q24.5; 16p12.1–16q12.3; 16q22.2; 18p21.2–18p23.2; 21q21.1–21q22.11; 8p12.1–8p23.2; 14q21.1</td>
<td>19</td>
</tr>
<tr>
<td>14</td>
<td>9p21.3; 17q21.3; 19p13.11–19p12; 12p13.1; 22q12.3</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>5q; 9p13.2–9p24.2; 11p11.12–11p12; 12p; 6p21.32; 11p15.2–11p15.4; 22q11.23</td>
<td>7</td>
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</tbody>
</table>

Homozygous deletions are shown in bold (amplitude ≤ −1).
ADAM3A locus was confirmed at the RNA level by TaqMan one-step quantitative RT-PCR, normalized to 18S mRNA levels (Fig. 4B). Normal bulbar conjunctival tissue from two eyes was used as an additional control. We observed that the ADAM3A gene was at least 100 times more highly expressed in five of the nine samples (cases 2, 3, 7, 12, and 13) where we detected high-level amplification by aCGH, as compared with cases 1 and 5, which showed genomic loss in this locus by aCGH. There was insufficient RNA to analyze samples 6, 8, 9, and 10 by real-time PCR. These five tumors with DNA amplification at the locus 8p11.22 also showed mRNA levels of ADAM3A approximately 100 times higher than non-neoplastic conjunctiva. One tumor (case 14) was found to have amplification of 8p11.22 by aCGH, but did not show increased expression of ADAM3A. Quantitative RT-PCR was also performed to determine the expression of ADAM5P gene, which maps in a region partially included in the 8p11.22 region of gain. However, in contrast to ADAM3A, ADAM5P mRNA was not detected in the tumor samples (data not shown).

FIGURE 3. Confirmation of chromosomal changes using NanoString. Comparison between the copy number variation (CNV) alterations identified by array CGH and NanoString platform shows that among the larger regions of DNA gain identified by aCGH, 14 (54%) of 26 were confirmed by all corresponding NanoString probes (complete confirmation), 9 (34.5%) of 26 were confirmed by some but not all of the corresponding probes (partial confirmation) and 3 (11.5%) were not confirmed. For the large deletions, 12 (34.3%) of 35 were completely confirmed, 19 (54.3%) of 35 were partially confirmed, and 4 (11.4%) were not confirmed. After normalization, NanoString copy number estimate values between 2.5 and 3.0 were scored as genomic gains, greater than 3.0 as high-level amplifications, between 1.0 and 1.5 as hemizygous deletions, and lower than 1.0 as homozygous deletions.
DISCUSSION

Ocular surface squamous neoplasms are most common in countries near the equator with increased exposure to sunlight, suggesting a role for DNA damage due to UV exposure.\(^\text{14,15}\) The incidence in the United States is 0.03 cases per 100,000 persons, whereas in Australia it is 1.9 cases per 100,000 persons.\(^\text{16}\) Conjunctival SCC also is common in Saudi Arabia, with one report noting that most patients spent significant time outdoors, and that advanced cases requiring orbital exenteration were common.\(^\text{17}\) A more recent study reviewing the KKESH tumor registry over the past three decades found that cSCC incidence has remained steady, and that it still represents the most common ocular malignancy diagnosed in adults at this tertiary eye hospital.\(^\text{18}\)

Using aCGH, we identified a number of recurring chromosomal aberrations in the analysis of 10 cSCC and 2 cCIS specimens. To our knowledge, no detailed chromosomal analyses of OSSN have been published, thus we are not able to compare these results to previous studies. However, we found that in 4 of the 12 tumor samples there was loss of one or more loci containing three of the eight genes responsible for the repair of the UV-induced DNA defects and altered in xeroderma pigmentosum. To the best of our knowledge, the genes XPA, XPE/DDB2, and XPG/ERCC5 have not been previously examined in sporadic cSCC, and their loss suggests DNA repair defects also may contribute to tumor formation or progression in nonsyndromic cases.

Less direct evidence implicating changes to DNA repair also were identified. At chromosome 22q11.23, five tumors showed DNA loss and two DNA gain. Deletions of 22q11.23 have previously been reported in cervical SCC, chronic lymphocytic leukemia, and ovarian adenocarcinoma.\(^\text{19-21}\) Interestingly, this locus contains several genes involved in glutathione metabolism, including Glutathione S-Transferase \(\tau\), whose deletion has been found in ovarian, cervical, and endometrial carcinoma.

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cells.22,23 Such deletion has been reported to predispose to mutations in the p53 gene in breast cancer.24 Alterations in p53 expression have also been detected in OSSN, and p53 is known to play a crucial role in the nucleotide excision repair, a pathway for repair of UV-induced DNA damage.25,26 Thus, these changes may relate to the defects in xeroderma pigmentosum genes described above.

A number of other regions of gain and loss we identified have been implicated in the pathogenesis of other tumor types. For example, chromosome 6p21.32 was lost in 3 of 12 of our cases. Deletions including this region also have been reported in nasopharyngeal carcinoma.27,28 Chromosome 14q13.2 was lost in 4 (33%) of the 12 OSSN samples, and this region contains a number of genes, including PAX9 and FOXG1, that play key roles in normal development and carcinogenesis.29 The locus 12p13.31 was deleted in 4 of the 12 samples and gained in 5. Interestingly, the region 12p13 has been found to be genetically unstable and fragile in patients with lymphoid and myeloid hematologic malignancies.30 We also found that the locus 1p31.1 was lost in two samples and gained in another three. High levels of loss of heterozygosity were found in 1p31 locus, along with other regions in the p arm of chromosome 1, in different human solid tumors.31

We also examined if any DNA changes in our cases could be linked to cutaneous SCC or HPV-associated cervical and oropharyngeal carcinomas, and a summary of relevant recurring chromosomal abnormalities is shown in Supplementary Table S4.32–42 Similarities include DNA gains that we observed in 3q22.3–3q28 and in 5p, as well as DNA losses in 9p, 13q, 17p, and 18q, which also are found in cutaneous SCC. A smaller number of changes, which were similar in our tumors and HPV-associated neoplasms outside the eye are also listed in Supplementary Table S4.

Our study was too small to tightly link clinical features with genetic changes. However, one of the four recurrent tumors, case 12, had the highest number of DNA gains (15) and the second highest number of DNA losses (19), thus an increased number of DNA alterations in some cases may be associated with more aggressive clinical behavior or tumor progression. Larger studies that include cases drawn from other ethnic groups and geographical regions will need to be performed to address this issue, as well as to confirm our genetic observations in this single-institution cohort.

The most frequent chromosomal variation was observed on chromosome 8, where 8p11.22 was amplified in nine tumors (75%). This region contains a group of genes coding for ADAM proteins known to be involved in the activation of oncogenic receptors and tumor formation and spread.33–35 The ADAMs are transmembrane proteins that contain a disintegrin and a metalloprotease domain, and have both cell adhesion and protease activities. Their functions include activation of receptors and tumor formation and spread.43 The ADAMs are implicated in mucous membrane carcinomas. Our studies thus confirm the importance of DNA repair in cSCC, and suggest that ADAM proteases might also play a role.

In summary, we found frequent DNA copy number alterations in cSCC samples, including gains and losses of large DNA regions and more focal changes encompassing a limited number of loci. The most frequent focal homozygous loss was found in 22q11.23, whose deletion predisposes to p53 mutation in breast cancer. Loss of loci encoding three genes altered in xeroderma pigmentosum also was found in four cases. The predominant amplification was observed at the 8p11.22 locus, where ADAM3A is located, and this family of genes has recently been implicated in mucous membrane carcinomas. Our studies thus confirm the importance of DNA repair in cSCC, and suggest that ADAM proteases might also play a role.

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References


