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Assessment of Chromosomal Gains and Losses in Oral Squamous Cell Carcinoma by Comparative Genomic Hybridisation

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Cytogenetic studies have demonstrated that oral squamous cell carcinomas (OSCCs) are usually characterised by complex karyotypes with many marker chromosomes. We analysed the genetic changes of six OSCC cell cultures by comparative genomic hybridisation (CGH). The CGH technique provides information on chromosomal gains and losses of the whole tumour genome in a single experiment and can therefore identify regions that harbour putative tumour suppressor genes (in the case of loss of chromosomal material) or oncogenes (in the case of gain or amplification of chromosomal material). Recurrent losses were detected at chromosome arms Xp and 3p (four cases). Gains consistently occurred at chromosome arms 8q and 9q (four cases) and at 1q, 3q, 5p, 7p, and 9p (three cases). The same six tumour cultures have previously been analysed by classical karyotyping. An important discrepancy between the two techniques was the number of losses detected: 55 with karyotyping versus 26 with CGH. On the basis of the cytogenetic complexity of these tumours and on FISH experiments that confirmed the CGH results, we conclude that genetic changes, particularly losses, can be more reliably detected by CGH analysis. © 1997 Elsevier Science Ltd. All rights reserved.

Key words: oral carcinoma, comparative genomic hybridisation, cytogenetics, fluorescence *in situ* hybridisation

INTRODUCTION

The multistep genetic pathway of oral squamous cell carcinoma (OSCC) development is still poorly understood. This is partly because OSCCs are among the most cytogenetically complex of all solid tumours; nearly all chromosomes have been reported to be involved in both numerical and structural aberrations. In order to identify which genetic changes are critically involved in the process of tumour initiation and progression, the combined data of many studies are needed. The most frequently reported non-random genetic changes are losses at 3p, 8p, 9p, 18q, and gains at 3q, 5p, 7p and 8q [1–5]. A limited number of chromosomal bands appear to be

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frequently involved in breakpoints, such as 1p13, 1p22, 3p13, and the centromeric regions of all chromosomes, which result in gains and losses of whole chromosome arms [4–6]. Structural variation in band 11q13 is often associated with amplification of the oncogenes FGF3, FGF4, cyclin D1 and EMS1 [7, 8]. Amplification of these genes has been reported to be associated to disease progression or clinical outcome [9, 10].

In an earlier study we have shown that centromeric breaks and fusions play an important role in contributing to chromosomal losses and gains in these tumours [5]. These data suggest that consistent gains and losses, rather than band-specific breakpoints may be a crucial type of event leading to tumour development in OSCC. In addition, unidentifiable marker chromosomes are commonly found in OSCC. It is therefore difficult to obtain an accurate estimate of the genetic changes using only cytogenetic techniques.

Recently a new technique has been developed, comparative genomic hybridisation (CGH) [11, 12], with which chromosomal gains and losses can be more rapidly investigated

than with classical karyotyping. In short, CGH uses tumour DNA and normal reference DNA which are differentially labelled, mixed and competitively hybridised to normal chromosome metaphases. Copy number changes in the tumour DNA are reflected in the ratio of the two hybridised DNA sequences along the normal metaphase chromosomes and are calculated and visualised by digital image processing. A genetic analysis of the tumour is then obtained without the need of the limiting variables of the presence of metaphases and the use of culture techniques. Using CGH, we measured six OSCC cultures and found a number of chromosomal loci that recurrently appeared to be over- or under-represented. In addition, we investigated how CGH compared to classical cytogenetic analysis for the detection of chromosomal gains and losses.

MATERIALS AND METHODS

Tumour samples were obtained from the Department of Oral Pathology within 1 h of resection. A slice of approximately $0.5 \times 2 \times 2$ cm of non-necrotic tumour tissue was carefully selected by the pathologist in order to exclude as much as possible from the surrounding normal tissue. OSCC cell cultures were established and karyotyped as described by Hermsen et al. [5], according to the ISCN guidelines [13].

Tumour DNA was prepared from the cell cultures at passages 7–15 using the QiAmp isolation kit (Qiagen GmbH, Hilden, Germany). Normal reference DNA was obtained by the same method from blood lymphocytes of a healthy individual. Tumour and reference DNA were labelled by nick translation with biotin-16-dUTP and digoxygenin-11-dUTP (Boehringer, Mannheim, Germany), respectively. Normal metaphase preparations were obtained from PHA-stimulated peripheral blood lymphocyte cultures from a healthy individual using standard procedures.

CGH was carried out essentially according to Kallioniemi et al. [14], following the modification described by De Meulemeester et al. [15]: The probes and the metaphase slides were denatured simultaneously for 22 s in a 130 kW Philips microwave oven at 60% power. Interactive karyotyping of the chromosomes and calculation of the green to red fluorescence ratio of each chromosome was performed with the Cytovision CGH software package (Applied Imaging). The averaged ratios of 10–20 chromosomes were plotted along ideograms of the corresponding chromosomes in a 'relative copy number karyotype', together with the 95% confidence intervals. Losses or gains were identified as those regions where the ratio profile and the complete 95% confidence interval were smaller or larger than 1.0.

In situ hybridisation and fluorescence detection on tumour metaphase preparations was carried out as described by Hoovers et al. [16] using biotinylated probes paint X and paint 3 (Cambio, Cambridge, U.K.), with simultaneous denaturation of probes and metaphase slides as described for the CGH. For digital image microscopy the Cytovision Probe system (Applied Imaging) was used.

RESULTS

The combined CGH results of the six tumour cell cultures are depicted in Fig. 1a. The major recurring gains were detected at chromosome arms 8q and 9q (four tumours) and at 1q, 3q, 5p, 7p, and 9p (three tumours). Amplification of band 11q13 was found in only one case. Consistent losses were found at Xp and 3p (four tumours).

The cytogenetic characteristics of the cell cultures used in the present study have been previously described by Hermsen et al. [5]. All six tumours had complex karyotypes with several marker chromosomes. The centromeric regions of nearly all chromosomes were involved in unbalanced translocations and deletions, resulting in gains and losses of whole chromosome arms. The major recurring gains were detected at chromosome arm 5p (four tumours) and at 9p, 9q and 14q (three tumours). A homogeneously staining region at band 11q13 was found in three cases. Losses occurred for Xp, Xq and 3p (four tumours) and for 8p, 9p, 10p, 18q, 19p and 22q (three tumours) (Fig. 1b). In total, karyotyping revealed 55 losses and 32 gains, while CGH identified 26 losses and 37 gains.

(a)

(b)
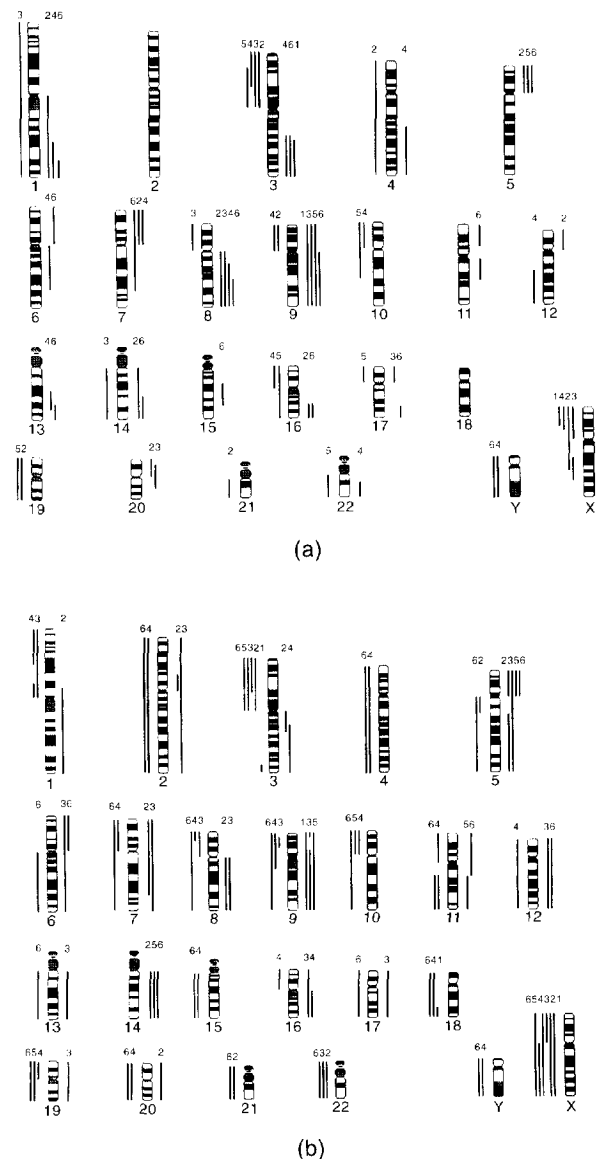
Fig. 1. Summary of all gains and losses detected by CGH analysis (a) and by cytogenetic analysis (b) in six OSCC cell cultures. The bars to the right of the pictograms represent gains and the bars to the left represent losses. The numbers at the top of the bars correspond to the following tumour cultures: 1 = 92VU040, 2 = 92VU059, 3 = 93VU094, 4 = 93VU96a, 5 = 93VU120, 6 = 93VU147. (For cytogenetic descriptions of these tumours see [5].)

FISH analysis with whole chromosome paint probes for chromosome X and 3 on tumour 1 (female) with the following karyotype: 46, X, −X, add(3)(q27), +9, add(12)(q24), add(18)(q22), showed that the addition on 3q contained chromosome 3 material and that the addition on 12q harboured chromosome X material (Fig. 2). These findings were in agreement with the CGH findings in this tumour.

DISCUSSION

CGH is a new and rapid technique which enables the identification of consistent chromosomal gains and losses throughout the whole tumour genome in a single experiment. CGH results can, in the case of consistent loss or gain of chromosomal material, point to the location of tumour suppressor genes or oncogenes, respectively, that are possibly involved in tumour development.

In this study we found the most frequent changes at chromosome 3 and 9, in all six tumours. The overall results were in agreement with previous CGH and cytogenetic studies on OSCC. Especially the loss of 3p and the gain of 3q material has been observed very frequently [4, 5, 17, 18]. Losses on 3p appeared most consistently at 3p14–22 and have also been

(a)

(b)   (c)
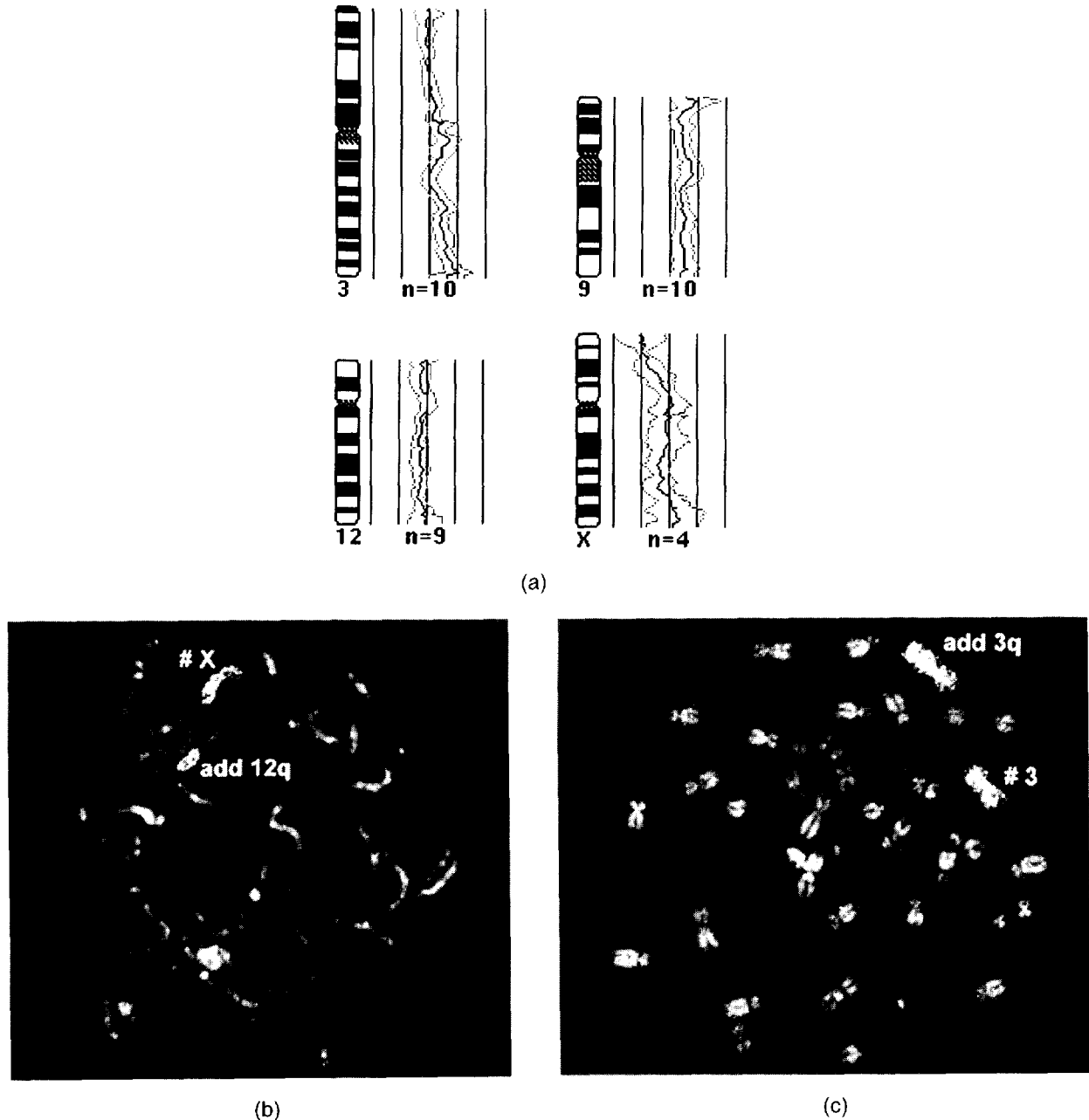
Fig. 2. (a) CGH profiles of chromosomes 3, 9, 12 and X of tumour 92VU040. The averaged profiles of *n* chromosomes are plotted on five straight vertical lines representing, from left to right, a green to red fluorescence ratio of 0.5, 0.75, 1.0, 1.25, and 1.5. The 95% confidence interval is depicted by the lines left and right of the average profile line. A gain or loss is significant when the average profile plus the confidence interval is larger or smaller than 1.0, in this case gain of 3qter and 9, and loss of Xpter. (b) and (c) FISH with whole chromosome paint probe for chromosome X and 3, respectively, showing a normal chromosome 3 and the add(3qter) completely painted by the chromosome 3 probe, and a normal X chromosome and the add(12qter) painted by the chromosome X probe.

detected by LOH study [19,20]. Virgilio *et al.* [21] claimed the *FHIT* gene (at chromosomal band 3p14.2) to be involved in oral cancer. This gene, earlier reported to be lost in primary tumours of the lung and the aerodigestive tract, was found to be abnormally transcribed in 15 out of 25 oral carcinoma cell lines. Up to now, its function is not known. Alterations at 3q26-qter have been found by both CGH and cytogenetic studies [5,17,18]. It may be that the zinc-finger encoding genes *BCL-6* or *LAZ3*, which have been suggested to be involved in translocations in lymphomas [22,23], are also involved in the development of OSCC. Also, the gains at 5p and 8q were in accordance with several other studies, indicating a possible role for these loci in OSCC. Homogeneously staining regions at the 11q13 region, found in three cases with cytogenetical analysis, was reflected only once as a gain of that region by CGH. In the other two cases, the homogeneously staining region was apparently not due to amplification of 11q13 material. The losses of Xp and gains of 9q, seen in four out of six cases in this study, were not observed in other studies.

In our cases, and in those by Brzoska *et al.* [17] and Speicher *et al.* [18] no recurrent losses at chromosome arms 8p, 9p, 10p and 18q were found, whereas these regions have been frequently reported to be lost in a number of cytogenetical studies [1,3–5]. It could be argued that CGH is not sensitive enough, but this is unlikely as losses of whole chromosome arms are large enough for CGH to detect copy number differences in. Alternatively, it cannot be ruled out that karyotyping produces false positive losses. To address this problem, we compared our CGH findings to the results obtained by cytogenetic analysis in the same six tumours. We noticed an overall agreement (Fig. 1), but there were some significant differences. In some cases, karyotyping found a loss where CGH detected a normal content or even gain of material (e.g. compare chromosome 18, or chromosome arm 7p for tumours 4 and 6). In total, karyotyping identified 55 losses and 32 gains, whereas CGH detected 26 losses and 37 gains. The differences in the number of losses were especially remarkable.

The discrepancy between karyotypic and CGH findings on these tumours can be due to the following. First, the cytogenetic findings of the six cell cultures in this study have been previously described by Hermsen *et al.* [5] using the system of *composite karyotypes* [13], meaning that all *clonal* abnormalities are taken into account, relative to the ploidy level, even though they may not be present in *every* tumour metaphase [5]. The assessment of chromosomal gains and losses found by karyotyping is based on these data. The second reason for discrepancies is the hampered identification of losses by the presence of unrecognisable marker chromosomes possibly containing material that is scored as lost or as being present in a normal amount [4]. In this situation, it is to be expected that karyotyping, in contrast to CGH which takes all genetic material into consideration, tends to overestimate losses and to underestimate the gains. This is what the numbers of gains and losses in the six tumours by the two techniques show.

We performed FISH on metaphases of tumour 1 in order to produce experimental evidence for the latter explanation. From the cytogenetic data, CGH would be expected to show a gain of chromosome 9, loss of chromosome X and losses of the distal regions of 3q, 12q and 18q (Fig. 1b). However, CGH analysis unexpectedly revealed a gain at 3qter and chromosome 9, a loss of Xp, and a normal diploid content of chromosome 12 (Fig. 1a). The FISH experiments showed that the additions at chromosomes 3 and 12 harbour chromosome 3 and chromosome X material, respectively (Fig. 2b,c). This confirmed that cytogenetically unrecognisable chromosomes or chromosome parts diminish the reliability of gains and losses evaluated by karyotyping while CGH is not hampered at all.

We conclude that in general CGH is faster and more informative than cytogenetic analysis, especially with the identification of chromosomal losses, as all genetic variation is taken into account. Therefore, CGH will be an important new tool in the recognition of which genetic regions are important in the development of OSCC.

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