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ORIGINAL PAPER

Chromosomal changes characterize head and neck cancer with poor prognosis

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Abstract It is well established that genetic alterations may be associated to prognosis in tumor patients. This study investigates chromosomal changes that predict the clinical outcome of head and neck squamous cell carcinoma (HNSCC) and correlate to characteristic clinicopathological parameters. We applied comparative genomic hybridization (CGH) to tissue samples from 117 HNSCC patients scheduled for radiotherapy. Genomic aberrations occurring in more than five patients were studied for impact on locoregional progression (LRP)-free survival. *p* values were adjusted by the Hochberg–Benjamini procedure and significant aberrations and clinical variables subjected to a stepwise backwards Cox proportional model. Significant alterations were further analyzed by array-CGH and fluorescence in situ hybridization (FISH). In multivariate survival analysis gains on 1q and 16q predict reduced LRP-free survival independently from known prognostic factors. Cluster analysis separated the HNSCC cases into two groups (cluster 1 and 2) that are characterized by significant differences for imbalances in 13 chromosomal regions. Moreover, it became apparent that cluster 1 correlates to nonanemic patients, while cluster 2 represents predominantly anemic cases. Array-CGH pinpoints 16q24.3 to be the region of interest on chromosome 16 which was further verified by FISH analysis where an increased copy number of *FANCA*, a member of the Fanconi anemia/breast cancer

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pathway, could be identified. This study demonstrates that chromosomal gains on 1q and 16q as well as chromosomal loss on 18q represent prognostic markers in HNSCC and that these alterations may explain to some extent the dismal course of a subgroup of patients.

Keywords HNSCC · Chromosomal imbalances · Array-CGH · FA/BRCA pathway · Prognostic marker

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the seventh most common cancer disease in the US with approximately 47,000 new cases per year [1]. Clinical features predict the individual patients' outcome, such as location, extent of disease (including nodal involvement), perilymphatic and perineural spread, and extent of surgery [2–3]. Finally, anemia negatively affects prognosis, independently from known prognostic factors [4]. However, recent studies report decreased disease control and survival in case of erythropoietin treatment for anemia correction [5–8]. Besides these clinicopathologic variables, malignant progression of HNSCC is characterized by the accumulation of genetic abnormalities [9–11] and distinct genetic changes associated to a progressing and recurrent disease [12, 13]. Frequent chromosomal imbalances in HNSCC have been reported on 3p, 4, 5q, 8p, 9p, 11, 13q, and 18q (losses) and 3q25–26, 5p, 8q24, 9q22–34, 11q13, 14q24, 16p, 19, 20q24, and 22q (gains) [14–15]. Several prognostic markers were identified in previous studies including DNA gains on 3q21–29, 11q13, and 12q24 and losses on 5q11–15, 6q14–21, 8p21–22, 18q22, and 21q11–21 [11, 16–18]. It has been suggested that gains on 3q26 and 11q13 and deletions on 8p23 and 22q could be valuable markers of aggressive disease [16, 19]. Also chromosomal aberrations have been related to treatment response; for example, loss of distal 11q has been associated with DNA repair and reduced sensitivity to ionizing radiation [20]. Although conventional comparative genomic hybridization (CGH) studies revealed promising prognostic markers, information about altered candidate genes is limited due to the low resolution of CGH of about 10 Mb [21]. Therefore, advanced approaches like array-CGH [22] may provide more detailed information on gene alterations as prognostic factors. This is of particular interest since pathways affected by genetic alterations can be discovered and investigated.

In this study, we confirm previous findings and furthermore identify new chromosomal markers in HNSCC that correlate with poor clinical outcome. Additionally, we show that HNSCC cases can be separated into two tumor groups based on distinct patterns of chromosomal abnormalities. We could demonstrate an association of these two tumor groups with the patients' anemia status. Finally, we were able to designate an amplified candidate gene as a prognostic marker from array-CGH analysis.

Materials and methods

Patient data, treatment, and tumor tissues

In total, 117 tumor samples from HNSCC patients were investigated. Sixty-eight tumor samples were derived from anemic HNSCC patients participating in a multicenter trial [5]. Inclusion criteria were anemia just before starting radiotherapy (blood hemoglobin level according to common definitions for anemia: female—lower than 12 g/dL, male—lower than 13 g/dL), age older than 18 years, histologically proven squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx, and scheduled treatment with radiotherapy only or postoperative radiotherapy for advanced disease (T3, T4, or nodal involvement). A further eligibility criterion was a Karnofsky score of 60 or more. Patients with any other simultaneous malignant disease, treatment with any cytostatic drug, pregnancy, or inadequate contraception were excluded. Additionally, we selected 49 nonanemic patients with HNSCC matched at the best for sex, location, stage, resection, and treatment compliance. The study was approved by the ethics committee of the Freiburg University Clinic and performed in accordance with the revised Declaration of Helsinki.

Standard planning and radiation techniques were used for both patient groups. The radiation volume included the tumor (or tumor bed) with a 2–3-cm safety margin and the regional lymph node areas. Six mega-electron-volt linear accelerators were used and standard dose fractionation protocols (five fractions of 2.0 Gy per week) were followed. Sixty Gy (allowable range, 56 to 64 Gy) were administered to regions for R0 or R1 resected disease and 70 Gy (allowable range, 66 to 74 Gy) for macroscopically incompletely resected tumor (R2) or primary definitive treatment. The spinal cord was shielded after 30–36 Gy. Patients were seen for first follow-up 6 weeks after completion of radiotherapy and, thereafter, every 3 months to assess locoregional tumor control and survival. Detailed patient data are summarized in Table 1.

Formalin-fixed and paraffin-embedded (FFPE) tissue sections were stained with hematoxylin/eosin according to standard procedures. The histopathological classification of each sample was evaluated according to the guidelines of the World Health Organization [23] and staging of tumors was performed according to the criteria of the International Union Against Cancer [24]. Microdissection was applied to

Table 1 Demography of HNSCC patients

	Total [%]	Alive without disease	Alive with tumor	Dead because of tumor	Dead because of other disease
Number of patients	117	47	7	29	34
Females	13 [11.1]	8	1	3	1
Males	104 [88.9]	39	6	26	33
Current smoker	56 [47,9]	20	3	13	20
Nonsmoker	46 [39,3]	17	4	14	11
n.s.	15 [12,8]	10	–	2	3
Hemoglobin level					
Nonanemic cases	49 [41,9]	28	–	4	17
Anemic cases	68 [58,1]	19	7	25	17
Radiotherapy	114 [97,4]	47	7	27	33
Recurrence of tumor after radiotherapy	24 [20,5]	–	4	20	–
Tumor localization					
Larynx	12 [10,3]	4	1	4	3
Hypopharynx	32 [27,4]	15	1	8	8
Oropharynx	44 [37,6]	17	3	9	15
Oral cavity	29 [24,8]	11	2	8	8
Staging					
pT1	14 [12]	8	–	2	4
pT2	35 [29,9]	15	4	7	9
pT3	23 [19,7]	11	1	7	4
pT4	42 [35,9]	11	2	13	16
n.s.	3 [2,6]	2	–	–	1
pN0	16 [12,8]	5	1	5	5
pN1	29 [24,8]	12	2	7	8
pN2	68 [58,1]	29	3	16	20
pN3	4 [3,4]	1	1	1	1
Grading					
G1	3 [2,6]	1	–	1	1
G2	62 [53]	24	3	21	14
G3	44 [37,6]	21	3	6	14
n.s.	8 [6,8]	1	1	1	5
Resection status					
R0	40 [34,2]	28	2	5	5
R1	13 [11,1]	6	1	3	3
R2	4 [3,4]	1	–	2	1
Primary definitive radiotherapy	60 [51,3]	12	4	19	25

n.s. Not specified

paraffin-embedded sections to enrich the tumor cell content to more than 90%. Genomic DNA was extracted from microdissected samples using a DNA extraction kit according to the manufacturer's protocol (Qiagen, Hilden, Germany).

Labeling of DNA

DNA extracted from paraffin-embedded tissues was indirectly labeled by nick translation using biotin-16-dUTP for tumor DNA and digoxigenin-11-dUTP for reference DNA which was isolated from peripheral lymphocytes of a healthy male or female donor. Nick translation of tumor and reference DNA was performed according to standard protocols.

CGH and image analysis

For each CGH hybridization, 1 μg tumor DNA, 1 μg reference DNA, 25 μg *Cot*-1 DNA (Invitrogen, Karlsruhe, Germany), and 20 μg herring sperm DNA (Sigma-Aldrich, Taufkirchen, Germany) were cohybridized to denatured metaphases for 72 h at 37°C. After hybridization, biotin-labeled tumor DNA was detected with avidin-fluorescein isothiocyanate (FITC) DCS (Vector Laboratories, Linaris, Wertheim-Bettingen, Germany) and digoxigenin-labeled reference DNA with anti-digoxigenin-rhodamine (Roche, Mannheim, Germany). Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in antifade solution (Vectashield, Vector Laboratories). Images

of at least ten metaphases were captured using a charge-coupled device camera (IMAC-CCD S30) and karyotyped after visualization with a Zeiss Axioplan 2 fluorescence microscope equipped with filter sets (single-band excitation filters for DAPI, FITC, and tetramethylrhodamine isothiocyanate (TRITC)) and 63× (N.A. 1,25) and 100× (N.A. 1,4) objectives (Plan-Neofluar, Zeiss). Averaged profiles were generated by CGH analysis software (ISIS 3, V2.84; MetaSystems, Altrussheim, Germany) from at least ten to 15 homologous chromosomes and interpreted according to published criteria [25] using statistical probability limits that adapt automatically to the real variability of the ratio values. The system calculates intra-experiment standard deviations (SDs) for all profile points in all chromosomes and combines them with an empirically defined position-independent inter-experiment standard deviation. From the resulting total SD's and the desired error probability, the width of the confidence interval is determined using Student *t* statistics. For the interpretation of the experiment, the position-dependent confidence interval around the normal value of 1 is plotted together with the averaged tumor/reference ratio profile, side by side with the chromosome ideogram. Chromosomal imbalances were defined as gains and losses if the averaged tumor/reference profile exceeds the position-dependent confidence interval. A chromosomal gain was classified as high-level amplification when the CGH ratio exceeded a value of 1.5 or when the FITC fluorescence showed a strong distinct signal by visual inspection and the corresponding ratio profile was diagnostic of overrepresentation.

Telomeric regions, heterochromatic regions, and the Y chromosome show strong interindividual variations and were therefore excluded from interpretation. Furthermore, deletions on 1p32-pter, 16p, 19, and 22 were eliminated from evaluation due to a high rate of apparently abnormal ratios in these regions in comparison of two normal DNAs [26].

Array-CGH

CGH of the entire genome identified six anemic patients with a particular poor clinical outcome that related to gain on 16q and did not depend on known prognostic factors. Having sufficient DNA samples available from five of these, we performed additional array-CGH analyses in order to locate more precisely the chromosomal imbalances. One megabyte BAC array slides were kindly provided by the Array Facility of the Wellcome Trust Sanger Centre (Hinxton/Cambridge, UK). They contain approximately 3,400 BAC clones spotted in duplicates onto aminoreactive slides (CodeLink, GE Healthcare, Buckinghamshire, England) and cover the whole human genome in 1 Mb distances [27]. In one case, a 1.4 Mb BAC array (HumArray 3.2) carrying 2,464 BAC clones from the UCSF Comprehensive Cancer Center (San Francisco, CA, USA) was used.

DNA was checked for quality in array-CGH experiments using a gene-specific multiplex polymerase chain reaction (PCR) [28]. Female reference DNA of 450 ng (Promega, Mannheim, Germany) and tumor DNA of 450 ng were labeled with Cy3-dCTP and Cy5-dCTP (PerkinElmer, Shelton, USA), respectively, using the BioPrime-Labelling Kit (Invitrogen, Karlsruhe, Germany) overnight at 37°C. One hundred thirty-five micrograms cot-1 DNA (Invitrogen), test, and reference DNAs were cohybridized using a hybridization station (HS400, Tecan, Crailsheim, Germany) for 23 h, washed, and dried. The slides were scanned (GenePix Personal 4100A, Axon Laboratories, Molecular Devices Corp, Chicago, IL, USA) and intensity ratios were measured with an array-analysis software (GenePix Pro 6.0, Axon Laboratories). Analysis was performed using the web-based array-CGH evaluation platform CAPweb [29] which is installed on a local via intranet accessible web server. After import of raw data, the normalization procedure which uses the R-package MANOR was performed using default parameters (exclusion of data points with a replicate deviation of >0.1 and/or a foreground to background signal ratio of <3). Segmentation of the dataset using the R-package GLAD was performed using default parameters with exception of the parameters GLAD.deltaN (set to one time standard deviation of the dataset) and GLAD.forceGL1/2 (set to 1.2 times SD of the dataset). Data have been exported to the array data visualization tool VAMP and analyzed for genomic copy number alterations. Common regions of alterations of the analyzed cases have been determined using the minimal alteration algorithm which is implemented to the VAMP software. Regions representing copy number alterations have been further analyzed using the web-based database Ensembl (www.ensembl.org) on the basis of National Center for Biotechnology Information build 36. For the array-CGH data, we followed the Minimum Information About a Microarray Gene Experiment guidelines and deposited them at the public repository "ArrayExpress" from the European Bioinformatics Institute under the accession number E-TABM-359.

FISH analysis using BAC clones

BAC clones from the 1 Mb BAC array mapping on chromosome 16q within the amplified region (RP11-21B21, RP11-354M24, RP11-533D19) were labeled with biotin or digoxigenin by nick translation according to standard protocols. Hybridization of BAC clones and of a commercially available digoxigenin-labeled human chromosome 16 satellite probe (Chrombios, Raubling, Germany) to paraffin-embedded tissue sections of the six

HNSCC cases carrying a DNA gain on 16q and detection of fluorescent signals were performed as described previously [30].

Screening for human papillomavirus

Extracted DNA from 68 anemic tumors was screened for the presence of human papillomavirus (HPV) of the high (HPV16, HPV18), medium (HPV31, 33), and low (HPV6, 11) risk subtypes according to a published protocol [31] with slight modifications: In brief, 2 μl of DNA were incubated with 4 μl of 10 mM dNTPs, 5 μl PCR buffer containing 15 mM $MgCl_2$, 1 μl of each forward and reverse primer (GP5, GP6, at 20 pM), 0.5 μl Amplitaq Gold, and water added up to a 50-μl reaction volume. The PCR was run with 95°C for 7 min, 40 cycles of 94°C/1 min, 40°C/45 s, 72°C/45 s, and a final step of 72°C for 7 min. Subsequently, 10 μl of the PCR product were run on a 2% agarose gel.

In parallel, a control PCR for β-globin was performed for all 68 anemic DNA samples as described previously [32] and yielded positive signals in 65/68 DNA samples. For the DNA samples with acceptable β-globin-specific (65/68) and a positive HPV-specific PCR signal (four out of 65), the HPV-specific PCR products were purified (PCR Purification Kit, Qiagen) and sequenced. For this, 1 μl of purified PCR product was cycled with 3.2 μl of GP6 or GP5 primer (at 1 μM), 4 μl of 5× buffer, 4 μl of BigDye 1.0 reagent (Appliedbiosystems), and water up to 20 μl, using a PCR program with 96°C for 1 min and 24 cycles of 96°C/10 s, 50°C/5 s, and 60°C/4 min. This was followed by purification of the cycle PCR products and their analysis on a capillary sequencer (ABI310, Appliedbiosystems). Resulting sequences were confirmed by Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST) analysis.

Statistical analysis

The statistical analysis was carried out in three parts: unsupervised cluster analysis of chromosomal changes, correlation tests of chromosomal changes with clinicopathological parameters, and analysis of locoregional progression (LRP)-free survival. Unsupervised hierarchical cluster analysis was done with R-package procedure *hclust* (www.r-project.org), using default methods (Euclidian distance and complete linkage). For cluster analysis, DNA copy number changes were reduced to chromosome arm resolution in order to get more clear arrangements. Gains were coded with 1, losses with −1, and normal regions with 0. For each of the two major top branches of cluster analysis, the frequency of chromosomal changes was compared and characteristic alterations for both clusters were defined, using cutoff values (total occurrence at least 20%, relation between the two clusters at least 3:1 or vice versa).

Potential associations of all chromosomal changes to clinicopathological factors were examined with nonparametric tests such as Jonckheere–Terpstra trend test for tumor size (p_T) and nodal involvement (p_N) and chi-square test for histological grading, localization, resection status, and anemia status [33].

LRP-free survival was analyzed for each chromosomal region which was altered in at least six patients. LRP was assumed when tumor size within the radiation volume increased by more than 25% and time to LRP was defined as the time between end of radiotherapy and locoregional recurrence of the tumor or death whichever was detected first. Patients were marked as censored if they were LRP-free and alive at the last follow-up. LRP-free survival curves were calculated using the Kaplan–Meier method [34]. The differences of the resulting two survival curves between cases with and without a chromosomal alteration were tested with the log-rank test [35]. Additionally, hazard coefficients of chromosomal abnormalities, tumor size (p_T), nodal involvement (p_N), histopathological grading, resection status, anemia status, and tumor localization were calculated using a univariate Cox proportional hazards regression model [36]. The survival-associated chromosomal alterations were also examined for pairwise co-alterations on other chromosomal sites using Fisher's exact test. Statistical significance for chromosomal alterations in the association tests for clinical parameters and in the univariate log-rank survival test was determined according to the Benjamini–Hochberg's false discovery rate (FDR) controlling procedure [37], allowing a FDR of q. If m is the total number of significance tests, p_i is the i-th smallest p value and k is the largest i for which $p_i <= q \times i/m$, then all smaller p values ($p_i <= p_k$) are indicating significance. Finally, chromosomal abnormalities and clinicopathological parameters were subjected together to one multivariate Cox regression model together with a stepwise variable selection procedure. Selected were only variables, chromosomal or clinicopathological, proven to be statistically significant in the univariate analyses.

Results

In total, 117 HNSCC patients were analyzed for chromosomal imbalances by CGH. The CGH results were subjected to cluster analysis to detect tumor groups with similar aberration patterns. This cluster analysis revealed two separated tumor groups (Fig. 2) that correlate significantly with the anemia status of patients (22 anemic cases in cluster 1, 46 anemic cases in cluster 2; $p<0.0001$).

Table 1 summarizes the demographic and outcome data. A subgroup of 65 patients was additionally screened for human papillomavirus subtype 16 to investigate the impact

of infection on patient's survival. No statistical computation was possible as the number of positive cases (four out of 65) was too low.

Correlation of outcome with CGH data and clinicopathological parameters

The clinical parameters staging, resection status, and anemia status showed a significant correlation to LRP (Table 2). In addition, chromosomal abnormalities that occurred in more than five patients were tested for a correlation to LRP-free survival. DNA gains on 1q43 and 16q23–24 as well as DNA loss on 18q22 predict outcome as visualized by Kaplan–Meier graphs (Fig. 1); this stays significant even after Hochberg–Benjamini adjustment (Table 3). Further, after multivariate survival analysis resection and anemia status (hazard ratio 2.3 per stage, $p<0.0001$ and hazard ratio 2.5, $p=0.001$, respectively) and gains on 1q43 (hazard ratio 2.6, $p=0.0076$) and 16q23–24 (hazard ratio 6.0, $p=0.0017$) remain as independent contributions to LRP-free survival.

Chromosomal imbalances in tumor subgroups

Cluster analysis of CGH data revealed two different groups of HNSCC. Cluster 1 consisted of 62 cases and cluster 2 of 55 cases (Fig. 2a). Minimal common regions of alterations were identified in both subgroups (cluster 1:27 DNA gains, 16 high-level amplifications, 22 DNA losses; cluster 2:29 DNA gains, 28 high-level amplifications, 24 DNA losses). DNA copy number changes and high-level amplifications of both tumor groups are summarized in Table 4.

Both subgroups demonstrate a different pattern of chromosomal changes (Fig. 2a), most striking differences were observed for imbalances on chromosomes 1q32 (gain), 1q43 (gain), 3p13 (loss), 3p24 (loss), 4p16 (loss), 4q26 (gain), 7q22 (gain), 9p22 (loss), 11q13 (gain), 13q31 (loss), 16q23–24 (gain), 18q22 (loss), and 21q21 (loss; Fig. 2b). Detailed CGH data from this study will be accessible at www.progenetix.net along with publication of this study. Loss of 16q23–24 was significantly associated with resection status of tumors (5.3% in R0–2, 31.7% in patients with primary definitive radiotherapy, FDR-adjusted $p=0.042$). A similar association with the anemia status of tumors was observed for chromosomal imbalances on chromosomes 1p22–31, 1q22–23, 3q12–13, 4q12–13, 4q24–26, 5q21, 6q13–14, 6q22–24, 1q13, 12q21, 13q21–31, and 19p/q (gains) and 3p13, 3p24, 4p15–16, 4q26–34, 9p22–24, 10p12–14, 11q23–24, 13q31–32, 16q12, 17p, 17q11–21, 18q12–23, 20q, and 21q21 (losses) with FDR-adjusted p values<0.05. None of the tested chromosomal changes were significantly associated with tumor localization, staging, lymph node metastasis, and grading (FDR-adjusted p values>0.05).

Correlations within CGH data

It is of note that co-alterations on 1q43 and 16q23–24 occurred in five cases ($p=0.0002$). We also found co-alterations of gain on 16q23–24 and loss on 3p14 in six cases ($p=0.0039$).

Array-CGH analysis, FISH analysis, and identification of candidate genes on 16q

BAC array analyses of tissues from five patients with gain on 16q verified the DNA gain on 16q24.3 in all cases. The BAC clones RP11-21B21, RP11-354M24, and RP11-533D19 localize to this minimal region of chromosomal gain (Fig. 3). Hybridization of these clones to paraffin-embedded tissue sections, finally, confirmed this observation in individual samples (Fig. 3). Clone RP11-354M24 (*FANCA* gene locus) showed amplified fluorescence in situ hybridization (FISH) signals in 49.4% (case 14452/00), 57% (case 28731/00), 70.6% (case 29783/99), 75.3% (case 44/00), 81.8% (case 24419/98), and 85% (case 8826/01) of tumor cells analyzed. Clones RP11-21B21 and RP11-533D19 demonstrated amplified FISH signals in 75.9% and 73.9% of cells, respectively (case 29783/99). Interestingly, the highly variable FISH signal numbers per cell indicate a distinct genetic heterogeneity within the tumor. Database analysis of these three BAC clones (www.ensembl.org), finally, tagged genes *CDT1*, *CBFA2T3*, *RPL13*, *DPEP1*, *FANCA*, *MCR1*, *TUBB3*, and *GAS8* to be most probably involved in the observed DNA amplification.

Table 2 Significant hazard ratios for clinicopathological parameters determined by univariate regression with the Cox proportional hazards model for locoregional progression-free survival

Parameter (levels)	Number of cases per level	Hazard ratio (per stage)	Log-rank test, degrees of freedom (*df*)	*p* value
Staging (pT0–pT3, pT4)	74/42	1.22	5.84, 1	0.016
Resection status (R0, R1–2, primary definitive radiotherapy)	40/17/59	1.95 per stage	19.9, 2	<0.0001
Nonanemic/anemic	49/68	2.24	9.04	0.0026

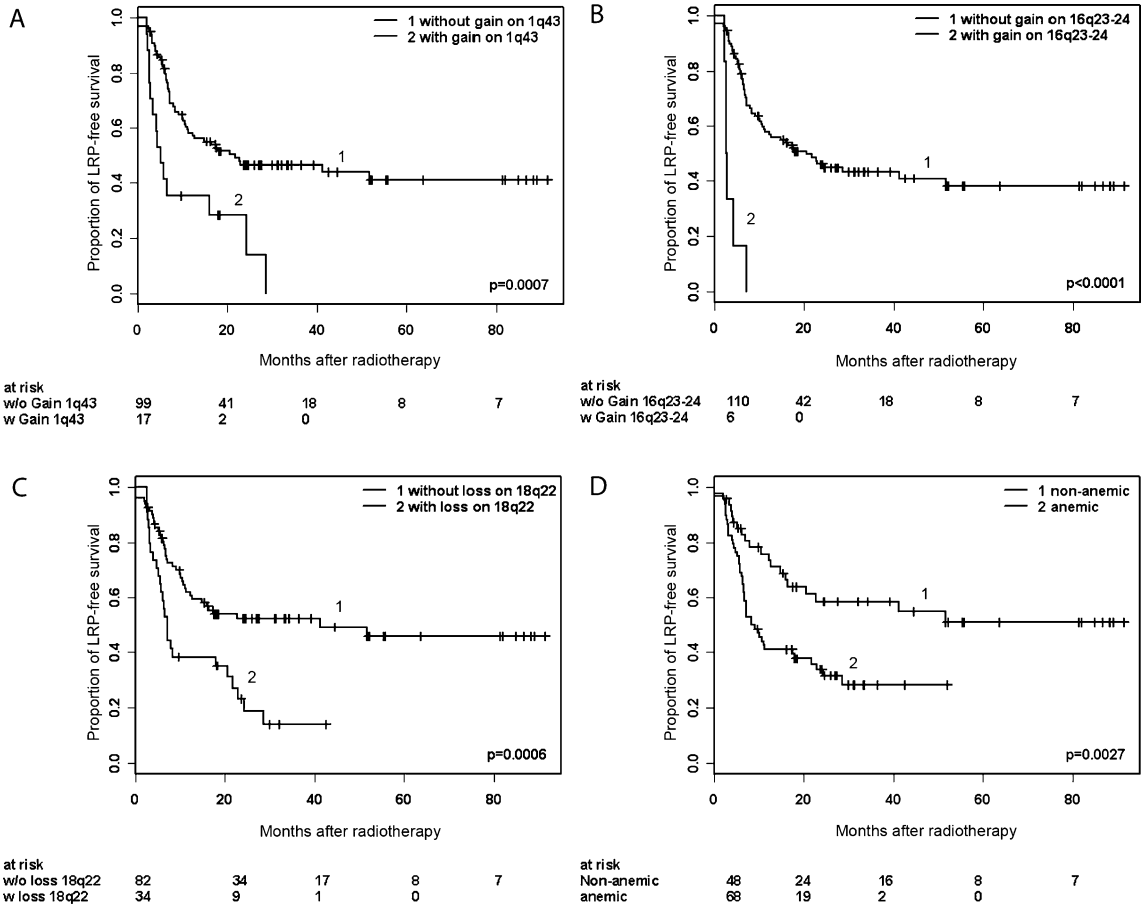

Fig. 1 Impact of gains on chromosome 1q43 (**a**) and 16q23–24 (**b**) as well as loss on chromosome 18q22 (**c**) and of the patient's anemic status (**d**) on LRP-free survival in HNSCC patients. *Ticks* represent censored patients

Discussion

In this study, we describe distinct chromosomal changes of tumor tissue from patients with HNSCC and prognostic markers that predict patients' outcome. We report for the first time that chromosomal imbalances on 1q43, 16q23–24, and 18q22 correlate significantly to LRP-free survival in HNSCC. Further, frequent and recurrent DNA gains on 3q and 11q13 and deletions on 3p, 4p, 9p, and 11q confirm previous reports [16, 19, 38–41]. Moreover, the pattern of chromosomal alterations of this study compares well to available data from the progenetix database ([42]; www.progenetix.net). This demonstrates the reliability of our CGH approaches used which have been performed at two

Table 3 Ten highest hazard ratios, determined by univariate regression with the Cox proportional hazards model for comparison of locoregional progression-free survival in patients with and without chromosomal imbalances

Chromosomal region	Abnormality	Hazard ratio	Log-rank statistic	Unadjusted *p* value	Adjusted *p* value[a]
16q23–24	Gain	8.43	31.1	<0.0001	0.0004[b]
1q43	Gain	2.71	11.59	0.0007	0.048[b]
18q22	Loss	2.32	11.64	0.0006	0.048[b]
1p36.2	Gain	2.24	3.68	0.055	0.74
20p11.2	Loss	2.20	2.41	0.12	0.74
1p21	Gain	2.01	3.17	0.075	0.74
5p15.3	Gain	1.94	3.21	0.073	0.74
10p14	Loss	1.94	3.17	0.075	0.74
15q15	Gain	1.90	2.30	0.13	0.74
9p24	Loss	1.83	3.87	0.049	0.74

[a] According to Benjamini–Hochberg's FDR controlling procedure
[b] Significant (FDR<0.05)

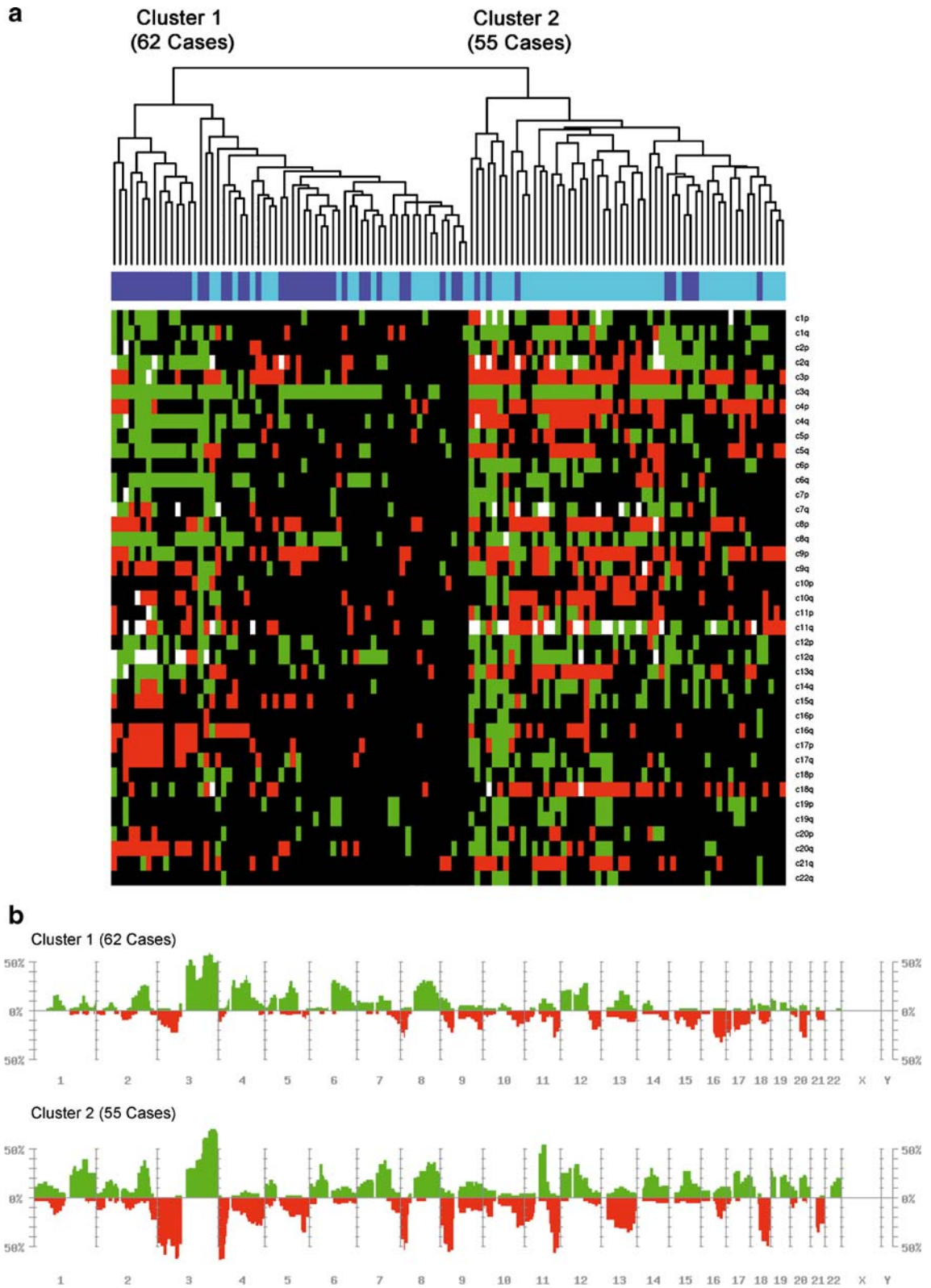

Fig. 2 Dendrogram of hierarchical cluster analysis based on complete linkage and Euclidian distance. *Colored bars* indicate tumors from anemic (*light blue*) and nonanemic (*dark blue*) patients. DNA gains (*green*) and losses (*red*) are arranged by tumor groups. High-level amplifications are indicated in *white* (**a**). Significant differences in the aberration pattern between both groups became apparent (**b**)

Table 4 Minimal common regions of chromosomal imbalances and high-level amplifications

DNA—gains			DNA—losses		
Chromosomal region	Frequency (%)	Tumor site	Chromosomal region	Frequency (%)	Tumor site
Cluster 1 (62 cases)					
1p22–31	16.4	HP/L/OC/OP	2q12–21	8.2	HP/L/OC/OP
1q31	14.8	HP/OC/OP	3p14–21	21.3	HP/L/OC/OP
1q44	8.2	HP/OC/OP			
2p15–16	8.2	HP/OP	7q36	11.5	HP/OC/OP
2q31–33	26.2	HP/L/OC/OP	8p22	26.2	HP/L/OC/OP
2q32–33	**1.6**	**HP**			
3q13.1–13.2	52.5	HP/L/OC/OP	9p13	21.3	HP/L/OC/OP
3q25–27	**21.0**	**HP/L/OC/OP**			
3q25–26.3	59.0	HP/L/OC/OP	9q34	18.0	HP/OC/OP
4q13	31.1	HP/L/OC/OP	10p13–14	4.9	OC/OP
4q26	36.1	HP/OC/OP			
4q28–31.2	**1.6**	**HP**			
5p13	16.4	HP/OC/OP	10q25	14.8	HP/OC/OP
5p13–14	**3.2**	**OC/OP**			
5q21	29.5	HP/OC/OP	11p15	9.8	HP/OC/OP
6q13–14	31.1	HP/L/OC/OP	11q1	9.8	HP/OC/O
6q22–23	24.6	HP/OC/OP	11q23	26.2	HP/OC/OP
7p14–22	9.8	HP/OC/OP	12q24.1–24.2	18.0	HP/L/OC/OP
7p13-pter	**1.6**	**OC**			
7q21	14.8	HP/OC/OP	13q31–33	9.8	HP/L/OC/OP
7q21–22	**4.8**	**HP/OP**			
8q21.1–23	31.1	HP/OC/OP	14q32	8.2	OC/OP
8q12–24.3	**1.6**	**OP**			
9p22–24	13.1	HP/OC/OP	15q13	11.5	HP/L/OC/OP
9p21–24	**1.6**	**OC**	15q23–24	18.0	HP/L/OP
9q21–34	4.9	HP/OC/OP	16q23	31.1	HP/L/OC/OP
9q21–34	**1.6**	**OP**			
10q21	6.6	HP/OP	17p12	24.6	HP/OC/OP
10q22	**3.2**	**OP**			
11q13	11.5	HP/L/OC/OP	17q12–21	19.7	HP/OC/OP
11q13	**4.8**	**HP/OC/OP**			
12p	**1.6**	**HP**	18q21–22	11.5	HP/OC/OP
12q21	27.9	HP/OC/OP	20q12–13.2	27.9	HP/L/OC/OP
13q21	19.7	HP/OC/OP	21q21–22	8.2	HP/OC/OP
14q21	9.8	HP/OP			
15q14–25	**1.6**	**OP**			
18p	**1.6**	**OC**			
18q11.2–12	6.6	HP/OC/OP			
18q11.2–21	**3.2**	**OC/OP**			
19p	11.5	HP/L/OC/OP			
19q	9.8	HP/OC/OP			
20p	**1.6**	**HP**			
20q12	6.6	HP/OC			
Cluster 2 (55 cases)					
1p34.3–36.2	16.1	HP/L/OC/OP	1p22–31	16.1	HP/L/OC/OP
1p34.2–36.1	**10.9**	**HP/L/OC/OP**			
1q31–32	39.3	HP/L/OC/OP	2p23–25	10.7	HP/L/OP
1q32–44	**10.9**	**L/OC/OP**			
2p15–22	17.9	HP/L/OC/OP	2q13–22	10.7	HP/L/OC/OP
2p14–16	**5.5**	**OP**	2q36–37	21.4	HP/L/OC/OP
2q32	37.5	HP/L/OC/OP	3p24	57.1	HP/L/OC/OP
2q24–32	**7.3**	**HP/L**	3p13–14	60.7	HP/L/OC/OP
3q26.1-qter	59.6	HP/L/OC/OP	4p15.3–16	62.5	HP/L/OC/OP

Table 4 (continued)

DNA—gains			DNA—losses		
Chromosomal region	Frequency (%)	Tumor site	Chromosomal region	Frequency (%)	Tumor site
3q24–29	**23.6**	**HP/L/OC/OP**			
4q24–26	7.1	HP/OC	4q32–34	26.8	HP/L/OC/OP
4q12–22	**1.8**	**OP**			
5p14–15.1	17.9	HP/L/OC/OP	5p14–15.1	8.9	HP/OC/OP
5p	**3.6**	**L**			
6p21.3	33.9	HP/L/OC/OP	5q32–34	33.9	HP/L/OC/OP
6p12–21.1	**7.3**	**HP/L/OP**			
6q22–24	10.7	HP/L/OC/OP	7q33–36	14.3	HP/L/OC/OP
6q22–23	**1.8**	**L**			
7p12–15	17.9	HP/L/OC/OP	8p22	51.8	HP/L/OC/OP
7q21–22	37.5	HP/L/OC/OP	9p13–21	53.6	HP/L/OC/OP
7q11.2–31	**10.9**	**HP/L**			
8q21.3–24.1	35.7	HP/L/OC/OP	9q21	14.3	HP/L/OC/OP
8q22–24.2	**10.9**	**HP/L/OC/OP**			
9p	**1.8**	**OP**	10p12–13	17.9	HP/L/OC/OP
9q22-qter	16.1	HP/OC/OP	10q25–26	28.6	HP/OC/OP
9q22–31	**7.3**	**HP/OC/OP**			
10p	8.9	HP/L/OC/OP	11p14–15	23.2	HP/L/OC/OP
10p	**3.6**	**OC/OP**			
11q13	53.6	HP/L/OC/OP	11q23–24	55.4	HP/L/OC/OP
11q13	**30.9**	**HP/L/OC/OP**			
12p11.2	30.4	HP/OC/OP	12q24.2–24.3	7.1	HP/OC/OP
12p	**5.5**	**HP/OC**			
12q13	33.9	HP/L/OC/OP	13q31–32	33.9	HP/L/OC/OP
12q13–21	**9.1**	**HP/OC**			
13q14–22	10.7	HP/OC/OP	16q22–23	19.6	HP/OC/OP
13q12–21	**1.8**	**HP**			
14q22	30.4	HP/L/OC/OP	17p12–13	5.4	HP/OC/OP
14q11.2–13	**7.4**	**HP/L/OC/OP**			
15q21–22	26.8	HP/L/OC/OP	18q22	48.2	HP/L/OC/OP
15q	**3.6**	**L/OC**			
16p	**3.6**	**OC/OP**	21q21	33.9	HP/L/OC/OP
16q22-qter	8.9	HP/L/OC/OP			
16q	**3.6**	**L/OP**			
17p12–13	10.7	HP/L/OC/OP			
17p	**5.5**	**OC/OP**			
Cluster 2 (55 cases)					
17q11.2–21	26.8	HP/L/OC/OP			
17q25	23.2	HP/L/OC/OP			
17q23–25	**7.3**	**OC/OP**			
18p11.3	21.4	HP/OC/OP			
19p13.3	30.4	HP/L/OC/OP			
19q	23.2	HP/L/OC/OP			
19q	**1.8**	**OP**			
20p	**1.8**	**L**			
20q12–13.2	23.2	HP/L/OC/OP			
20q	**1.8**	**OP**			
22q13	19.6	HP/L/OC/OP			
22q12–13	**3.6**	**OC/OP**			

Bold—high-level amplifications
OC Oral cavity, *OP* oropharynx, *L* larynx, *HP* hypopharynx

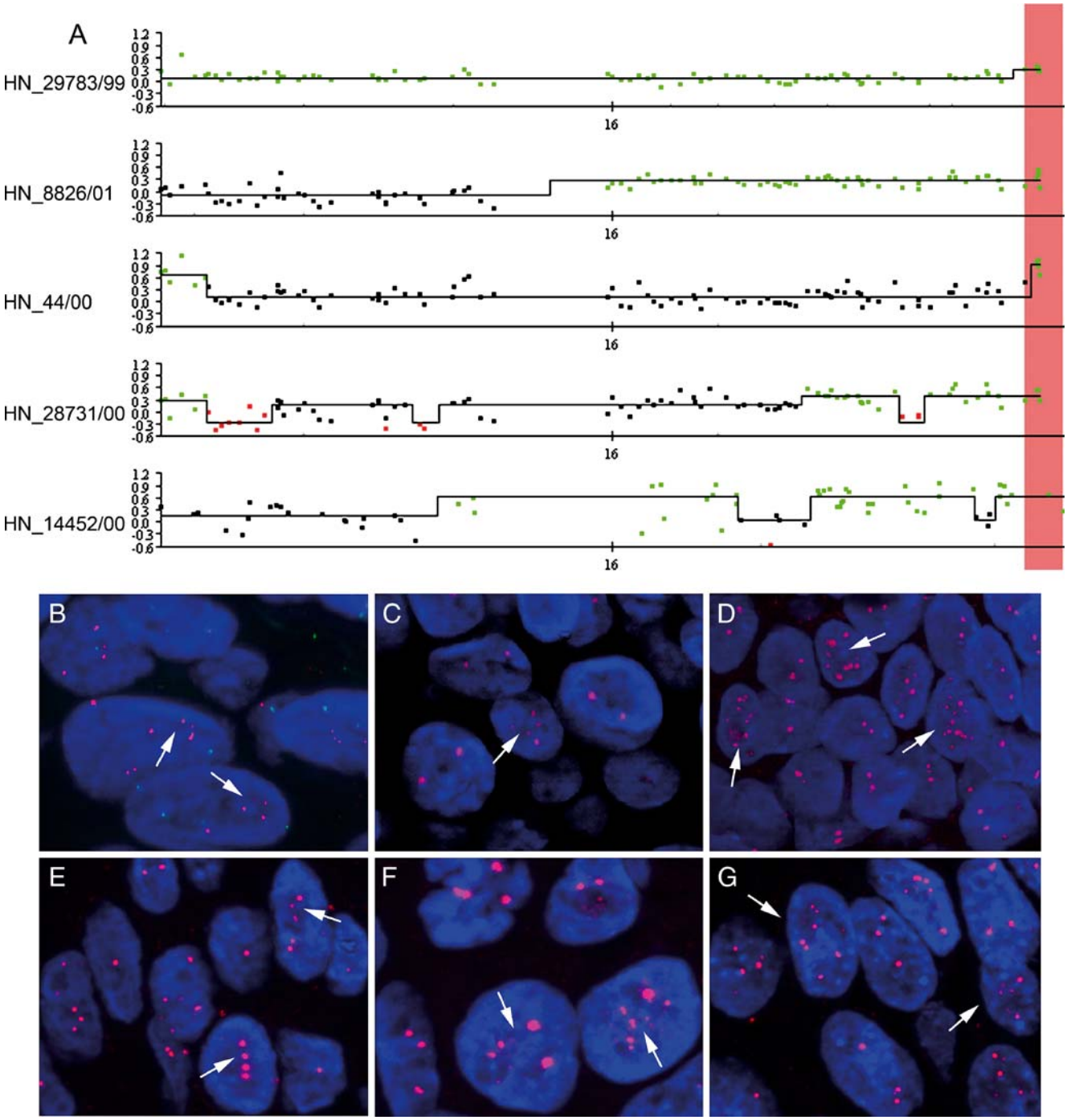

Fig. 3 Array-CGH and FISH on 16q23–24. **a** Array-CGH profiles displaying a minimal amplified region on 16q24.3 (*red bar*); *dots*—log2-ratios of BAC clones indicating no alteration (*black*), deletion (*red*), DNA gain (*green*). *FANCA* FISH analysis with clone RP11-354M24 (*red signals*, *arrows* if amplified) and chromosome 16 centromeric region (*signals in green*) for anemic HNSCC cases 29783/99 (**b**), 28731/00 (**c**), 14452/00 (**d**), 44/00 (**e**), 8826/01 (**f**), 24419/98 (**g**)

different resolution levels (5 to 10 Mb for conventional CGH and approximately 1 Mb for array-CGH on selected cases). We also validated exemplary array-CGH findings by FISH on FFPE sections.

It is noteworthy that two subgroups of HNSCC could be identified from our CGH data by cluster analysis. Characteristic changes can be attributed to each tumor group which suggests that different routes of tumor progression exist. Interestingly, these two tumor groups also correlate with the anemia status of patients indicating a specific aberration pattern, if anemia has developed. This observation of distinct genetic changes in anemic HNSCC patients

suggests that particular chromosomal aberration patterns favor a more malignant phenotype. Thus, we can imagine anemia to bring about tissue hypoxia that, on the other hand, may promote progressing genetic changes and select a more malignant and treatment resistant progeny [43]. This interpretation is also supported by the highly significant association between LRP-free survival and the anemic status of patients (Table 2).

Among the above mentioned prognostic markers, we considered the DNA gain on 16q23–24 as most relevant because (1) it shows the most significant effect on survival (Table 2), (2) it could be confirmed in multivariate testing, and (3) it appeared as a co-alteration with 1q43 gain (a second prognostic marker in this study) and 3p14 loss (a common alteration in squamous cell cancers) suggesting a strong impact in tumor development. For these reasons, we decided to investigate the 16q23–24 gain further for the involvement of particular candidate genes.

It became obvious from the array-based analyses that 16q24.3 harbors *FANCA*, a key regulator of the Fanconi anemia (FA)/breast cancer (BRCA) pathway controlling homology-directed DNA repair [44]. FANCA cooperates with FANCC, FANCE, FANCF, FANCG, and BRCA1 and BRCA2, RAD51, and the MRE11/RAD50/NBS1 complex. Large deletions on 3p, 9q, 11q, and 13q, respectively, suggest FANCD2, FANCC, MRE11, BRCA2/FANCD1 to be additionally involved (Table 4). Furthermore, array-CGH analysis revealed amplified regions on 5q and 17q (data not shown) mapping for candidate genes RAD50 and BRCA1 which are also part of the FA/BRCA pathway. To validate the significance of changes along the FA/BRCA pathway in HNSCC, more detailed analyses at mRNA and protein expression level are essential. The reported findings from this study were obtained from a small subunit of tumors. Therefore, a larger number of cases must be investigated for confirmation of these changes which require a FA/BRCA pathway-specific array or an appropriate multiplex ligation-dependent probe amplification PCR approach.

The other prognostic markers of poor survival, gain on 1q43 and loss on 18q22, also harbor tumor-related candidate genes that might affect the malignancy of tumor cells. There are several genes located on 18q22 (cadherin 7, cadherin 19, and *DNAM-1* (*CD226*)) which mediate cell–cell adhesion and thus could influence tumor invasion and metastasis [45, 46]. Also the suppressor of cytokine signaling (*SOCS6*) is affected by the deletion which presumably influences the Janus kinase/signal transducers and activators of transcription cascade and thus regulation processes of signal transduction and cell growth [47]. The amplified region on 1q43 contains galectin 8 which is implicated in processes such as development, differentiation, cell–cell adhesion, and growth regulation [48]. Within the same region also exonuclease 1 and *MTR-5* are located, which are both implicated with increased risk for colon cancer and HNSCC, respectively [49, 50].

In conclusion, chromosomal imbalances discriminate subgroups of HNSCC that correlate to the anemic status of patients. Chromosomal gains on 1q and 16q as well as loss on 18q confer to some extent to the particular poor prognosis of these patients. It is likely that anemia promotes malignant progression by the accumulation of genetic changes and the selection of a treatment-resistant phenotype.

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