ORIGINAL ARTICLES

Analysis of Cytogenetic Abnormalities in Squamous Cell Carcinoma by Array Comparative Genomic Hybridization

R. Salgado,* A. Toll,* B. Espinet,* E. González-Roca,* C. L. Barranco,* S. Serrano,* F. Solé,* and R. M. Pujol‡
*Servicio de Anatomía Patológica, Laboratorio de Citogenética y Biología Molecular and ‡Servicio de Dermatología, Hospital del Mar, Barcelona, Spain

Abstract. Introduction. Few conventional cytogenetic studies of squamous cell carcinoma (SCC) have been performed to date. The introduction of cytogenetic techniques such as comparative genomic hybridization (CGH) has resolved some of the problems associated with conventional cytogenetics. The aim of this study was to analyze the presence of genetic abnormalities in a series of patients with SCC using the technique of array CGH.

Material and methods. The study included 8 patients (7 men and 1 woman; mean age, 75 years) diagnosed with primary SCC. DNA was extracted from frozen tissue and analyzed by array CGH.

Results. All cases had genetic alterations, with gains more frequent than losses. The chromosomal regions with gains, in descending order of frequency, were as follows: 5p15.2, 9q31.3-q33.2, 13q, 18q22, 1p21-p22, 1q24-q25, 3p13, 4q33-q34 (HMGB2, SAP30), 20p12.2 (JAG1), 21q21.1, and Xq21.33. The region 9p13.1-p13.3 was the only one to display recurrent loss. No correlation was observed between the presence of gains or losses and the clinical and pathologic characteristics of the tumors.

Conclusions. This is the first study to use the technique of array CGH to analyze genetic alterations in SCC. The finding of certain previously described aberrations (gain of 5p) suggests the existence of recurrent abnormalities. Likewise, the observation of alterations in small regions of chromosome 1 highlights the sensitivity of the technique to detect small changes. Application of the technique to a larger series of cases will provide greater insight into the genetic abnormalities implicated in the process of tumorigenesis in SCC.

Key words: array, comparative genomic hybridization, cytogenetics, cutaneous squamous cell carcinoma.

ESTUDIO DE LAS LESIONES CITOGENÉTICAS EN EL CARCINOMA ESCAMOSO CUTÁNEO MEDIANTE LA TÉCNICA DE ARRAYS DE HIBRIDACIÓN GENÓMICA COMPARADA

Resumen. Introducción. Los estudios de citogenética convencional realizados en el carcinoma escamoso cutáneo (CEC) son escasos. La introducción de las técnicas de citogenética, como la de hibridación genómica comparada (HGC), solventan algunos de los inconvenientes planteados por las técnicas de citogenética convencional. El objetivo de este estudio es analizar la presencia de alteraciones genéticas mediante la técnica de array–HGC en una serie de CEC.

Material y métodos. Se estudiaron un total de 8 pacientes (7 varones/una mujer; edad media: 75 años) diagnosticados de CEC primario. Se realizó extracción de ADN a partir de material congelado y se realizó la técnica de array–HGC.

Correspondence:
Agustí Toll Abelló
Servicio de Dermatología, Hospital del Mar
Passeig Marítim, 25-29
08003 Barcelona, Spain
93628@imas.imim.es

This study was funded by a grant from the Health Research Fund (Fondo de Investigación Sanitaria) of the Spanish Ministry of Health (grant PI041728).

Manuscript accepted for publication September 13, 2007.
Introduction
Cutaneous squamous cell carcinoma (SCC) represents approximately 20% of all malignant skin cancers. Its incidence is increasing and some studies situate it at between 40 and 200 cases per 100,000 individuals per year. SCC is usually observed in elderly individuals. It has been suggested that 14% of men and 8% to 9% of women will develop SCC over the course of their lifetime. It has the potential for distant metastasis, with an overall mortality estimated at 2,500 cases per year in the United States of America. Its etiology is multifactorial and is affected by both environmental and host-related factors.

Among the most important extrinsic or environmental factors is chronic sun exposure (UV radiation). Other factors less frequently implicated in the development of SCC include exposure to ionizing radiation (x-rays), arsenic, hydrocarbons, tobacco (SCC of the lip), chronic inflammation (burn scars, chronic ulcers, chronic fistulas, etc), and some viral infections (human papillomavirus). Age, skin phototype, immune status, and the presence of constitutive genetic abnormalities are also relevant factors. Individuals who undergo chronic immunosuppressant therapy (organ recipients) have a greater risk (3 to 4 times greater than the general population) of developing SCC.

Genetic Lesions and Cutaneous Squamous Cell Carcinoma
The development and metastasis of SCC is a complex process that includes transformation, proliferation, neovascularization, and invasion. Despite the unquestionable advances that have taken place in recent years in terms of our understanding of the pathogenic mechanisms involved, the genetic lesions responsible for SCC are still unknown. The identification of genetic abnormalities in specific regions of the genome in these tumors may facilitate future identification of the genes (oncogenes and tumor suppressor genes) involved in the pathogenesis of the disease.

It has been demonstrated that 25% to 80% of SCC are associated with aneuploid lesions, with little variation in the degree of aneuploidy (unlike in the precursor lesions), suggesting a clonal origin. Various experimental and epidemiologic findings have revealed a direct correlation between the development of SCC and exposure to UV radiation. UV radiation leads to characteristic point mutations and small deletions in the DNA. Exposure of cultured keratinocytes to low doses of UV-A and UV-B led to the appearance of micronuclei, cytoplasmic inclusions of a nuclear nature that correspond to genetic material that was not correctly incorporated in the daughter cells during cell division.

Various approaches involving cytogenetic techniques of differing complexity have been used to identify the genetic alterations that are characteristic of SCC.

Conventional Cytogenetics
Conventional cytogenetics is a frequently used tool in the identification of genetic alterations in hematologic
malignancies, in which metaphase chromosomes can be easily obtained. In hematology, this technique is very useful for the detection of recurrent chromosomal aberrations, which affect both the diagnosis and the prognosis and course of many such malignancies. The difficulties associated with obtaining metaphase chromosomes from tumor cells in cultured SCC explain why few cytogenetic studies of cutaneous tumors have been performed.

Casalone et al analyzed 3 patients with SCC using conventional cytogenetics in direct preparations (24 hours) and short-term cultures (10 to 28 days). Direct preparations showed gains of chromosomes 6, 8, 9, 11, 16, and 21, and loss of chromosomes 1 and 14, while these abnormalities were not observed in short-term cultures. However, Jin et al identified numerous cytogenetic abnormalities in short-term cultures of neoplastic cells, the most frequent being loss of chromosomes 2, 4, 8p, 9, 11, 13, 14, 18, 21, X, and Y, and gain of chromosomes 1q, 7, and 8q.

In addition to numerical chromosomal changes (gains and losses), other authors have observed structural rearrangements in the centromeric regions of chromosomes 1, 8, and 9, including the formation of isochromosomes—i(1p), i(1q), i(9p), i(9q), and i(9q)—and deletions and translocations involving the entire chromosomal arm.

Fluorescent In Situ Hybridization

The technique of fluorescent in situ hybridization (FISH) allows detection and localization of specific DNA sequences in chromosome, cell, or tissue preparations. This method is based on the hybridization of a fluorescently labeled specific DNA sequence (probe) to the DNA of the sample.

Few studies have used interphase FISH in SCC, probably due to the technical problems associated with sample processing (presence of keratin, adipose tissue, etc). A case has been described in which this technology revealed the presence of trisomy 7 in SCC. In another study, a 3p21 deletion was identified in 67% of cases and a surprising gain of 17p13 (TP53) was observed in 27% of cases of SCC studied.

Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique based on 2-color FISH that allows analysis of overall losses and gains of genomic material in a single hybridization step without the need to obtain dividing cells.

It involves competitive hybridization between tumor DNA (test) and normal DNA (reference) on normal metaphase chromosomes in the presence of an excess of human Cot1 DNA. The tumor cell DNA from the chromosomal regions with increased copy numbers (gains and amplifications) binds the metaphase chromosomes proportionally more than DNA from healthy cells, while the DNA from regions that are present at lower copy numbers (losses) will bind proportionally less to normal chromosomes. Prior to hybridization, the DNA from the tumor and the healthy tissue are labeled with different fluorochromes (eg, the tumor DNA in green and the normal DNA in red), allowing subsequent identification by fluorescence microscopy. Digital analysis of the captured images of metaphase chromosomes allows quantification of the proportion of green and red fluorescence in each chromosome, such that gains of genomic material would lead to an increase in green fluorescence in that area, and loss of genomic material would lead to a reduction in green fluorescence, thereby leading to a relative increase in red fluorescence. Subsequent karyotyping is performed by staining of the chromosomes with DAPI (4′-6-diamidino-2-phenylindole).

This technique has been very useful in the cytogenetic characterization of solid tumors, which do not allow easy preparation of metaphase chromosomes for conventional karyotyping and multicolor FISH on chromosome preparations. Studies of SCC by CGH have allowed detection of genomic gains on chromosome arms 3q, 17q, 4p, 14q, Xq, 5p, 9q, 8q, 17p, and 20q, and losses on 9p, 3p, 13q, 17p, 11p, 8q, and 18p.

Array Comparative Genomic Hybridization

In recent years, the technique of array CGH has been developed. The principle of the technique is the same as for conventional CGH but involves the use of genetic sequences spotted on glass slides (genomic sequences derived from P1 artificial chromosomes [PAC], bacterial artificial chromosomes [BAC], etc) instead of conventional chromosome spreads. The arrays are cohybridized with DNA from the sample and with control markers labeled with 2 different fluorochromes (generally Cy3 and Cy5), and the results are analyzed using specific software. This technique allows a higher resolution to be obtained (1 megabase pair [Mb] compared with a resolution of 10 Mb obtained with conventional CGH). In addition, the technique is much more sensitive than conventional CGH, since it allows the regions containing gains and losses to be defined more accurately.

In this study, we describe the analysis of 8 primary SCC by array CGH in an attempt to identify new genes or chromosomal regions implicated in the development and progression of this cutaneous tumor.
Materials and Methods

Material

Patients

Eight patients with a diagnosis of primary SCC were included in the study. The group comprised 7 men and a woman aged between 61 and 97 years (mean, 75 years). The clinicopathologic characteristics of the lesions are shown in Table 1.

The lesions were located in photoexposed areas in 6 patients and in photoprotected skin (legs) in 2. The mean diameter of the tumors measured at the widest point was 2.2 cm.

In terms of morphology, 6 lesions displayed a multinodular histologic pattern, and the remainder displayed crateriform (keratoacanthoma-like) morphologic characteristics. Six lesions had changes that were compatible with actinic keratosis in the epidermis adjacent to the tumor. The mean depth of the tumor by histology was 3.14 mm. None of the tumors displayed perineural, vascular, or skin adnexal invasion.

Samples for Analysis

A sample of the tumor taken from each patient was embedded in optimum cutting temperature (OCT)
compound (Tissue Tek OCT, Sakura, Zoeterwoede, The Netherlands) to be stored in the tissue bank. Histologic sections were obtained from the tumors and stained with hematoxylin-eosin to confirm the presence of at least 70% tumor cells, which is essential for array CGH.

Methods

DNA Extraction

For extraction of genomic DNA, 10 to 14 sections (14 µm) were obtained and stored in a 1.5 mL sterile tube. Extraction was performed using the QiaAmp DNA Tissue Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer’s protocol. If samples were contaminated with RNA or protein, purification was performed using the traditional method of DNA precipitation with phenol-chloroform-isoamyl alcohol (Sigma-Aldrich, St Louis, Missouri, USA). DNA was quantified by spectrophotometry (Nanodrop, Wilmington, Delaware, USA). A minimum of 50 ng in 50 µL was established in order to obtain the amount necessary for the analysis.

Array CGH

The DNA samples from the 8 patients selected were hybridized individually on a microarray, consisting of a glass slide spotted with 2621 BAC clones with a mean resolution of 1 Mb (Spectral Chip 2600, Spectral Genomics, Houston, Texas, USA). All clones were spotted on the microarray in duplicate and their position was provided by the manufacturer. Hybridization was performed with 2 µg samples of genomic tumor and control DNA (Promega, Madison, Wisconsin, USA), which were separately labeled with deoxycytidine triphosphates (dCTPs) carrying the fluorochromes Cy5 (Cy5-dCTP, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and Cy3 (Cy3-dCTP, Amersham Biosciences) using the Bioprime Genomic Random Labeling Kit (Invitrogen, Carlsbad, California, USA). Both sets of labeled DNA probes (both direct labeling—tumor DNA-Cy5 and control DNA-Cy3—and dye-swap—tumor DNA-Cy3 and control DNA-Cy5) were mixed, coprecipitated with isopropanol, washed, and resuspended in hybridization solution (Spectral Genomics, Houston, Texas, USA). The DNA mixture was denatured at 72°C for 10 minutes, prehybridized at 37°C for 30 minutes, and hybridized with the microarrays for a minimum of 16 hours at 37°C, according to the manufacturer’s instructions. Following hybridization, the microarrays were washed with 50% formamide in 2X salt-sodium citrate (SSC), 2X SSC containing Nonidet P-40, and 0.2X SSC.

Images and signal intensity were obtained with a G2565BA scanner (Agilent Technologies, Santa Clara, California, USA) using GenePix 6.0 image software (Axon Instruments, Union City, California, USA). Raw data were filtered and normalized using BacAnal, a local web server allowing access to the Limma statistical package (Bioconductor).

The threshold to consider values as statistically significant for gains or losses was independently established for each patient and the final reference values were established based on the mean ± 2 SD of the proportions of all of the BAC spots.
Results

All of the tumors included in the study were primary cutaneous SCC, 6 located on sunlight-exposed skin and 2 in areas not exposed to sunlight. The tumors varied both in size and depth of the lesion, as well as in histologic pattern.

Array CGH revealed that genomic gains and losses occurred in all of the hybridized samples, although they did not affect all patients equally (Table 2).

Table 3 shows the characteristics of the lesions studied in relation to the different clinicopathologic characteristics. The gains most often observed affected the clones mapped to 5p15.2 (Figure), 9q13.3-q33.2, 13q, 18q22, 1p21-p22, 1q24-q25, 3p13, 4q33-q34 (HMGB2, SAP30), 20p12.2 (JAG1), 21q21.1, and Xq21.33. The 9p13.1-p13.3 region was the only one to display recurrent loss (Table 4).

<table>
<thead>
<tr>
<th>Mean Gains</th>
<th>Mean Losses</th>
<th>Mean Total Alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic Pattern</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multinodular (n = 6)</td>
<td>9</td>
<td>4.5</td>
</tr>
<tr>
<td>Similar to keratoacanthoma (n = 2)</td>
<td>5.5</td>
<td>3</td>
</tr>
<tr>
<td>Depth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 3 mm (n = 3)</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>&lt; 3 mm (n = 5)</td>
<td>7.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 1 cm (n = 6)</td>
<td>8.5</td>
<td>4.5</td>
</tr>
<tr>
<td>&lt; 1 cm (n = 2)</td>
<td>8</td>
<td>3.5</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photexposed (n = 6)</td>
<td>9</td>
<td>4.5</td>
</tr>
<tr>
<td>Photoprotected (n = 2)</td>
<td>5.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 4. Most Common Alterations

<table>
<thead>
<tr>
<th>Gains</th>
<th>Losses</th>
</tr>
</thead>
<tbody>
<tr>
<td>5p15.2 (3/8)</td>
<td>9p13.1-p13.3 (2/8)</td>
</tr>
<tr>
<td>9q13.3-q33.2 (2/8)</td>
<td></td>
</tr>
<tr>
<td>13q (3/8)</td>
<td></td>
</tr>
<tr>
<td>18q22 (2/8)</td>
<td></td>
</tr>
<tr>
<td>1p21-p22 (2/8)</td>
<td></td>
</tr>
<tr>
<td>1q24-q25 (2/8)</td>
<td></td>
</tr>
<tr>
<td>3p13 (2/8)</td>
<td></td>
</tr>
<tr>
<td>4q33-q34 (HMGB2, SAP30) (2/8)</td>
<td></td>
</tr>
<tr>
<td>20p12.2 (JAG1) (3/8)</td>
<td></td>
</tr>
<tr>
<td>21q21.1 (2/8)</td>
<td></td>
</tr>
<tr>
<td>Xq21.33 (2/8)</td>
<td></td>
</tr>
</tbody>
</table>

Figure. Results of array comparative genomic hybridization on chromosome 5 in case 2. A, Normal chromosome 5. B, Gain on the short arm of chromosome 5.
Discussion

Few conventional cytogenetics studies have been performed with solid tumors, probably as a consequence of technical difficulties in obtaining dividing cells. The introduction of CGH techniques, which allow detection of variation in copy number over the entire genome in a single experiment, has resolved some of the difficulties presented by conventional cytogenetics techniques. CGH requires only small quantities of DNA and detects gains and losses of genetic material without the need for cultured cells. Loss or gain of fragments of genetic material can imply critical functional changes in genes involved in a variety of physiologic processes associated with cell proliferation and differentiation. Therefore, the analysis of genetic alterations can yield valuable information on the genes involved in both the onset and progression of a tumor.

Analysis of mutations in the tumor suppressor gene TP53 (17p13) revealed a high rate of mutations induced by UV radiation, both in actinic keratosis (30%-53%) and SCC (58%-69%). Other chromosomal loci (3p, 9p, 9q, 13q, and 17q) have also been observed to have genomic losses with similar frequencies in actinic keratosis and SCC.

Numerical chromosomal aberrations have also been detected in chromosomes 1, 11, 8, 9, 5, 3, and 7 and isochromosomes 1q, 8q, 5p, 1p, 9p, and 9q in SCC. Analysis of SCC by interphase FISH revealed a common deletion at 3p21 in 67% of cases and a surprising gain at 17p13 (TP53) in 27% of cases. Using CGH techniques, Ashton et al detected genomic gains on chromosome arms 3q, 17q, 4p, 14q, Xq, 5p, 9q, 8q, 17p, and 20q, and losses on 9p, 3p, 13q, 17p, 11p, 8q, and 18p. In that study, they also observed a significant loss of 18q in SCC but not in actinic keratosis.

Studies of SCC cell lines using complementary DNA microarrays revealed an increase in the expression of genes regulated by nuclear factor κB (NF-κB). Activation of NF-κB has been found to be associated with an increase in the aggressive and metastatic potential of SCC. Inactivation of genes in the CDKN2A locus, located in chromosome region 9p21, either by allelic loss or mutation (induced by UV light), can have a marked effect in aggressive forms of SCC through inactivation of p16 and p14 proteins.

At some particular sites (SCC of the penis), gains have occasionally been observed at 8q24, 16p11-12, 20q11, 13, 22q, 19q13, and 5p15, and deletions at 13q21-22, 4q21-32, and the X chromosome. The development of array CGH has led to an increase in the resolution of the analysis compared with conventional CGH. Another advantage of the technique is the rapid mapping within the complexity of the genome, thus facilitating the search for candidate genes with potential pathogenic roles.

In our study, undertaken in a small series of patients with SCC, we found that all cases had twice as many gains as losses. Due to the small number of cases analyzed, it was not possible to establish an unequivocal relationship between histologic subtype and depth of the lesion and an increased or decreased number of genetic alterations. Likewise, patients with lesions larger than 1 cm did not have a greater number of genetic alterations than those with lesions smaller than 1 cm.

The observation of previously described genetic alterations (gain of 5p) indicates that there may be recurrent lesions that could be informative. In addition, detection of small cytogenetic changes such as those involving chromosome 1, which were not observed previously using conventional CGH, highlights the capacity of the technique to detect small changes.

This study represents a preliminary appraisal of the use of array CGH in the cytogenetic characterization of SCC. Detailed analysis of each of the lesions described, both in the patients studied and in new cases, should help to identify the underlying genetic lesions responsible and the genes involved in the development of SCC, as well as helping to provide a better understanding of the pathogenic mechanisms involved.

Conflicts of Interest

The authors declare no conflicts of interest.

References

of 73 basal cell and three squamous cell carcinoma: different
findings in direct preparations and short-term culture. Cancer
et al. Nonrandom karyotypic features in squamous cell
 carcinomas of the skin. Genes Chromosomes Cancer. 1999;26:
295-303.
translocation of chromosome 14 in a skin squamous cell
carcinoma from xeroderma pigmentosum patient. Cancer
13. Worsham MJ, Carey TE, Benninger MS, Gasser KM, Kelker
W, Zarbo RJ, et al. Clonal cytogenetic evolution in a squamous
cell carcinoma of the skin from a xeroderma pigmentosum
14. Cheville JC, Bromley C, Argenyi ZB. Trisomy 7 in
keratoacanthoma and squamous cell carcinoma detected by
546-50.
E. Deletion monitoring in skin tumors by interphase-FISH
JW, Waldman F, et al. Comparative genomic hybridization
17. Ashton KJ, Weinstein SR, Maguire DJ, Griffiths LR.
Chromosomal aberrations in squamous cell carcinoma and
solar keratoses revealed by comparative genomic hybridization.
Arch Dermatol. 2003;139:876-82.
18. Cai WW, Mao JH, Chow CW, Damani S, Balmain A,
Bradley A. Genome-wide detection of chromosomal
2002;20:393-6.
comparative genomic hybridization and its applications
20. Brash DE, Rudolph JA, Simon JA. A role for sunlight in
skin cancer: UV-induced p53 mutations in squamous cell
21. Rehman I, Takata M, Wu Y-Y, Rees JL. Genetic change in
22. Dong G, Loukinova E, Chen Z, Gangi L, Chanturita TI,
Liu ET, et al. Molecular profiling of transformed and metastatic
murine squamous carcinoma cells by differential display and
cDNA microarray reveals altered expression of multiple genes
related to growth, apoptosis, angiogenesis, and the NF-κB
23. Sunwoo JB, Chen Z, Dong G, Yeh N, Bancroft CC, Sausville
E, et al. Novel proteasome inhibitor PS-341 inhibits activation
of NF-κB, cell survival, tumor growth and angiogenesis in
O, Spandidos DA. Mutational analysis of CDKN2A genes
in patients with squamous cell carcinoma of the skin. Br J
Ornellas AA, et al. Genetic imbalances in 26 cases of penile
squamous cell carcinoma. Genes Chromosomes Cancer.
2001;31:48-53.