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Chromosomal changes in relation to clinical outcome in larynx and pharynx squamous cell carcinoma

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Abstract. Invasive head and neck squamous carcinomas are among the cytogenetically most complex tumors. Perhaps for this reason, there is little consensus on the prognostic value of specific chromosomal aberrations. Here we present results of CGH analysis of 56 clinically well-characterized set of head and neck cancers, consisting of larynx and pharynx only. The aim was to find possible associations with clinical outcome. The major chromosome arms showing gains were (in decreasing order): 3q, 7q, 8q, 5p, 11q13, 17q and 18p, and losses occurred at 3p, 11qter, 4p, 18q, and 5q. The segments most frequently amplified were 3q26-qter, 11q13, 11q22, 3q12–13, 18p11.3, 18q11.2 and 8q24.3. Tumors with stages III and IV, and lymph node positive tumors had a worse clinical outcome. Surprisingly, no specific chromosomal abnormality correlated with disease-free survival. The only aberration that correlated to one of the clinico-pathological parameters was amplification 11q13, that occurred solely in lymph node positive, stage IV tumors. However 11q13 amplification did not correlate with disease-free survival. These results seem to indicate that genetic alterations at the level of chromosomes have limited prognostic value in patients with invasive larynx and pharynx squamous cell carcinomas.

1. Introduction

Squamous cell carcinoma (SCC) of the head and neck is the sixth most common cancer in the Western world. In Spain the incidence is 10–18 and in the region of Asturias 24 per 100,000 inhabitants [7,22]. In the last decades, this incidence has increased due to a high exposition to risk factors, such as tobacco and alcohol, and to increased life expectancy [4,29]. Long-term exhibition of the epithelium to factors such as tobacco and alcohol induces biologic and morphologic changes, ranging from pre-neoplastic hyperplasia and dysplasia to carcinoma *in situ* and invasive carcinoma. This spectrum of changes is accompanied by accumulating chromosomal aberrations.

Several genetic studies on head and neck SCC using cytogenetic analysis, FISH and comparative genomic hybridization (CGH), have shown a consistent set of chromosome regions to be frequently altered: gains on 3q, 5p, 8q and 11q13 and losses on 3p and 9p [3,5,9–13,27,28,33]. A model of tumorigenesis has been proposed in which deletion of 9p21, the locus of the cell cycle inhibitor p16, would be the primary event, followed by deletion of 17p13 (the p53 locus) and 3p21, and subsequent deletion of 11q13, 13q21, and 14q32 in an undetermined order [6,8].

Despite this knowledge, there is little consensus on the prognostic value of specific chromosomal aberrations. Here we present a study on chromosomal aberrations in a clinically well-characterized set of head and neck cancers, consisting of larynx and pharynx only. The aim was to find possible associations between recurrent chromosomal changes and clinical outcome.

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2. Materials and methods

All samples analyzed in the present study were primary tumors (34 larynx and 22 pharynx squamous cell carcinomas) from male patients, that were seen in the Otolaringology Department of the Valle del Nalón Hospital in Asturias, a coal mining region in Asturias, Spain. Twenty-nine of the 56 patients were or had been coal miners or metal and chemistry industry workers. All patients regularly used tobacco and alcohol. The mean age was 62 years (range 44–81). All patients underwent radical surgery (i.e. laryngectomy or pharyngectomy) and in all cases, resection margins were free of tumor. Snap frozen samples of the primary tumor were obtained from surgical resection specimens of non-necrotic tumor areas and stored in liquid nitrogen. The median disease-free survival was 49 months (range 0–78). Twenty-two patients received radiotherapy after surgery. Clinical characteristics are listed in Table 1. Forty-two tumors of this series have been studied previously, aiming to find new chromosomal regions with amplification and this work has been published [10].

DNA ploidy was measured by flow cytometry as described before in detail [24]. In short, fresh/paraffin embedded tissue was disaggregated mechanically, suspended in citrate-phosphate-buffered solution, and stained with propidium iodide [30]. Specimens were measured with the Cytoron flow cytometer (Ortho Diagnostic Systems), and results analysed according to the guidelines for implementation of clinical DNA cytometry [25].

For CGH, tumor DNA was extracted using phenol/chloroform extraction following standard DNA isolation protocols, and labeled by standard nick translation reaction with biotin-16-dUTP (Roche Diagnostics, Almere, The Netherlands). Normal DNA was extracted from normal tissue of a healthy female donor using the QiAmp Tissue Kit (Qiagen GmbH, Hilden, Germany) and labeled with digoxigenin-11-dUTP (Roche Diagnostics, Almere, The Netherlands). CGH was performed as described by Weiss et al. [32]. The green to red fluorescence ratio along the chromosomal axes was calculated by dedicated image analysis software (Cytovision 3.5, Applied Imaging, Newcastle Upon Tyne, United Kingdom). Chromosomal gain or loss was interpreted when the average ratio was significantly higher or lower than 1.0, as evaluated by the 95% confidence interval. High level amplifications were interpreted as discrete chromosomal segments showing a ratio significantly higher than 1.5. Chromosome regions 1pter, 16p and 19 were excluded from interpretation for reasons of unreliability due to a high proportion of repetitive sequences.

The statistical analyses was carried out using Kaplan–Meier statistics, Student's t-test and the Chi-square test. P-values < 0.05 were considered significant. Both the DNA ploidy and CGH results, i.e. total number of chromosomal aberrations and individual chromosomal changes, were tested for correlations with the following histopathological and clinical characteristics: tumor location, patient age, clinical stage and histological differentiation, lymph node status and disease-free survival.

3. Results

Eighteen tumors were DNA diploid, 30 were DNA aneuploid and 8 cases could not be analysed. The mean DNA index of the 30 aneuploid cases was 1.7, ranging from 1.4 to 3.0. CGH revealed chromosomal changes in all cases. DNA diploid and DNA aneuploid cases showed a comparable high number of chromosomal alterations: 19.5 on average, ranging from 1 to 34 alterations. Figures 1a and 1b show representative CGH results of a diploid and an aneuploid tumor. Gains occurred more frequently than losses, with an average of 11.1 and 8.4, respectively. The most recurrent gains and losses are presented in Table 2. Thirty-five tumors (DNA diploid as well as aneuploid) contained high level amplifications, those occurring in two or more cases are given in Table 2. An overview of all CGH results is presented in Fig. 2. Table 1 lists the results of all cases, together with their clinico-pathological and follow up data. Gains and losses rarely concerned whole chromosomes, but whole chromosome arm imbalances were frequently found, up to 36% of all events (Fig. 2). Moreover, in a substantial number of cases simultaneous gain of the one and loss of the other chromosome arm, indicative of isochromosome formation, was observed: 3p loss/3q gain (24 cases), 5p gain/5q loss (10 cases), 8p loss/8q gain (13 cases), 9p loss/9q gain (4 cases), 17p loss/17q gain (6 cases), 18p gain/18q loss (7 cases).

Larynx and pharynx tumors of comparable clinical stage were found to have similar characteristics with regard to lymph node status, DNA ploidy and pattern of chromosomal abnormalities. Therefore, all cases were grouped together in the statistical analyses. As expected, tumor stage and lymph node status correlated with disease-free survival (Table 3). No correla-

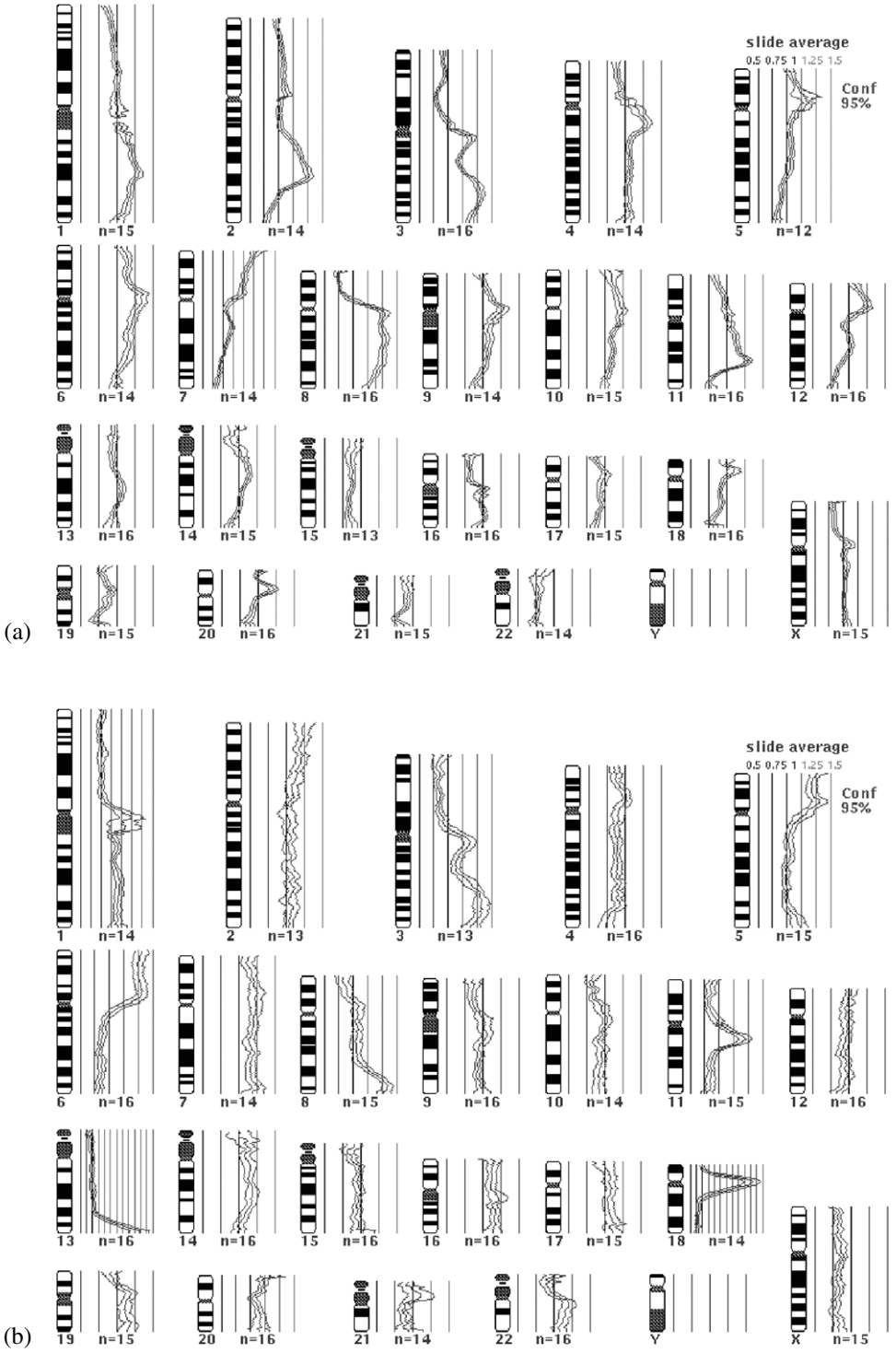

Fig. 1. (a) Example of a CGH result of a laryngeal squamous cell carcinoma with a diploid DNA histogram. Gains were found at 1q, 2p, 2q, 3q, 4q, 5p, 6p&q, 7p&q, 8q, 10p&q, 11q, 12p, 14q and 20p, and losses at 1p, 2q, 3p, 4p, 5q, 7q, 8p, 11p, 11q, 12q, 15q, 16p, 17p, 17q, 18q, 19p&q, 20q, 21q, 22q and Xp. (b) Example of a CGH result of a laryngeal squamous cell carcinoma with an aneuploid DNA histogram (DI = 1.55). Gains were found at 1q, 2p, 3q, 5p, 5q, 6p, 7p&q, 8q, 11q, 13q, 14q, 16p&q, 16q, 18q, 19q, 20p&q, and 22q, and losses at 3p, 4p, 4q, 5q, 6q, 8p, 9p, 10p, 11p, 11q, 18q, 21q and X.

Table 1
Clinicopathological features, clinical outcome and CGH results of all 56 patients

Patient data			Tumor data				Clinical data				Genetic data			
Case	Site	Age	Stage	TNM	Histopat.	Lymph nodes	Radio-therapy	Follow up (months)	Recurrence/ metastasis	Status patient	DNA index	Gains	Losses	Amplifi-cations
1	L	67	I	T1bN0	w	−	−	45	−	a	nd	16	10	1
2	L	54	I	T1bN0	m	−	+	67	−	a	1	5	6	0
3	L	70	I	T1N0	m	−	−	24	−	a	nd	16	11	5
4	L	63	I	T1N0	w	−	−	78	+	dd	1.8	10	8	3
5	L	72	I	T1N0	m	−	−	48	−	a	1.9	14	17	3
6	P	65	I	T1N0	m	−	−	51	−	a	nd	15	18	2
7	L	71	I	T1N0	p	−	−	72	−	a	1.8	17	11	2
8	L	67	I	T1N0	w	−	−	41	−	a	1	13	16	2
9	L	65	I	T1N0	w	−	−	55	−	a	nd	6	16	0
10	L	80	II	T2N0	w	−	−	39	−	doc	1	6	5	0
11	L	61	II	T2N0	p	−	−	77	−	a	1.5	8	4	0
12	L	55	II	T2N0	m	−	−	74	−	a	1	15	6	1
13	L	58	II	T2N0	m	−	−	69	−	doc	1.5	9	8	0
14	L	70	II	T2N0	w	−	−	72	−	a	1.6	14	7	0
15	P	60	II	T2N0	w	−	−	49	−	a	1.4	23	7	3
16	L	72	II	T2N0	p	−	−	1	−	doc	nd	10	12	1
17	L	79	II	T2N0	m	−	−	9	+	dd	nd	9	17	2
18	L	56	II	T2N0	w	−	−	65	−	a	1	9	8	0
19	L	74	II	T2N0	w	−	−	72	−	a	1	14	20	6
20	P	54	II	T2N0	w	−	+	28	−	doc	1	5	12	0
21	L	44	II	T2N0	p	−	−	20	+	dd	1	4	12	0
22	L	46	II	T2N0	w	−	−	37	−	a	nd	14	11	1
23	L	60	III	T2N1	m	+	−	19	+	dd	1.4	11	0	1
24	P	74	III	T2N1	m	+	−	16	+	doc	1.5	6	0	1
25	L	74	III	T3N0	m	−	−	7	+	dd	1.8	13	10	1
26	P	44	III	T3N0	w	−	−	43	+	dd	1.7	11	6	1
27	P	62	III	T3N0	m	−	−	49	−	a	1.7	15	11	1
28	L	70	III	T3N0	m	−	+	14	+	dd	1.7	14	8	0
29	P	68	III	T3N0	m	−	−	25	−	doc	1.6	9	3	1
30	L	52	III	T3N1	m	+	+	13	−	doc	1.8	6	2	0
31	P	72	III	T3N1	p	+	+	15	+	dd	1	5	2	0
32	L	48	IV	T1N2b	m	+	+	67	−	a	1	19	14	3
33	P	56	IV	T1N2c	p	+	+	14	+	dd	1.9	14	10	1
34	P	58	IV	T2N2	p	+	+	19	+	dd	1.7	13	8	1
35	P	64	IV	T2N2	m	+	−	0	−	doc	1.8	10	1	0
36	P	63	IV	T2N2	p	+	−	18	+	dd	nd	18	13	2
37	L	67	IV	T2N2	m	+	+	8	+	dd	1	14	9	0
38	L	64	IV	T2N2a	m	+	−	0	−	doc	1.5	17	11	7
39	L	70	IV	T2N2b	p	+	+	22	−	doc	1.5	6	5	2
40	L	73	IV	T2N2b	w	+	+	22	+	dd	1	18	9	2
41	L	64	IV	T2N2c	m	+	+	33	−	doc	1.6	1	10	0
42	L	76	IV	T2N2c	m	+	+	68	−	a	1.8	15	12	5
43	P	50	IV	T2N3	p	+	+	9	+	dd	1	1	0	0
44	L	49	IV	T3N2b	m	+	+	13	+	dd	1.5	11	10	2

Table 1
(Continued)

Patient data			Tumor data				Clinical data				Genetic data			
Case	Site	Age	Stage	TNM	Histopat.	Lymph nodes	Radio-therapy	Follow up (months)	Recurrence/ metastasis	Status patient	DNA index	Gains	Losses	Amplifi-cations
45	P	71	IV	T3N2b	m	+	+	12	+	dd	1.7	6	1	0
46	P	69	IV	T3N2b	m	+	−	35	+	dd	1.7	11	4	0
47	P	66	IV	T3N2c	w	+	−	4	+	dd	1	6	5	2
48	L	66	IV	T3N3	w	+	+	6	+	dd	1	5	6	0
49	P	64	IV	T3N3	w	+	+	2	+	dd	1.7	20	13	3
50	P	61	IV	T4N0	w	−	−	10	+	dd	1.7	6	5	0
51	L	48	IV	T4N0	m	−	−	44	−	a	1	10	0	0
52	P	57	IV	T4N0	m	−	−	30	−	a	1.5	12	6	4
53	L	66	IV	T4N2b	m	+	+	19	−	doc	1	16	9	4
54	P	50	IV	T4N2b	m	+	+	11	+	dd	1	8	7	1
55	P	48	IV	T4N2b	m	+	+	7	+	dd	3.0	18	13	4
56	P	49	IV	T4N2c	p	+	−	0	−	doc	1.6	6	8	1

L: larynx; P: pharynx; histopat: histopathological degree of differentiation; w: well-differentiated; m: moderately differentiated; p: poorly differentiated; gains: total number of gains detected by CGH; losses: total number of losses detected by CGH; a: alive; dd: died of disease; doc: died of other causes; nd: not done.

Table 2
Listing of the chromosomal regions most frequently involved in copy number changes

Loss		Gain		Amplification	
3p13–24	44 cases, 79%	3q25–27	48 cases, 86%	3q26-qter	20 cases, 35%
11q23–25	25 cases, 45%	7q21–31	38 cases, 68%	11q13	7 cases, 13%
4p15–16	16 cases, 38%	8q11-qter	34 cases, 61%	11q22	5 cases, 9%
18q21–23	16 cases, 38%	5p12–14	30 cases, 54%	3q12–13	5 cases, 9%
5q31–35	21 cases, 36%	11q11–13	28 cases, 50%	18p11.3	5 cases, 9%
8p21–22	19 cases, 34%	2q24–32	24 cases, 43%	18q11.2	5 cases, 9%
21q22	19 cases, 34%	12p11–12	24 cases, 43%	8q24.3	3 cases, 5%
17p12–13	18 cases, 32%	17q11–23	23 cases, 41%	1p31	2 cases, 4%
4q31–34	17 cases, 30%	6q12–15	21 cases, 38%	2q32–33	2 cases, 4%
9p21–23	17 cases, 30%	4q12–13	20 cases, 36%	4q11–13	2 cases, 4%
		18p11-pter	20 cases, 36%	5p11–14	2 cases, 4%
				8p11	2 cases, 4%
				13q33–34	2 cases, 4%
				22q13	2 cases, 4%

tion was found between DNA ploidy and disease-free survival, clinical stage or lymph node status. More surprisingly, neither of the chromosomal regions recurrently involved in loss, gain or a high level amplification were related to disease-free survival. We looked separately at 35 patients that had not received radiotherapy and 21 patients that had, but again we could not find correlations between disease-free survival and DNA ploidy or specific chromosomal amplifications, gains or losses.

Amplification 11q13 was related to clinical stage; all six amplifications 11q13 occurred in stage IV tumors (Pearson Chi^2, $p = 0.04$), and all six cases were lymph node positive (Fisher exact, $p = 0.007$). Amplification of 18p11.3 occurred in four of five cases in stage IV tumors and in lymph node positive cases, but this did not reach significance.

4. Discussion

In this paper we investigated the prognostic value of genetic aberrations in larynx and pharynx squamous cell carcinomas detected by CGH. First we confirmed

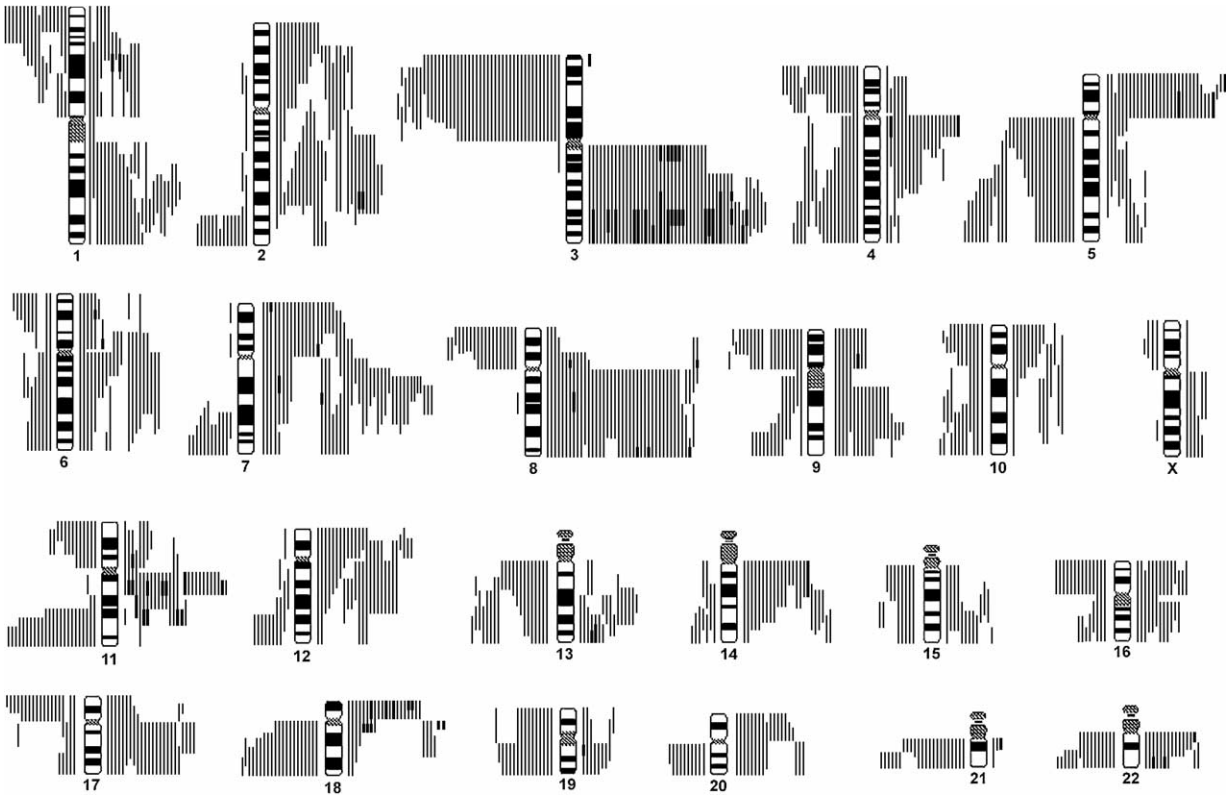

Fig. 2. Overview of CGH results of all 56 larynx and pharynx squamous cell carcinomas. Bars to the right of the ideograms represent gains and bars to the left represent losses. Amplifications are shown by bold bars.

Table 3
Clinical and genetic characteristics in relation to disease-free survival

	Disease-free	Reccurrence/metastasis	*p*-value
Stage I	8	1	Stage 1,2 vs 3,4:
Stage II	11	2	0.0001
Stage III	4	5	(log rank 20.9)
Stage IV	10	15	
Lymph node negative neanenegative	23	7	0.0001
Lymph node positive	9	17	(log rank 24.9)
DNA diploid	10	8	0.8891
DNA aneuploid	16	14	(log rank 0.02)
Amp 11q13: no	23	21	0.7068
Amp 11q13: yes	4	3	(log rank 0.14)
Amp 18p11.3 : no	28	23	0.5754
Amp 18p11.3: yes	4	1	(log rank 0.31)

Statistical evaluation was done by Kaplan–Meier analysis. This table shows that only the tumor stage (when comparing stages 1 and 2 versus 3 and 4), and the lymph node status were significantly correlated to disease-free survival, and none of the genetical characteristics of the tumor.

that our series of cases showed the general pattern of chromosomal abnormalities, as described in literature, i.e. losses at 3p, 8p, 11qter, 17p, 18q, and gains at 3q, 5p, 7q, 8q, 11q13, 18p (Fig. 1). Next we checked if our cases were representative of larynx/pharynx cancers as a whole in terms of established prognostic factors like lymph nodes status and clinical stage. We found that these two clinical features correlated strongly with disease-free survival (Table 3), as was expected from literature data.

However, when evaluating the recurrent chromosomal abnormalities as detected by CGH, no statistical correlation to disease-free survival could be found. Moreover, neither of the gains and losses correlated to clinical stage or lymph node status. Only 11q13 amplification correlated to a high clinical stage and positive lymph node status (all six amplifications 11q13 occurred in stage IV tumors, and all six cases were lymph node positive).

This was a surprising result. In the literature, a number of chromosomal loci involved in losses have been proposed to have prognostic value for head and neck cancers. Partridge et al. [19] studying mainly stage I and II tumors, found prognostic value for LOH on 3p24–26, 3p13 and 9p21, i.e. the same regions found to be of importance for progression from premalignant tumor to invasive carcinoma [16,31]. Other investigators proposed LOH on 8p23 and 18q21 [21,26], and CGH losses on 3p, 4q, and gains on 9q [15] to be related to clinical outcome. In addition, CGH losses on 3p, 5q, and 18q [14] and CGH losses on 10q and 11p [2] have been reported to occur more frequently in lymph node metastasizing tumors compared to non-metastasizing tumors.

Amplification of 11q13 has been associated with poor survival in several studies [1,17,20,23]. However, in other studies this association could not be confirmed [13,18]. Our data on 11q13 amplification seem to be intermediate: there was a correlation with prognosticators as lymph node status and clinical stage, but there was no direct correlation with disease-free survival (Table 2). In a recent study using conventional cytogenetic analysis, Jin et al. [13] did not find any relation between overall karyotypic complexity or specific recurrent changes and tumor stage or differentiation (data on clinical outcome were not given). This is in agreement with our findings.

An explanation for this lack of prognostic value is difficult to find. One possibility is that the progression step from premalignant tumor to invasive cancer is accompanied by the immediate acquisition of a multitude of at random chromosomal abnormalities. Support for this idea may come from the observation in this study that stage I tumors harbour as many chromosomal abnormalities, including high level amplifications, as stage IV tumors. Preliminary data of one case of carcinoma *in situ* and one case with severe dysplasia (unpublished results) also revealed a large number of chromosomal abnormalities. We intend to continue these studies on premalignant tumors in order to increase our knowledge on the process of chromosomal instability in the progression of larynx and pharynx squamous cell carcinoma, and the possible implications for clinical decision-making.

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