

Genetic classification of oral and oropharyngeal carcinomas identifies subgroups with a different prognosis

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Abstract. The common risk factors for oral and oropharyngeal cancer are tobacco smoking and alcohol consumption, and recently the human papillomavirus (HPV) was shown to be involved in a subgroup. HPV-positive and -negative carcinomas can be distinguished on basis of their genetic profiles. Aim of this study was to investigate patterns of chromosomal aberrations of HPV-negative oral and oropharyngeal squamous cell carcinomas (OOSCC) in order to improve stratification of patients regarding outcome. Thirty-nine OOSCCs were classified on basis of their genetic pattern determined by array comparative genomic hybridization (aCGH). Resulting groups were related to patient and tumor characteristics using the Fisher's exact test and in addition to survival with the Kaplan–Meier and log rank tests. Classification distinguished three groups, one characterized by hardly any chromosomal aberration ($N = 8$) and another by a relatively high level ($N = 26$), and one with a very high level ($N = 5$) of chromosomal aberrations. This classification was significantly ($p = 0.003$) associated with survival, with the best survival in the genetically 'silent' group and the worst survival in the most aberrant group. The silent profile was significantly ($p < 0.05$) associated with wild-type *TP53*, an absence of alcohol consumption and a female gender. These carcinomas were negative for microsatellite instability. This classification of OOSCC was confirmed in an independent set of 89 oral carcinomas. In conclusion, the discovery of these new classes of oral and oropharyngeal cancer with unique genetic and clinical characteristics has important consequences for future basic and clinical studies.

Keywords: Array CGH, head and neck cancer, HPV, oral cancer, prognosis, *TP53*

Abbreviations

- aCGH: Array comparative genomic hybridization,
BAC: Bacterial artificial chromosome,
OOSCC: Oral and oropharyngeal squamous cell carcinoma,
HPV: Human papillomavirus.

1. Introduction

Oral and oropharyngeal squamous cell carcinomas (OOSCC) develop in the mucosal linings of the oral

cavity and oropharynx and constitute with hypopharyngeal and laryngeal carcinomas the group of head and neck squamous cell carcinomas. Despite significant advances in loco-regional control, long-term survival of OOSCC patients has only moderately improved during the last 20 years. The identification of biological markers will be essential to make headway in detecting this malignancy at an early stage and developing novel therapies.

The major and classical risk factors for OOSCC are exposure to tobacco and alcohol, but it has recently become clear that also an infection with human papillomavirus 16 (HPV16) plays an important role in a subgroup of these tumors. There is convincing evidence that HPV-infected tumors form a completely distinct group. These tumors differ from non-infected tumors with respect to risk factors [13], absence of a *TP53* mutations [38,42], a low level of allelic losses [5] and

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mRNA expression profiles [30]. This distinct pathological entity may be the reason for a better response rate and survival [11,43].

Genetic analysis of cancers with array comparative genomic hybridization (aCGH) makes it nowadays possible to analyze DNA copy number variations with high resolution [12]. With aCGH it could be shown that HPV-infected tumors are also distinct from non-infected OOSCC regarding DNA copy number changes [31].

We hypothesized that other subgroups of non-HPV-involved carcinomas exist that differ regarding their pattern of genetic alterations and that this might have consequences for prognosis. Previously, it was shown that breast cancers and leukemia could be divided into prognostic relevant subgroups using expression array analysis [18,40]. These classifications are based on algorithms using the *T*-statistics as expression data are normally distributed. Only recently, cluster algorithms like WECCA, tailor-made for ordered aCGH data sets, became available [7,41]. The present study aims to identify distinct groups of non-HPV OOSCC by means of aCGH analysis and unsupervised cluster analysis. Different genetic groups were identified and could be linked to clinical parameters.

2. Material and methods

2.1. Patients and tumor specimens

We obtained tumor specimens from 39 patients who underwent surgical treatment for a carcinoma in the oral cavity or oropharynx at the VU University Medical Center. This group was randomly selected from our tissue collection that contains snap-frozen carcinomas, gathered during the period from 1997 to 2001. To exclude the presence of HPV, detection of viral DNA and expression analysis of the oncogenes E6 and E7 was performed as previously described [31,38]. The mutational status of *TP53* of these carcinomas was determined in the evolutionarily conserved regions, exons 5 to 9 [39]. When no mutations were found in these exons, the remaining coding exons 2, 3, 4, 10 and 11 were sequenced in addition. Mutations were also classified in disruptive or non-disruptive mutations, according to the criteria used by Poeta et al. [27]. In brief, these criteria are based on the location of the mutation and the type of predicted amino acid alteration. For all studies microdissection was performed to enrich for carcinoma tissue. The study had been approved

by the Institutional Review Board of the VU University Medical Center, and written informed consent was obtained from all patients. Staging was performed according to the classification of the International Union Against Cancer (UICC) [33] and information on patient tobacco and alcohol use was obtained from the medical files. Patients were classified as never, current or former tobacco smokers. Pack-years were taken as a measure of cumulative tobacco consumption. It is calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked. Patients were classified as never, current or former alcohol drinkers. Unit-years were taken as a measure of cumulative alcohol consumption and were calculated as the number of years drinking multiplied by the number of units per day. A unit is defined as one alcoholic beverage (equivalent to approximately 15 ml of alcohol).

2.2. Array comparative genomic hybridization

Test DNA (300 ng), extracted as described previously [39] and reference genomic DNA (300 ng) isolated from a random panel of blood donors were labeled with a random primer elongation kit (Invitrogen, Breda, The Netherlands). Arrays of Bacterial Artificial Chromosomes (BACs) were prepared and hybridized as described previously [31].

2.3. Image acquisition and data analysis

Image analysis, the exclusion of bad spots and the calculation of the mean log₂ ratios of triplicate Cy3 and Cy5 signals were performed as previously described [31]. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO), and are accessible through GEO series accession number GSE11929.

Chromosome X-clones were discarded from further analysis, since all tumor samples were hybridized to reference DNA of the opposite gender. Data of a BAC was only included in the analysis when at least 80% of the tumors showed a value for that particular BAC. A total number of 4062 clones could be analyzed for all samples. The log₂ ratios are segmented by means of 'DNACopy' [25], and called by means of 'CGHcall' [36]. The calling results in the so-called call probabilities, a probability vector for each feature on the array: a probability of a loss, a probability of 'no-aberration', and a probability of a gain. The maximum probability indicates the most likely aberration. The use of call

probabilities in down-stream analyses prevents the loss of information associated with the use of calls. Finally, genomically adjacent features not separated by a breakpoint have the same call probability signature over the samples. Therefore, the data are reduced to the unique, non-spurious call probability signatures by means of 'CGHregions' [37].

The samples are clustered by means of a modified version of WECCA [41]. WECCA is a hierarchical clustering method tailor-made for called aCGH data. The modified version accommodates the use of call probabilities instead of calls. The use of the call probabilities in the unsupervised analysis will give a more subtle picture of the similarities and differences between the samples. The modified version of WECCA defines the distance between two features as the absolute difference between the cumulative call probability distributions. The distance between the call probability profiles of two samples is then defined as the average of these differences over all features. In the construction of the dendrogram we used Ward's linkage as it yields compact and well-separated clusters. Chromosomal breakpoints were calculated from the segmented data.

Differences between groups as for frequencies of patient and tumor characteristics were performed with the 2×2 and 2×3 Fisher's exact test. Continuous variables were compared with the Mann-Whitney (two groups) or the Kruskal-Wallis (three groups) test. Survival characteristics were determined according to the Kaplan-Meier method and survival curves were compared with the log-rank test. All tests were two-sided and differences were considered to be significant if the p -value was below 0.05.

2.4. Micro Satellite Instability (MSI)

MSI was detected using a MSI analysis system (Promega corporation, Madison, USA), according to instructions of the manufacturer. In short, this system uses fluorescently labeled primers (marker panel) for co-amplification of seven markers for analysis of the MSI-high (MSI-H) phenotype, including five nearly monomorphic mononucleotide repeat markers (BAT-25, BAT-26, MON0-27, NR-21 and NR-24) and two highly polymorphic pentanucleotide repeat markers (Penta C and Penta D). These markers are recommended by the National Cancer Institute (NCI) workshop for identifying MSI-type colorectal cancer [35]. Amplified fragments were detected by a sequence analyzer (model 3100 from Applied Biosystems BV, Nieuwerkerk a/d IJssel, The Netherlands).

3. Results

To investigate whether classification on the basis of genetic aberrations is possible, we performed aCGH on 39 OOSCC and analyzed the data. Unsupervised clustering enabled the discovery of two distinct groups at the top of the tree (Fig. 1). Group 1 consisted of 8 tumors that showed a significantly lower level of chromosomal aberrations, when compared to the other group, labeled 2 (average number of chromosomal breakpoints 2.8 and 33.7, respectively; Table 1). The few changes that were observed in group 1 were never shared by two or more tumors of that group. Group 2 was characterized by a higher level of chromosomal aberrations. Aberrations that were present in this group in more than 50% of the tumors, were gains at chromosomes 3q, 7q, 8q and 11q13 and losses at chromosomes 3p, 5q, 11q23 and 18q (Fig. 1). Regions that showed numerical changes in even more than 80% of carcinomas; loss at 3p (total p-arm), gain at 3q26.2-qtter (29 Mb) and gain at 8q24.13-q24.21 (3 MB). Typical examples of a CGH profile of tumors of groups 1 and 2 are shown in Fig. 2.

Looking further down the tree it appeared that group 2 consists of two further subgroups; one (2a) of 26 tumors with a relatively high level of aberrations (average number breakpoints of 30.0) and another (2b) of five tumors with an even higher level of aberrations (average number of breakpoints 52.8).

Comparison of the clinical parameters between the identified groups revealed that group 1 (with infrequent aberrations), significantly differed from groups 2a and 2b, as it was characterized by the absence of alcohol consumption, female gender and wild type *TP53* in the carcinoma (Table 1). Regarding the mutational status in *TP53*, the differences between group 1 and 2 were more pronounced when the mutations were classified as disruptive vs. non-disruptive and wild-type (Table 1). A remarkable feature of group 2b was the gender dysbalance, as only males were observed in this group.

Analysis of overall survival between the groups revealed a significant difference between the groups (log rank test with a p -value of 0.003), with the best survival in group 1 and the worst survival in group 2b (Fig. 3). We next investigated classification after inclusion of the dataset of 12 HPV-positive OOSCC from a previous study [31]. Seven out of twelve HPV-positive OOSCC appeared to cluster as a separate group within group 2a. Five HPV-positive tumors belonged to group 1 in

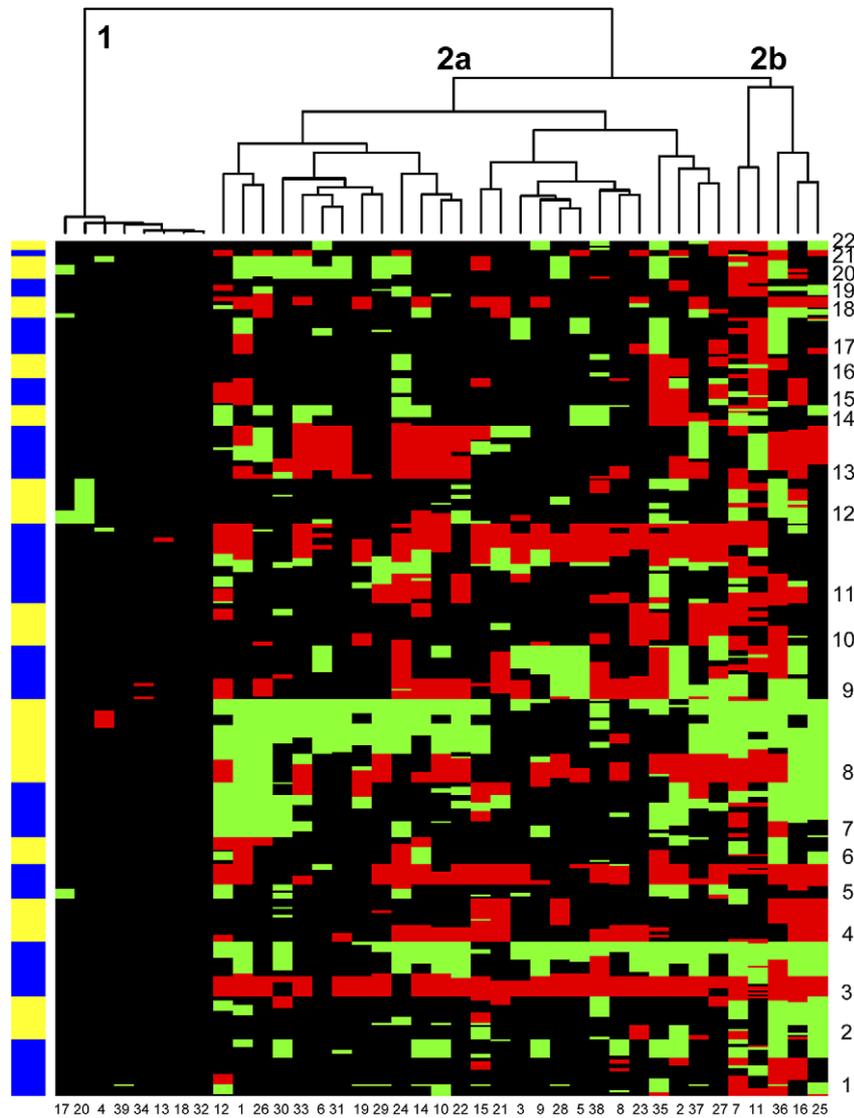


Fig. 1. WECCA heatmap of 39 OOSCCs without HPV involvement. At the top of the tree two tumor groups can be distinguished: a group consisting of 8 OOSCC with in general genetically silent profiles and another group of 31 tumor with many genetic aberrations. In group 2 downwards, two subgroups (a and b) can be distinguished with 2b as the group with the most genetic aberrations. The x -axis represents tumor numbers and the y -axis chromosome numbers.

which hardly any numerical change was observed (see Suppl. Fig. 1: <http://www.qub.ac.uk/isco/JCO>).

It was tried to confirm the findings by analysis of an independent and external aCGH dataset of 89 oral squamous cell carcinomas described by Snijders et al. [32]. Also in this material two main groups were identified (Suppl. Fig. 2: <http://www.qub.ac.uk/isco/JCO>). One group of oral carcinomas ($N = 33$) was characterized by a low level of chromosomal aberrations (average breakpoints: 11.5 ranging from 0 to 27) and a group of oral carcinomas ($N = 56$) with a high level

of chromosomal aberrations (average breakpoints 28.1 ranging from 7 to 53). Similar to our dataset also in this external set a subgroup of tumors ($N = 18$) with a much higher level of chromosomal aberrations (average breakpoints 34.7 ranging from 18 to 53) could be recognized. Unfortunately, no clinical parameters were available of these patients. The *TP53* mutation status of most of the tumors was described by Snijders et al. [32]. Similar to our data we found a significant correlation between mutation status and classification as 2 out of 25 tumors in group 1, and 15 out of 34 tu-

Table 1
Patient and tumor characteristics in relation to genetic classification

Characteristic	Group			Difference (<i>p</i> -values)	
	1	2a	2b	1 vs. 2	1 vs. 2a vs. 2b
Number	8	26	5		
Age (in years)				0.772	0.930
Average	58.6	59.0	61.4		
Range	43–81	38–76	42–79		
Gender				0.235	0.021
Female	6	14	0		
Male	2	12	5		
Tumor site				0.652	0.554
Oropharynx	1	7	2		
Oral cavity	7	19	3		
Pathological tumor stage					
I + II	6	13	3	0.426	0.567
III + IV	2	12	2		
Unknown	0	1	0		
Pathological nodal stage					
N–	2	10	2	0.683	0.781
N+	6	15	3		
Smoking [§]					
Current + former	5	22	5	0.302	0.524
Never	2	4	0		
Unknown	1	0	0		
Pack-years				0.644	0.680
Average	37	31	39		
Range	0–84	0–65	23–61		
Alcohol [‡]					
Current + former	2	20	5	0.014	0.014
Never	5	6	0		
Unknown	1	0	0		
Unit-years				0.199	0.230
Average	103.57	136.0	138.0		
Range	0–405	0–450	120–244		
<i>TP53</i>					
Mutated	1	15	2	0.049	0.076
Wild-type	7	11	3		
Disruptive mutation	0	11	2	0.035	0.061
Non-disruptive/Wild-type	8	15	3		
Average number of breakpoints				<0.001	<0.001
Average	2.8	30.0	52.8		
Range	0–6	12–49	31–80		

Notes: Characteristics of the three groups as classified with WECCA (Fig. 1) are listed. Group 1 tumors show the lowest and group 2b the highest level of aberrations. *p* Values are two-sided and for the 2 × 2 and 2 × 3 frequency comparisons the Fisher's exact test was used and for the mean comparisons, the Mann–Whitney or the Kruskal–Wallis test. Significant differences (*p* < 0.05) are shown in bold.

[§]Patients were classified as never, current or former tobacco smokers. Pack-years were taken as a measure of cumulative tobacco consumption. It is calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked.

[‡]Patients were classified as never, current or former alcohol drinkers. Unit-years were taken as a measure of cumulative alcohol consumption and were calculated as the number of years drinking multiplied by the number of units per day. A unit is defined as one alcoholic beverage (equivalent to approximately 15 ml of alcohol).

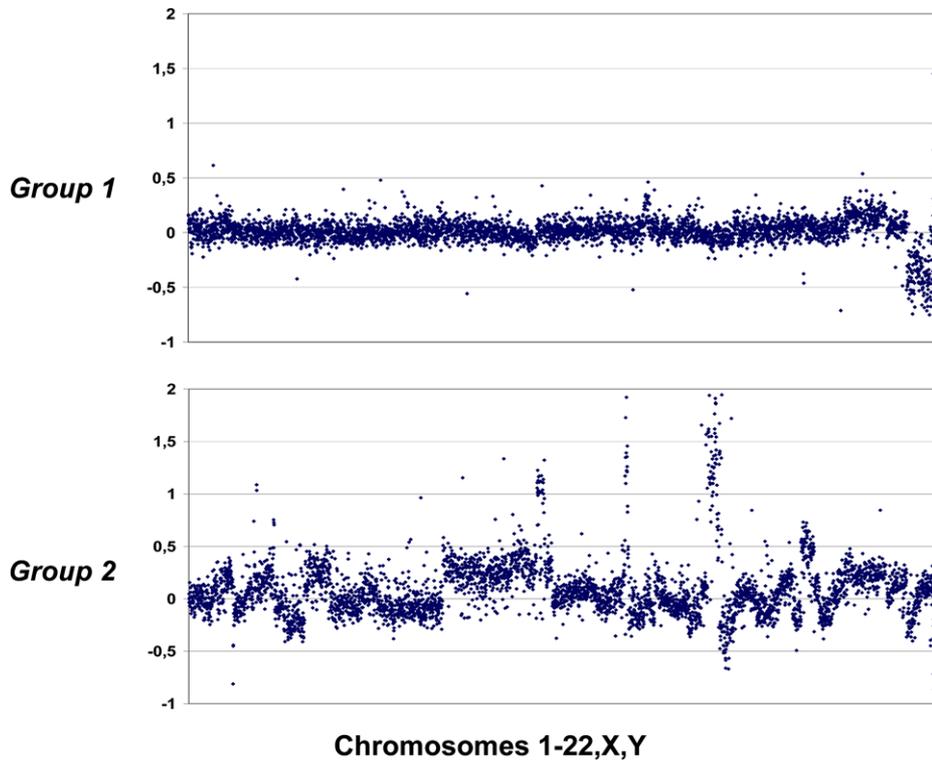


Fig. 2. Typical examples of an aCGH profile of each group. Upper panel represents a tumor with a silent profile and wild-type *TP53*, showing just a few aberrations. Lower panel shows a typical profile of a tumor of group 2 showing many aberrations, e.g. with gains at 3q, chromosome 8, 11q13, 17q, chromosome 20, and losses at 3p, 11q23 and 13q.

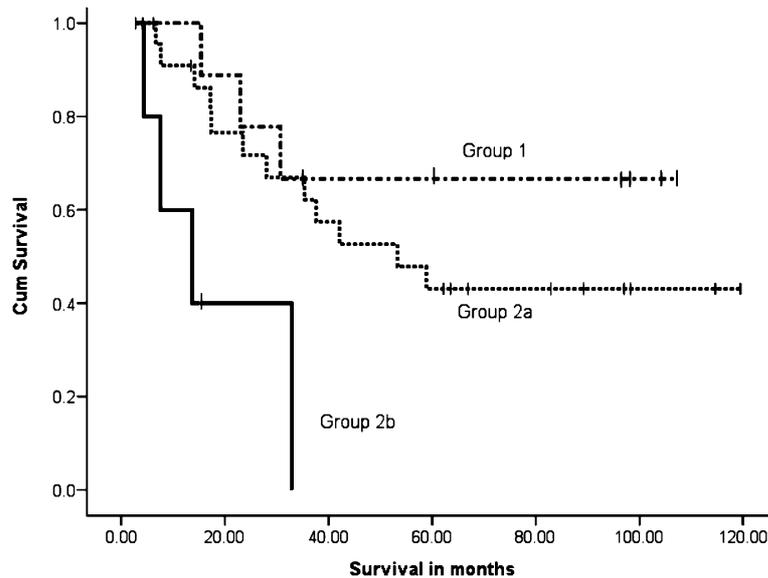


Fig. 3. Kaplan-Meier curves of clusters 1, 2a and 2b. Cumulative survival is shown in relation to time in months. Group 1 ($N = 8$) are OOSCC with hardly any chromosomal aberrations; group 2 contains the OOSCC with a relatively high level of chromosomal aberrations, divided in b ($N = 5$) with the highest and a ($N = 26$) with a lower level of aberrations. Log-rank analysis yielded a p -value of 0.004.

mors of group 2 contained mutated *TP53* ($p = 0.02$). It was noteworthy that all aberrations in our dataset were present in the dataset of Snijders et al. at high frequency as well.

Tumors with hardly any chromosomal aberrations are also known in colorectal cancer. In these tumors microsatellite instability (MSI) is mostly the driving force for progression [10]. We evaluated this possibility on this subgroup and found no indications for MSI in all evaluated markers (data not shown).

4. Discussion

The present analysis of a large number of HPV-negative OOSCC by high resolution CGH revealed a large number of DNA copy number changes. This complex genetic pattern is a well-known characteristic of OOSCC and confirms other CGH-studies [2,3,12,16,24,26,32,34,45]. Copy number changes were relatively frequent at chromosomes 3, 5p, 8 and 11q, involving over 50% of the tumors in group 2. Aberrations at these chromosomal locations have also been described in other studies, but at lower frequencies [2,3,12,16,24,26,32,34,45]. Three chromosomal regions were more pronounced and found to be altered in more than 80% of these tumors; loss at 3p, gain at 3q26 and gain at 8q24. The gain of 8q24.21 comprising 3 MB was identified by defining the smallest region of overlap. Gain at 8q, in particular 8q24 has also been reported by others in an unselected group OOSCC [3,44]. In this region *c-Myc* is located, a forceful oncogene that is commonly found to be overexpressed by genomic amplification in OOSCC [19,14,23] and that has the capability to transform primary oral epithelial cells to cancer cells *in vitro* [15].

By virtue of WECCA, a new classification tool for aCGH data, we were able to identify separate tumor groups. A group with a remarkably low and a group with a relatively high level of chromosomal aberrations could be discriminated; a subgroup within that latter group showed a very high level of chromosomal alterations. To find confirmation of the present findings, the data from a previously published study [32] was investigated in more detail with the WECCA cluster algorithm. Analysis of the data revealed again the presence of two major tumor groups divided on the basis of the number of aberrations. The average number of aberrations in the genetically silent group was somewhat higher than in our tumor panel and this group was somewhat larger (37.1% vs. 20.5% in our mater-

ial). Nevertheless the high similarity between the two datasets, including the identification of the subgroup with the rather high number of aberrations, is noteworthy. Unfortunately the survival data were not available from that group. It has to be realized that there are geographical difference regarding the patient populations that were compared. In addition, the fact that only oral carcinomas had been included and that some cases could be HPV-positive [32], may explain some of the differences.

OOSCC harboring a genome with few chromosomal aberrations were earlier recognized in cytogenetic studies as tumors with simple karyotypic changes [20]. In fact, our sensitive aCGH method confirms in a way and extends in much greater detail of what was found with this 'older' crude method. The tumors with such a simple karyotype comprised approximately 36% of the study population, but were never discussed as a separate tumor group and this characteristic was not correlated with clinical parameters like prognosis.

When comparing the presently classified tumor groups regarding clinical and biological parameters it was found that survival, alcohol drinking history, gender and *TP53* mutation status were significantly different, which can be interpreted as independent evidence to support the relevance of the findings. It can be appreciated that the tumors showing a silent genetic profile were all *TP53* wild type. This notion was also supported by the results of the external aCGH dataset of 89 OOSCC [32]. The question remains whether this is a non-random association or that the *TP53* status plays a causative role on the genetic profile. An argument against the latter is that some tumors of group 2 with a high level of chromosomal changes also showed a wild type *TP53* gene while the gene was sequenced from exons 2–11. Notwithstanding, exon deletions could have been missed and we cannot exclude that the p53 pathway is impaired in an alternative way. Genes modifying p53 function might be mutated, abrogating p53 function indirectly [4,17].

Six of the eight patients with a silent tumor profile, never had consumed alcohol. In addition, an excessive alcohol consumption appeared to be correlated with the presence of a *TP53* mutation and thereby an increased genomic instability. Although, a causal relation between both tobacco and alcohol abuse and OOSCC is well established [9,22], there is controversy about the relationship between levels of tobacco and alcohol exposure vs. the number and type of genetic aberrations in the tumor. Koch et al. [21] found a distinct clinical and molecular entity of head and neck

squamous cell carcinoma in non-smokers, while Singh et al. [29] did not. In addition, Brennan et al. found an association between excessive alcohol/tobacco consumption and the presence of mutated *TP53* [6]. It has recently become clear that alcohol can be a carcinogen on its own [28], but its role in generating a mutation in *TP53* needs further attention.

We found evidence that the level of DNA copy number changes is associated with patient outcome. The lowest level of aberrations was associated with the best and the highest level with the worst survival. This finding is in line with what could be expected: patients with carcinomas without a *TP53* mutation have a better survival [13] and a relatively low level of genetic instability has been reported to be related to a better outcome, for head and neck [1] and breast cancer [8]. Although the difference in survival between the groups was significant, it has to be added that the numbers were small. A larger study on OOSCC is needed to definitively prove the clinical value of this classification.

The genetically silent group (number 1) is for a large part characterized by tumors that are *TP53* wild type and contain hardly any chromosomal aberrations. It is tempting to speculate on the molecular mechanism that might drive carcinogenesis in these tumors. This subgroup may be the result of a defect known as microsatellite instability (MSI), related to a deficiency in the mismatch repair machinery. A proportion of colorectal carcinomas show this phenomenon as well that is reflected in relatively few chromosomal aberrations [10]. We evaluated this possibility on this subgroup, but found that all were MSI negative. It has to be added that there is a possibility that MSI was missed; a set of markers was tested that may not be ideal for OOSCC, as it is normally used for colon carcinoma. Second, other forms of DNA alterations, in particular loss of heterozygosity (LOH) without numerical changes and epigenetic alterations such as promoter hypermethylation, might play a role in these tumors and might even abrogate the p53 pathway. Finally, tumor heterogeneity at the cellular level may explain the absence of specific aberrations in these OOSCC. Gains in some cells could be counterbalanced by losses in other. However, it seems unlikely that this results in normal genetic profiles, but without more detailed investigations this remains unclear.

Taken together, our main finding is the identification by array CGH of a previously unrecognized group of OOSCC with a remarkably almost normal genome, regarding DNA copy numbers. Data of the current study indicate that this genomic subgroup of OOSCC shows

a different outcome and patient profile; these findings and the unraveling of the possible mechanisms driving its carcinogenesis warrant additional studies in a larger OOSCC cohort. At this moment, at least three different OOSCC groups can be discriminated: (1) an HPV-positive group that was discussed in a previous study [31], (2) a 'normal DNA' group without *TP53* mutation that we now reported on, and (3) a group with a high to very high level of chromosomal aberrations, characterized by loss of 3p, 5p, 11q23 and gains at 3q, chromosomes 8 and 11q13. These findings underline the clinical importance of biological classification of OOSCC. Stratification in groups is critical when analyzing the role of potential cancer genes, early detection of tumor markers, and selection of treatments.

Acknowledgement

This work was supported by the EU-sixth framework project: DISMAL, Contract No. LSHC-CT-2005-018911.

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