Short Communication

Genetic Imbalances with Impact on Survival in Head and Neck Cancer Patients

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Chromosomal imbalances in 113 primary head and neck squamous cell carcinomas (HNSCCs) determined by comparative genomic hybridization were correlated with patients survival using custom-made computer software which enabled the assessment of individual chromosomal loci. The Kaplan-Meier analysis revealed that overrepresentations of 2q12, 3q21-29, 6p21.1, 11q13, 14q23, 14q24, 14q31, 14q32, 15q24, 16q22, and deletions of 8p21-22 and 18q11.2 were significantly associated with both shorter disease-free interval and disease-specific survival in this tumor collective. Multivariate Cox proportional hazards regression models consistently identified the gains of 3q21-29, 11q13, and the loss of 8p21-22 as independent prognostic markers carrying a higher significance than the nodal status as the only clinico-pathological parameter with statistical importance. In addition, these three markers allowed a molecular dissection of the patients with low clinical risk (pN0 and pT2 tumors). Thus, the genomic data being derived from the evaluation of primary HNSCC enabled a stratification of the patients into subgroups with different survival highlighting the necessity of a genetically based tumor classification for refining diagnosis and treatment of HNSCC patients. (*Am J Pathol*, :369-375)

Histopathological tumor classification is still considered the gold standard for the characterization of solid tumors. However, it is well known that these established parameters do not satisfactorily predict the clinical outcome in individual cases. This is primarily because of the different biological behavior of the tumors that partially escape the conventional histological analysis. In head and neck squamous cell carcinoma (HNSCC) the nodal status is

the best prognostic parameter as for many other tumor types.¹⁻⁴ It reflects a specific biological behavior being the capacity for lymphangiogenic dissemination. However, for its correct determination an extended operation is necessary. Although surgery is the major option for initial treatment, primary radiochemotherapy has shown promising results in HNSCC.⁵ For the improvement of different treatment modalities there is an increased demand for a refined initial characterization which is generally performed by a small biopsy of the primary tumor. So far, these specimens are only used to establish the diagnosis of the disease and a semiquantitative estimate of its malignancy expressed by the tumor grade. In an ideal setting, the pathologist should also answer the question whether or not the tumor carries the potential for metastatic spread or if it is resistant to radiation and/or chemotherapy and he should provide a statement on the patient's prognosis.

Multiple genetic changes have been reported in HNSCC which include both activation of proto-oncogenes and inactivation of tumor suppressor genes.⁶ Until now, the genetic alterations underlying the malignant behavior and progression of these tumors are only partially understood. The only alteration that has been repeatedly associated with worse prognosis in HNSCC is the amplification of chromosome 11q13 harboring the *cycD1* gene.⁷

To get a more comprehensive picture about which additional chromosomal alterations are associated with prognosis in HNSCC, we correlated our comparative genomic hybridization (CGH) data of 113 primary carcinomas with patients survival using computer software that allowed the assessment of individual loci by Kaplan-Meier analysis. The study highlighted several imbalances with prognostic impact and with each carrying a higher significance than the classical parameters.

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Materials and Methods

Patients and Tumor Samples

The study comprised 113 patients with single primary HNSCC. None of the patients had previous malignancies or received treatment before initial tumor biopsy. They were all treated for cure by surgical removal of the primary carcinoma along with a neck dissection being complemented by adjuvant postoperative radiation in advanced stages. All specimens were obtained from surgical resections of the primary tumors that were operated on at the Department of Otorhinolaryngology, Charité Hospital, Humboldt University in Berlin during the period 1994 through 1996. Operation specimens were transferred to the Institute of Pathology of the Charité University Hospital within 1 hour after surgical removal. One aliquot of tumor tissue was frozen in liquid nitrogen and kept at -80°C until DNA extraction. DNA was extracted from several 30- μm cryostat tissue sections by proteinase K digestion and phenol-chloroform extraction and which was verified to consist of a minimum of 70% tumor cells in each case. A second aliquot was submitted for formalin fixation and paraffin embedding. The histopathological diagnosis was established in every case according to the World Health Organization guidelines on hematoxylin and eosin (H&E)-stained tissue sections and the tumors were staged using the tumor-node-metastasis classification forwarded by the International Union Against Cancer (UICC).⁸

Follow-up of the patients, performed on an ambulant basis after completed therapy, lasted until November 1, 1999. The median duration of follow-up was 44 months overall (95% confidence interval, 40.14 to 48.76). The causes of death were determined at autopsy or by clinical examination at the Charité University Hospital. The distribution of clinicopathological and survival data of all cases is summarized in Table 1. None of the patients had distant metastases (pM = 0) at the time of diagnosis.

CGH

DNA labeling, hybridization, and detection were performed as previously described.^{9,10} The protocols are also available at our web site <http://amba.charite.de/cgh>.

Digital Image Analysis

The image acquisition and digital image analysis have also been described in detail.^{11,12} At least 10 and generally even 15 metaphases/karyograms were analyzed per case calculating CGH sum karyograms and mean ratio profiles with confidence intervals. The DNA imbalances were determined according to a statistical procedure as described.^{10,11} Briefly, the deviations of the mean fluorescein isothiocyanate/tetramethylrhodamine B isothiocyanate profiles from the normal ratio of 1.0 were

Table 1. Clinicopathological Data of the Study Cohort

	Total	A	AWT	DBT	DOD
No. of patients	113	70	10	33	7
Females	26	14	5	7	1
Males	87	56	5	26	6
Tumor sites					
Larynx	41	31	2	8	3
Supraglottic	17	13	2	2	1
Glottic	23	17	—	6	2
Subglottic	1	1	—	—	—
Hypopharynx	29	16	3	10	1
Oropharynx	31	18	4	9	3
Oral cavity	12	5	1	6	—
Stage					
pT1	6	6	—	—	—
pT2	29	13	4	12	1
pT3	45	33	3	9	4
pT4	33	18	3	12	2
pN0	41	33	1	7	4
pN1	22	11	4	7	2
pN2	48	25	5	18	1
pN3	2	1	—	1	—
Grade					
G1	3	3	—	—	—
G2	86	56	5	25	7
G3	24	11	5	8	—

A, alive without disease; AWT, alive with tumor; DBT, dead because of tumor; DOD, dead because of other disease.

tested for significance by a two-sided Student's *t*-test. Deviations of the ratio profile with at least 99% significance in the Student's *t*-test were scored as DNA gains or losses, ie, only those imbalances in which the ratio profile with its 99% confidence interval exceeded the line of the normal ratio 1.0 to the same side were included in the evaluation. This procedure is rather sensitive for scoring chromosomal alterations by CGH. Pronounced DNA gains and losses of HNSCC shown in the histogram of Figure 2 were defined by those alterations exceeding the ratio values 1.5 and 0.5, respectively.¹³ They most likely correspond to high copy amplifications of multicopy deletions. Individual ratio profiles of all tumors are accessible at our CGH Online Tumor Database at <http://amba.charite.de/cgh>.

Statistical Methods

Survival analysis was applied to all 113 patients comprising clinicopathological as well as genetic parameters. The clinical parameters included tumor site, tumor size (pT), lymph node metastases (pN), histopathological grade, and UICC stage. The genetic parameters consisted of a comprehensive set of chromosomal alterations determined by CGH corresponding to the 400-band ISCN nomenclature. All noninformative bands, ie, centromeres, heterochromatic regions, satellites, and the sex chromosomes were excluded from the analysis, which in total led to 303 individual bands. A genetic imbalance was scored if at least 75% of the digital chromosome segments were affected by an imbalance.

Survival analysis of the data were performed with SPSS

software (SPSS, Munich, Germany) controlled by custom-made software. Values of $P < 0.05$ were considered to be statistically significant. Deaths from causes other than the index tumor or recurrence/metastases were not considered treatment failures, and these patients were censored in each analysis involving the length of survival. Kaplan-Meier analysis¹⁴ was sequentially performed on each of the 303 bands by using the criteria for a genetic alteration as described above. Because the criterion is binary (alteration/no alteration) it subdivided the set of 113 patients into two subgroups. By this way the relationship between the single chromosomal alterations and the overall survival and disease-free interval was performed. The differences of the survival curves were tested for statistical significance with the log rank test.

Cox proportional hazards regression models¹⁵ were used to examine the relative impact of the variables demonstrated to be statistically significant in univariate analysis. The stepwise backward/forward procedures provided by the SPSS software were used to further reduce the number of variables in the Cox models. For assessing and comparing the Cox models a Wald test with a significance level of 0.05 was used for both inclusion and exclusion of variables.

Results

Survival Analysis—Correlation with Clinicopathological Parameters

The clinicopathological data for all patients are summarized in Table 1. Survival was analyzed for the parameters tumor site, stage (pT, pN, UICC), and grade with respect to the occurrence of metastasis/recurrence (disease-free interval) and death from HNSCC (disease-specific survival), respectively. In the univariate Kaplan-Meier analysis statistically significant differences were observed for the pN stage only when comparing nodal-negative cases (pN0) with nodal-positive tumors (pN1, pN2, or pN3), the tumor site comparing pharyngeal *versus* laryngeal carcinomas and the pT stage.

For the pT stage, however, the best prognosis was associated with the pT1 tumors followed by pT3, pT2, and pT4 carcinomas. Surprisingly, the pT2 carcinomas had a worse outcome than pT3 tumors. However, this can be explained by the composition of both subgroups regarding the location of the primary tumor. The pT2 group consisted mainly of pharyngeal carcinomas (26 pharyngeal *versus* three laryngeal carcinomas) carrying a worse prognosis than laryngeal carcinomas. For the pT3 group, in contrast, the carcinomas were almost equally distributed (22 pharyngeal *versus* 23 laryngeal tumors).

For the nodal stage, the log rank test indicated significance in the correlation with the disease-free interval ($P = 0.011$) as shown in Figure 1D whereas there was only a tendency for significance regarding the disease-specific survival ($P = 0.078$). Similarly, for the tumor site there was a significant P value only for the disease-free

interval ($P = 0.048$) in contrast to the disease-specific survival ($P = 0.136$).

Survival Analysis—Correlation with Chromosomal Imbalances

Kaplan-Meier analysis was performed separately for 303 chromosomal imbalances representing the chromosomal bands and subbands of all autosomes. Representative diagrams of chromosomal imbalances showing a statistically significant correlation with patients survival are illustrated in Figure 1, A–C. All significant alterations are listed in Table 2. Overrepresentations of 2q12, 3q21-29, 6p21.1, 11q13, 14q23, 14q24, 14q31, 14q32, 15q24, 16q22, and deletions of 8p21-22 and 18q11.2 had a statistically significant association with both the shorter disease-free interval and the shorter disease-specific survival in this patient population.

Table 2 indicated only those loci harboring DNA gains or losses in at least 20 of the 113 cases. The overall incidence of imbalances at specific chromosomal sites can be deduced from the histogram of the 113 primary HNSCC shown in Figure 2. A frequency of $>50\%$ along with significance in the survival analysis was observed for the deletions on 8p21-23, 11q23-25, and 18q as well as the DNA overrepresentations on chromosomes 3q13.3-qter and 11q13. Individual ratio profiles with confidence intervals as well as the histogram are also available at our CGH Online Tumor Database at <http://amba.charite.de/cgh> where the incidences can be assessed interactively by opening the Infoscreen and pointing at specific chromosomal sites using the computer mouse. Chromosomal imbalances were observed in each case using our methodology for checking the normal cell contamination.

Survival Analysis—Multivariate Regression

Cox proportional hazard models were used to examine the relative impact of the variables identified by the univariate analysis. As a first step, chromosomal loci of Table 2 were preselected for those imbalances being present in at least 40 tumors and showing $\leq 55\%$ of censored cases with respect to the disease-free interval. Applying these criteria five chromosomal locations remained, ie, gains of 3q21-29, 11q13, and losses of 8p21-22, 11q23-25, and 18q21. The presence or absence of lymph node metastases (pN0 *versus* pN1/pN2/pN3) was included as the only clinical parameter in the multivariate analysis resulting totally in six binary variables. For the disease-free interval, the backward and the forward procedure for variable inclusion/exclusion determined the same set of remaining variables in the Cox model which are listed in Table 3, ie, nodal status, gains of 3q21-29 and 11q13, as well as deletions of 8p21-22. The Cox model for the disease-specific survival using the backward procedure resulted in the same variables; however, the forward procedure further reduced it to the gains of 3q21-29 and 11q13.

Figure 1. A–C: Kaplan-Meier plots comparing disease-specific survival in patients whose tumors showed gains at 3q21-29 (A), gains at 11q13 (B), deletions at 8p21-22 (C) represented by **solid lines** with that of patients whose tumors did not exhibit the specific chromosomal imbalances (**stippled lines**), respectively. D: Kaplan-Meier plot comparing disease-specific survival in patients whose tumors had already spread to cervical lymph nodes with that of patients whose tumors did not metastasize at time of diagnosis (**stippled line**).

Impact of Genetic Markers in Clinicopathological Subgroups

To evaluate the influence of the genetic markers in conventionally defined tumor subgroups we performed Kaplan-Meier analysis for each of the five imbalances that were assessed in the multivariate analysis in patients with pT2, pT3, pT4, pN0, pN1, and pN2 stage, respectively. In particular for the pN0 subgroup, the analysis confirmed the validity of the three independent markers, ie, 3q21-29 gain, 11q13 gain, and 8p21-22 loss, yielding significant *P* values of the log rank test ranging from <0.001 up to 0.045 both for the disease-specific survival and disease-free interval. Other significant *P* values (<0.05) were observed for the following combinations: pT2-3q21-29 gain (disease-specific survival and disease-free interval), pT2-11q13 gain (disease-free interval), pT3-8p21-22 loss (disease-specific survival), and pN1-3q21-29 gain (disease-free interval).

Discussion

Analyzing primary tumors by CGH we identified several chromosomal imbalances that are associated with bad prognosis of HNSCC patients. Although it is a very powerful tool to screen a tumor genome for chromosomal imbalances most similar CGH studies in other tumor entities gave rather disappointing results often indicating that only a higher overall number of alterations is associated with shorter survival. This discrepancy can be mainly explained by methodological considerations. First, we collected our tumor samples in the context of a prospective study performing hybridizations exclusively with DNA from fresh-frozen tumor samples checking the amount of contaminating normal tissue in each specimen.^{9,10} Second, for the calculation of the ratio profiles and confidence intervals at least 10 metaphases were included in the digital image analysis using a statistical

Table 2. Chromosomal Sites Associated with Disease-Free Interval and/or Disease-Specific Survival

Chromosome	Type	Log rank (p)	Number of cases with/without alteration	Censored cases with/without alteration
1q32	Gain	0.049	33/80	16/55
1q42-43	Gain	0.034	27/86	13/58
2p24-25	Gain	0.013	27/86	12/59
2q12	Gain	<0.001 (<0.001)	32/81	14/57 (16/64)
3q21-29	Gain	0.007 (0.006)	66/47	35/36 (40/40)
6p21.1	Gain	0.010 (0.008)	26/87	13/58 (14/47)
6p22-24	Gain	0.006	20/93	9/62
7p22	Gain	0.045	31/82	15/56
8p21-22	Loss	0.014 (0.008)	43/70	22/49 (25/55)
11q13	Gain	0.005 (0.013)	56/57	29/42 (34/46)
11q23-25	Loss	0.042	41/72	21/50
14q23	Gain	0.041 (0.021)	21/92	12/59 (4/85)
14q24	Gain	0.003 (0.000)	21/92	11/60 (11/69)
*14q31	Gain	0.024	21/92	13/67
*14q32	Gain	0.030	20/93	12/68
15q24	Gain	0.005 (0.005)	23/90	10/61 (11/69)
16q22	Gain	0.015 (0.001)	24/89	11/60 (11/68)
*18q11.2	Loss	0.034	25/88	14/66
18q21	Loss	0.043	64/49	35/36

Values for disease-specific survival are indicated in brackets.
*Values for disease-free interval and disease-specific survival were identical.

evaluation for the determination of chromosomal imbalances which has been reported to be superior to the classical evaluation scheme by fixed ratio thresholds.^{16,17} Third, previous studies usually scored the alterations with a resolution that is far behind the power of CGH counting a specific genetic change as an imbalance of the entire chromosome arm. This is understand-

able because of the complexity of the statistical analysis if each chromosomal subregion is analyzed separately. To overcome this methodological shortcoming we developed computer software enabling the assessment of individual loci and here applied it for the first time on our tumor collective of head and neck carcinomas. The fact that we confirmed several changes that have been pre-

Figure 2. Summary of chromosomal alterations in 113 HNSCC in a histogram representation. The chromosomal imbalances are shown as incidence curves along each chromosome. Areas on the left side of the chromosome ideogram correspond to loss of genetic material; those on the right side to DNA gains. The frequency of alterations can be determined from 0.5 (50%) and 1.0 (100%) incidence lines depicted parallel to the chromosome ideograms. DNA changes with 99% significance are colored in gray, additional changes with 95% significance are depicted in light gray. The proportion of pronounced DNA gains and losses being defined as imbalances for which the ratio profiles exceeded the thresholds of 1.5 and 0.5, respectively, are visualized in black.

Table 3. Results of the Cox Multivariate Analysis (Disease-Free Interval)

Variable	B	P	Exp (B)	95% CI for Exp (B)	
				Lower	Upper
pN	0.978	0.015	2.658	1.213	5.824
3q21-29	1.021	0.006	2.775	1.339	5.753
8p21-22	0.642	0.043	1.901	1.022	3.538
11q13	0.877	0.008	2.403	1.258	4.592

B, the estimation for the regression coefficient β ; P, probability (rejection of the null hypothesis if it is true); Exp(B), the hazard ratio which measures the relative survival experience of every two compared groups; CI, confidence interval.

viously reported using other methodologies supports the validity of our findings.

Chromosome 11q13 gains have repeatedly been reported to be associated with bad prognosis in HNSCC.^{18,19} The main candidate gene is the *cycD1* proto-oncogene which drives the cell from the G1 into the S phase of the cell cycle. However, overexpression of this gene seems to be an independent prognostic factor because it is not strictly related to gene amplification.⁷ Thus, there must be other genes on chromosome 11q13 which are responsible for poor survival. This is well perceivable because deregulation of the cell cycle alone is probably not sufficient to explain the biological behavior responsible for tumor progression that is mainly characterized by increased invasiveness and the capability of metastasis formation.

Chromosome 3q gain has been defined as the key lesion in the transition from the preinvasive to invasive stage of squamous cell carcinomas of the uterine cervix²⁰ and was correlated with bad prognosis in our study. Interestingly, losses on the short arm of chromosome 3 did not affect the patients' outcomes. This is consistent with the observation that 3p deletions along with 9p loss constitute a very early change in tumor development being detectable in precursor lesions.⁵

Consistently with our findings, allelic loss on chromosome 8p have been associated with advanced tumor stages and poor survival²⁰⁻²² similar to 11qter and 18q deletions.^{10,23,24} However, our study also revealed further loci on several other chromosome arms.

For chromosomes 14q and 15q, the histogram in Figure 2 indicated small regions of deletion that does well correspond with the previously reported minimal regions identified by allelotyping. However, these studies suggested that deletions rather than DNA gains are associated with bad prognosis or tumor progression.^{11,25,26} This apparent contradiction can be explained by the consideration that deletions of one allele may be associated with the numerical gain of the second chromosome. Such duplication would result in a shift of the ratio profile, eg, indicating a gain of the telomeric chromosome part instead of a loss of the centromeric region. The histogram curves showing deletions preferentially of the centromeric part as well as overrepresentations in the distal regions are consistent with this hypothesis which however needs to be substantiated by further studies. The findings for chromosomes 14 and 15 also need to be

interpreted cautiously because the number of cases carrying these alterations is relatively low compared with other loci. In general, it is important to be aware of the methodological pros and cons of CGH compared to microsatellite analysis being particularly the capacity for the detection of amplifications but the lack of information on the allelic status.

A major result of our study is the fact that the genetic changes, ie, gains of 3q21-29, 11q13, and loss of 8p21-22, were independent markers and carried a higher significance than the classical clinicopathological parameters. In addition, these markers enabled a molecular dissection of conventionally defined subgroups, particularly the patients with pN0 tumors. Importantly, the findings were derived from the investigation of primary tumors. Thus our data clearly point to the feasibility and necessity of a genetic characterization of HNSCC for a refined stratification of patient subgroups which will hopefully pave the way for an individualized treatment finally improving the prognosis of this often lethal disease.

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