Prognostic significance of mitotic and apoptotic index and the DNA cytometry in head and neck cancer*

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The lack of suitable criteria to predict the response to chemo- and or radiotherapy for individual patients with squamous cell carcinoma of the head and neck (HNSCC) remains still a major problem. This study was conducted to analyze prognostic significance of mitotic and apoptotic index and the DNA flow cytometric analysis of HNSCC to the recurrence-free survival time and to the overall survival. The analysis was carried out in a set of 56 patients suffering from carcinoma of the pharynx and supraglottis. Most patients (96.7%) underwent neoadjuvant chemotherapy, followed by surgery and postoperative irradiation. Besides routine examinations, flow cytometric analysis was performed, as well as p53 and Ki-67 markers and mitotic and apoptotic index were established by means of immunohistochemistry. Event-free survival (EFS) and overall survival (OS) were accepted as primary endpoints for the prognostic analyses. All the examined potential markers entered standard Kaplan-Meier survival analysis and Cox regression modeling. Statistical significance of prognostic factors was first examined in univariate models and all the parameters subsequently entered multivariate models.

The analyses revealed significant prognostic position of advanced clinical stage (III+IV) and increased proliferative activity as primary risk factors ($p<0.01$) that typically positively correlate with increased mitotic activity and G2/M cell fraction. Better survival results obtained for grade 3–4 as compared to grade 1–2 were caused by molecular parameters that make these samples similar to less risk cases. Cytokinetic parameters and proliferation activity were found as important predictors of the second level (after recognizing stage, grade and DNA status of the tumor). Multivariate combination of these markers contributed namely to the prognosis of early risk event: a ratio S phase cell fraction/G2M cell fraction was found to be the key prognostic factor ($p<0.01$). Early risk events are associated with increased mitotic activity, decreased apoptic rate, decreased S phase cell fraction and significantly increased G2/M fraction.

Key words: head and neck cancer, risk prognosis, mitotic/apoptotic index, DNA cytometry

5-years survival in operable advanced forms of the Waldeyer's ring carcinomas is given in 30–40%, despite radical surgical treatment and postoperative radiotherapy [14]. One of the possibilities how to improve the cure rate of patients with the head and neck carcinoma is to find suitable predictors of the tumor development. If a patient can be classified into a group with better prognosis, a suitable, less radical method of therapy can be chosen, which means for a patient the improved life quality with maintaining of the survival period. However, the clinical applicability and prognostic importance of many predictive biomarkers have been unclear; sometimes the results of trials are highly controversial.

The purpose of this study was to analyze the prognostic significance of p53, Ki-67, mitotic and apoptotic index and the DNA flow cytometric analysis of head and neck cancer to the recurrence-free survival time and to the overall survival.

Material and method

The analysis was carried out in a set of 56 patients suffering from carcinoma of the pharynx and supraglottis treated within 1991–1999. Most patients (96.7%) underwent neoadjuvant chemotherapy, followed by surgery and postopera-

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tive irradiation. Besides routine examinations, there were performed: flow cytometric analysis of DNA, immunohistochemical assessment of p53 and Ki-67 markers, mitotic and apoptotic rate (%) and ratio mitosis/apoptosis. Representative samples were taken from tumors tissue and processed using standard formol-paraffin technique. 50-microns thick sections determined for flow cytometry and 5-micrometers thick sections for immunohistochemical staining determination of the cell proliferation were prepared from the representative blocks. The standard sections were stained with routine hematoxylin-eosin. The histologic typing and grading of squamous carcinomas was made according to the degree of differentiation of the tumor (the Broders histopathological grading) as follows: G1, well differentiated; G2, moderately well differentiated; G3, poorly differentiated; and G4, undifferentiated.

Other sections were used for the immunohistochemical establishing of an antibody against Ki-67. Ki-67 labeling index (proliferation index) was assessed as a percentage of positive out of 1000 neoplastic nuclei, while the parts of preparations with the most conspicuous expression ("hot spots") were selected. The second parameter was the expression of protein p53 evaluated as binary code according to threshold value 10%. Cases with expression less then 10% neoplastic cells were classified as p53 negative, remaining as p53 positive. The cases with evidently higher or lower expression than the threshold 10% of neoplastic cells, were evaluated without using morphometry. In questionable cases nearby the threshold value, the percentage of positive nuclei was calculated again in at least 1000 tumor cells. In both cases, the original enlargement of the microscope was 400x, cellularity and the number of positive nuclei were established by means of system image analysis LUCIA G. A method in Hedley's modification was applied for the DNA flow cytometric analysis. According to this method, suspension of cell nuclei was obtained. After the lavage and centrifugation, the nuclei were re-suspended in a staining solution of propidium iodide (50 microl/ml, Sigma Co.) for the total volume of 200 microlitres of solution. About 10,000 nuclei were analysed. Measurements were carried out with EPICS Profile flow cytometer (Coulter Electronics, USA). DNA histograms were interpreted according to the criteria accepted conventionally.

Standard descriptive statistics were used to express differences among subgroups of cases (median and MIN/MAX values; arithmetic mean supplied with 95% confidence limits or relative frequencies). Common univariate statistical techniques were used to test differences between chosen subgroups of patients: Fisher exact test for binary outcomes, ML chi-square test for ordinal categorical variables, Mann-Whitney U test for non-normally distributed continuous variables and one-way ANOVA technique for normally distributed continuous variables. A value $p<0.05$ was taken as a universal indicative limit for statistical significance in all analyses. Three principal endpoints were defined and used in all the analyses (1) event-free survival (EFS), (2) overall survival (OS), (3) early risk event coded as binary variable for cases with relapse/progression till 6th month of follow-up, with subsequent death. For statistical analysis, the cases were separated into two groups according to the histopathological grade: better differentiated tumors (G1–2) and worse differentiated tumors (G3–4). Examined cohort of patients was observed for sufficient follow-up period that allowed exact evaluation of at least 5-yr survival. For that reason, the cases were separated into three groups according to the risk of disease development: group R1: highly risk development with early event till 6th month of follow-up and subsequent early death, group R2: medium risk group with risk event in the follow-up period from 6–60 month, group R3: long-term survivors with event free interval exceeding 60th months, no death event was detected in this group. Stratified Kaplan-Meier product-limit method was applied to discriminate survival rates between two or more subgroups given by potential predictors. Peto-Prentice generalized log-rank test was used as comparative statistical test. All the potential predictors associated probability with patients at risk (according to study endpoints) were examined separately by univariate Cox regression models (for time-to event endpoints: EFS, OS) and relative risk ratio with 95% confidence limits was estimated. Potential influence of risk factors on early risk event was examined by logistic regression models. Odds ratio with 95% confidence limits was estimated.

Results

Basic characteristic of the cohort is given in Table 1. Patients were divided into two basic groups according to localization of primary tumor. Patients with cancer of nasopharynx and the lateral wall of the oropharynx (n=20; 35.7%) were assigned to the first group, patients with cancer of the tongue root, supraglottis and cancer of hypopharynx (n=36; 64.3%) were assigned to the second group. The investigated tumors were mostly advanced (stage III–IV in 83.9%). Median EFS was 18.8 months, median OS was 51.3 months. Summary statistics of apoptotic rate, mitotic rate, prolipheration index, cell phase fractions, Ki-67, ploidy and p53 positivity are displayed in Table 1. Full spectrum of common clinical stratification criteria was included (T-N-M categories, clinical stage, grade) and supplied with molecular and cytokinetic markers as additional set of potential predictors. All important factors including p53 status, ploidy and cytokinetic parameters provided sample distribution suitable for predictive analyses, i.e. symmetric occurrence of values in the whole range and sufficient incidence of positive findings (Tab. 1). Tumors with grade 3–4 were characterized with higher share of aneuploid cells (without statistical significance).

Patients were followed for sufficient time and available data allowed exact analysis of long-term survival including stratification of patients according to risk factors (Fig. 1).

Table 1. Basic characteristics of the cohort

Parameter[1]	Initial values (n= 56)
Patients and disease	
Sex	Men: n = 42 (75.0%); Women: n = 14 (25.0%)
Age (years)	52 (32; 71)
Follow-up time (months)	106 (73; 152)
Tumour localization	
Nasopharynx, oropharynx (C090,C091, C099, C102, C110-113)	n = 20 (35.7%)
Tongue root, suppraglottis (C01, C019, C021, C024, C020, C321, C329)	n = 30 (53.6%)
Hypopharynx (C129, C133, C139)	n = 6 (10.7%)
Clinical stage	
I – II	n = 9 (16.1%)
III – IV	n = 47 (83.9%)
T categories	
T1	n = 7 (12.5%)
T2	n = 18 (32.1%)
T3	n = 19 (33.9%)
T4	n = 12 (21.4%)
N categories	
N0	n = 13 (23.2%)
N1	n = 17 (30.4%)
N2	n = 17 (30.4%)
N3	n = 9 (16.0%)
Grade	
1 – 2	n = 40 (71.4%)
3 – 4	n = 16 (28.6%)
Therapeutic results[2]	
Median event-free survival	18.8 months
Median overall survival	51.3 months
No. of events (relapse, disease progression)	n = 44
No. of death events	n = 41
Examined cytokinetic parameters	
Apoptic rate (A;%)	0.54 (0.07; 1.8)
Mitotic rate (M;%)	1.14 (0.28; 2.03)
Prolipheration index (PI;%)	7.9 (2.4; 21.6)
Cell phase fractions (%)	
Go/G1	92.1 (78.4; 97.6)
S phase fraction	4.8 (1.4; 16.9)
G2/M	3.0 (0.5; 15.7)
KI-67 (%)	41.8 (2.3; 78.5)
Aneuploidy	n = 19 (33.9%)
DNA index (n = 19 aneuploid samples)	1.42 (1.14; 1.97)
p53 positivity	n = 28 (50.0%)

[1] Continuous parameters are characterized by median and MIN/MAX values (in brackets). Discrete parameters are characterized by sample (n) and relative occurrence (in %) that refer to the given category

Stratified analyses of event-free survival provided very similar outputs as analyses of overall survival only with necessarily lower median of survival time. That is why, only event free survival and early risk event were used as principal endpoints for predictive risk analyses, as summarized in Table 2. Comparing tumor characteristics, T categories (size of tumor) were found to be more predictive in relation to survival events than clinical stage system. Although relative risk logically increases with increasing stage of tumor, statistical significance for this influence was not proved. Similarly, relative risk is increased for risk tumor localization (tongue root – supraglottis, hypopharynx), but again without statistical significance (Tab. 2).

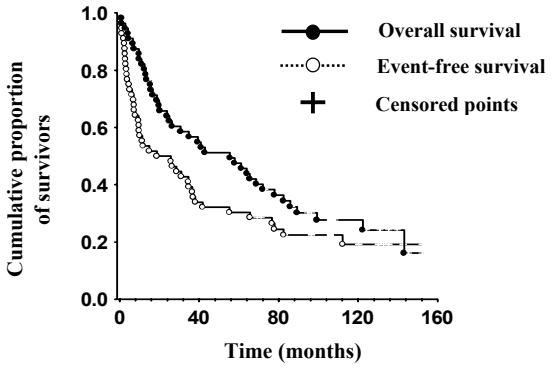


Figure 1. Reached therapeutic results: overall survival (OS) and event-free survival (EFS) in Kaplan-Meier curves (n=60).

None of the factors examined in univariate risk analyses (Tab. 2) contribute significantly to the prediction of early risk events, although odds ratio indicated increased relative risk in association to this endpoint. Also survival analyses depicted in figures 2–4 confirmed decreased capability of tumor size, ploidy and p53 status to separate survival curves in the early phase of development. We can conclude that risk development of the disease during first 6 months appears to be independent on common clinical categories, namely staging and localization, and thus represents specific problem masking overall predictive power of these factors. That is why an additional complex set of molecular and cytokinetic markers was included in the analyses.

Special attention had to be paid to grade degree of tumors due to unexpected influence on survival development, in the case of less advanced tumors (T categories 1–2). Tumors with grade 1–2 revealed significantly better survival than tumors with grade 3–4 within this group. This pattern was not significantly apparent in more advanced disease status, because there is relatively low space for other predictive factors like grade (Tab. 2, Fig. 3). Although rather controversial impression seems due to better prognosis for grade 3–4, grade categories significantly separated survival curves also in the region of early risk events (Fig. 3). This indicates that parameters associated with grade could contribute to the prognosis of early risk development of the disease.

Molecular markers were related to the separated risk categories of patients (R1–R3) and provided significant separation of early risk group (R1) from the others (Fig. 5). Early risk events are associated with significantly increased mitotic activity (M) and decreased apoptotic rate (A). Relative ratio of M/A was found to be more conclusive than mitotic rate itself. Early risk cases were further characterized by decreased S phase cell fraction and significantly increased G2/M fraction, which again indicated increased proliferation activity.

The results displayed in figure 5 confirmed the key role of

molecular and cytokinetic markers in the prognosis of the disease development, namely in early risk phases where most of the common clinical characteristics failed (see also Tab. 2). Furthermore, analyses of the molecular factors explained better prognosis reached in cases with high grade tumors. The stratification according to combined grade degree and ploidy (both potentially prognostic factors) led to significantly changed profiles of cytokinetic parameters as it is shown in Figure 6. Diploid tumor samples are similar in proliferation markers both in low grade and high grade cases. Diploid tumors of grade 3–4 further revealed increased S phase cell fraction and decreased proportion of G2/M phase in cell cycle, i.e. both factors with positive prognosis for disease development (see also Fig. 5). Better survival results obtained for grade 3–4 as compared to grade 1–2 were therefore highly probably caused by molecular parameters that made these samples similar to less risk cases.

The molecular markers significantly separated aneuploid tumors with grade 3–4 as the most important risk category, with increased mitotic activity and very significantly reduced apoptic rate and S phase cell fraction of cell cycle (Fig. 6).

Discussion

Prognostic factors of HNSCC have been discussed in several reports. Identification of aggressive biological behavior and the tendency to metastasize is very important in the management of treatment choice [2, 22, 24, 26]. The prognosis of the head and neck tumors depends, besides others, on the following: age, sex, smoking, risk factors, state of nutrition, immunological capability, response to therapy, primary location of a tumor, TNM stage, histological differentiation, lymphatic invasion, perineural invasion, metastases into lymph nodes, extra capsular spreading of a tumor, tumor angiogenesis and predictive biomarkers as well [13, 25].

TNM classification is of high prognostic value but it is bur-

Table 2. Relative risk associated with common clinical categories that defines disease status

Parameter	Predefined endpoint					
	Event-free survival (EFS)[1]			Early risk event[2]		
	Relative risk (95% CI)	p value	Median EFS (months)	Odds ratio (95% CI)	p value	Early risk event (occurrence in %)
Clinical stage						
I-II	0.88 (0.39; 1.99)	0.749	65.6	0.61 (0.13; 2.79)	0.507	17.6 %
III	1.35 (0.68; 2.69)	0.405	27.5	1.47 (0.80; 2.51)	0.133	38.5 %
IV	1.40 (0.89; 2.86)	0.642	10.1	1.03 (0.88; 1.20)	0.395	33.3 %
Tumor localization						
Nasopharynx, oropharynx	0.82 (0.55; 1.41)	0.513	13.4	0.86 (0.24; 3.11)	0.821	20.0 %
Tongue root, suppraglottis, hypopharynx	1.23 (0.66; 2.27)		36.8	1.15 (0.32; 4.13)		27.8 %
Grade(G) x tumor size (T)						
T (1-2) + G (1-2)	1.41 (1.01; 2.63)	0.034	26.4	0.78 (0.20; 3.02)	0.714	23.5 %
T (1-2) + G (3-4)	0.37 (0.14; 0.95)	0.019	106.1	0.29 (0.03; 2.71)	0.212	11.1 %
T (3-4) + G (1-2)	1.60 (1.07; 3.16)	0.013	9.5	1.98 (0.58; 6.73)	0.261	34.8 %
T (3-4) + G (3-4)	1.08 (0.45; 1.81)	0.423	34.8	1.11 (0.18; 6.69)	0.909	28.6 %
Ploidy						
Aneuploidic tumors	1.88 (1.05; 3.55)	0.031	Aneuploid: 9.7 Diploid: 29.8	2.25 (0.72; 5.12)	0.208	Aneuploid: 31.6% Diploid: 20.6%
Grade (G) x Ploidy categories						
G(1-2) + diploid	0.85 (0.45; 1.25)	0.406	26.2	0.89 (0.26; 1.89)	0.451	20.0 %
G(1-2) + aneuploid	1.65 (0.83; 3.28)	0.176	9.7	1.50 (0.45; 4.15)	0.269	30.0 %
G(3-4) + diploid	0.15 (0.04; 0.49)	0.021	96.2	0.32 (0.09; 0.75)	0.012	0.0 %
G(3-4) + aneuploid	1.78 (1.02; 3.26)	0.046	11.3	2.31 1.19; 5.89)	0.048	42.9 %
Status in p53						
p53 positivity	1.72 (0.95; 2.51)	0.071	p53 positive: 13.2 p53 negative: 32.7	1.21 (0.45; 3.55)	0.756	p53 positive: 28.7% p53 negative: 21.4%

[1] Relative risk estimated from univariate Cox regression models

[2] Early risk event was defined as relapse or disease progression that occurred till 6th month of follow-up. Early risk events were coded as binary dependent variable for univariate logistic regression models.

dened, to a certain extent, with subjective application of the classification guidelines. Result of our analysis is consistent with generally accepted predictive value of TNM categories.

Our results seem to be partly in controversy with the results of Broders grading. As for histopathological grading, in most cases, the tumor has the more malignant character, it differs most from mother tissue [23, 32]. Broders initiated quantitative grading of carcinomas with a system based on the proportion of highly differentiated cells within the entire tumor. The Broders classification ignores tissue atypia and thus lost part of its impact. Differentiation, especially on the cell level is further more poor and a subjectively defined term and therefore it is not very reproducible [23]. The traditional grading system of Broders is still a widely used method, but it has consistently proven unsatisfactory in predicting survival and prognosis [11, 31]. Studies have demonstrated a high degree of intra- and inter-observer differences in the histologic grading of a tumor. Pathologists tend to evaluate most of the head and neck cancers as moderately differentiated and a number of recent studies find that patient survival is poorly related to conventional histologic grading [13]. For better results there is morphologic tumor front grading, which analyzes morphologic features in the most invasive zones of the tumor. BRYNE et al compared Broders's method of grading with a modification of malignancy grading system recommended by Anneroth on 68 biopsy specimens of oral squamous cell carcinomas. The univariate survival analysis shows the new malignancy grading to be the only significant prognostic factors ($p<0.05$), whereas Broders' grade and size of tumor were of no prognostic value. A reported lack of correlation between Broders' grade and the prognosis of HNSCC has been explained by the fact that HNSCC usually exhibit a heterogenous cell population with probable differences in invasive and metastatic behavior [4]. Microscopic features of tumors are only the indirect reflection of their biological behavior. ALBUQUERQUE et al did not find correlation between c-erbB-2 positivity and histological malignancy grading [1]. Quantitative characteristics of the tumor are directly related to the growth and metastatic potential and allow far more precise estimation of the tumor behavior. Better survival results obtained for grade 3–4 as compared to grade 1–2 were apparently caused by molecular parameters that make these samples similar to less risk cases. The degree of the tumor cell growth is influenced particularly by the balance between cellular proliferation and apoptosis [6, 29, 30]. That is why "predictive biomarkers" have been studied intensively [7, 8, 9]. P53 overexpression was found as an independent prognostic factor (together with tumor bulk) for local control in early-stage glottic cancer treated with curative radiotherapy [18, 28]. Result of our analysis is consistent with predictive value of proliferative and apoptotic rate of tumor cells. SILVESTRI et al found out direct relation between density of immunoreaction Ki67/p53 and prognosis. Some studies show that the proliferation rate is better indicator of tumor aggressiveness than tumor stage. No correlation was found in their study between the proliferation rate and tumor stage; however, all recurrent tumors showed high proliferation scores [27].

The content of DNA, estimated by means of flow cytometry, seems to be another important independent prognostic factor [5, 31]. In HNSCC, there is aneuploidy in

Table 3. Three groups of patients categorized according to risk development and their structure[1]

Parameter and T/G categories	Group R1 (n = 14)	Group R2 (n = 24)	Group R3 (n = 18)	p level [2]
	– % calculated within columns –			
Clinical stage				
I-II	17.6 %	50.0 %	32.4 %	
III	38.5 %	46.2 %	15.4 %	0.093
IV	33.3 %	11.1 %	55.6 %	
Tumor size (T)				
T(1-2)	19.2 %	38.5 %	42.3 %	0.297
T(3-4)	30.0 %	46.7 %	23.3 %	
Tumor localization				
Nasopharynx, oropharynx	20.0 %	35.0 %	45.0 %	0.313
Tongue root, suppraglottis, hypopharynx	27.8 %	47.2 %	25.0 %	
Tumor grade (G)				
G(1-2)	27.5 %	50.0 %	22.5 %	0.042
G(3-4)	18.8 %	25.0 %	56.3 %	
T x G categories				
T(1-2) + G(1–2)	23.5 %	47.1 %	29.4 %	
T(1-2) + G(3–4)	11.1 %	22.2 %	66.7 %	0.251
T(3-4) + G(1–2)	34.8 %	52.2 %	17.4 %	
T(3-4) + G(3–4)	28.6 %	28.6 %	42.9 %	
Ploidy				
Diploid cases	20.6 %	44.1 %	35.3 %	0.475
Aneuploid cases	31.6 %	47.4 %	21.0 %	
G x ploidy categories				
G(1-2) + diploid	20.0 %	56.0 %	24.0 %	
G(1-2) + aneuploid	30.0 %	50.0 %	20.0 %	p = 0.04
G(3-4) + diploid	0.0 %	11.1 %	88.9 %	
G(3-4) + aneuploid	42.9 %	42.9 %	14.3 %	
p53 status				
p53 positive cases	21.4 %	32.1 %	46.4 %	0.168
p53 negative cases	28.6 %	53.6 %	17.8 %	

[1]Group R1: highly risk development with early event till 6th month of follow-up and subsequent early death. Group R2: medium risk group without early risk events but with risk event in the follow-up period from 6 – 60 month. Group R3: long-term survivors with disease free interval exceeding 60th months, no death event was detected in this group.

[2]Significance level of M-L Pearson Chi-square test that was applied to detect significant association between specified parameter and risk groups.

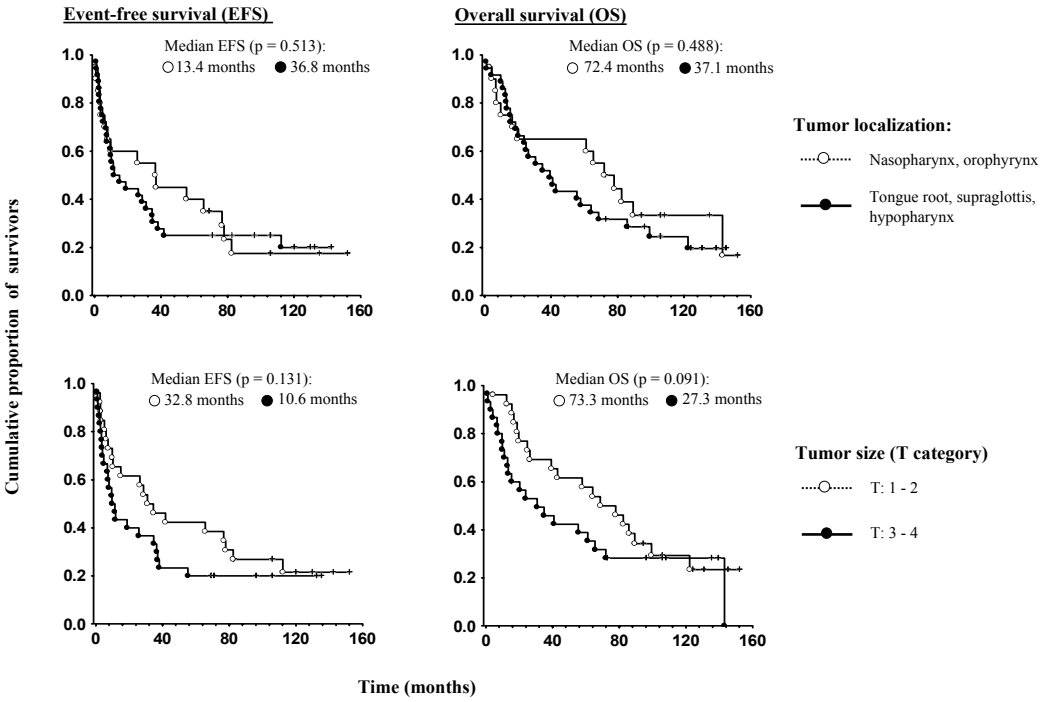
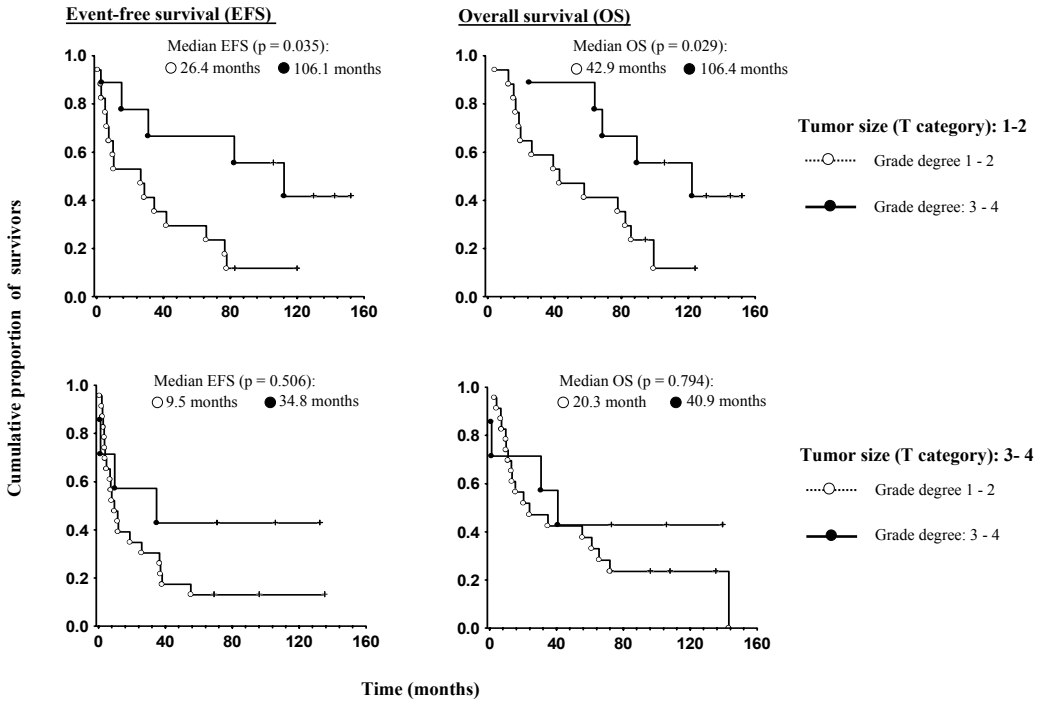


Figure 2. Survival of patients stratified according to tumor localization and tumor size (T category). P values refer to the log-rank test that was applied for comparison of stratified variants.



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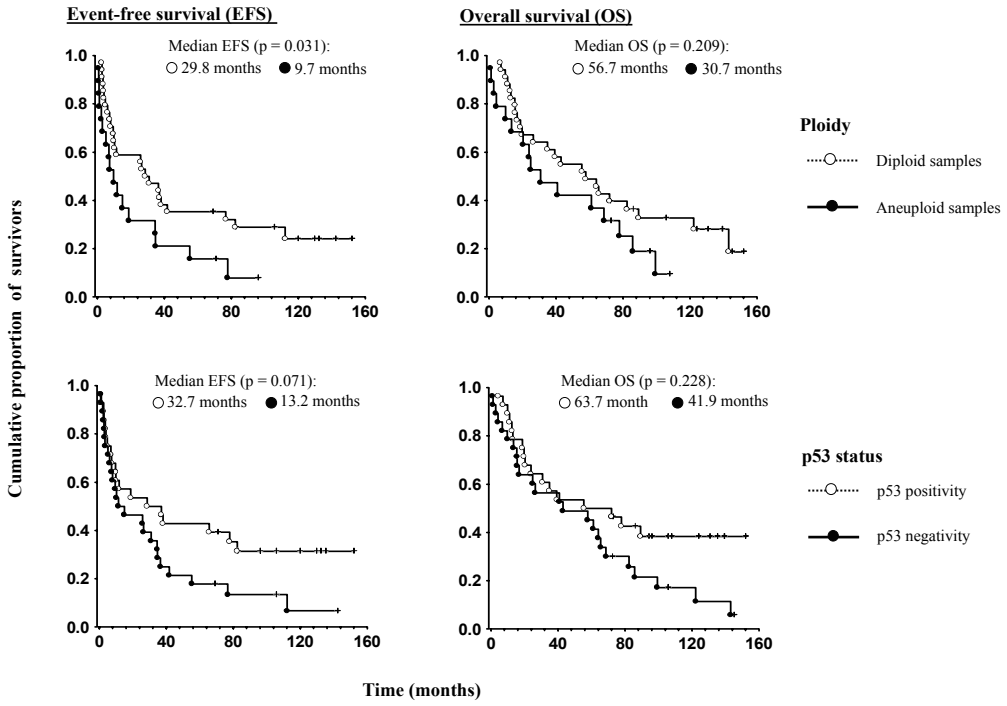
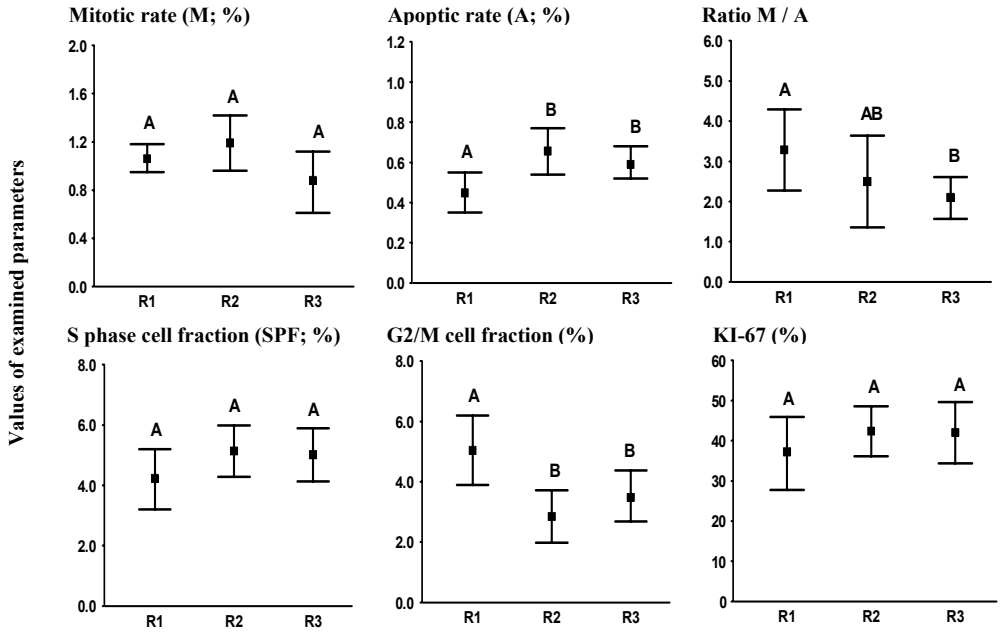


Figure 4. Survival of patients stratified according to tumor ploidy and p53 status. P values refer to the log-rank test that was applied for comparison of stratified variants.



Group R1: highly risk development with early event till 6th month of follow-up and subsequent early death.**Group R2:** medium risk group without early risk events but with risk event in the follow-up period from 6 – 60 month. **Group R3:** long-term survivors with disease free interval exceeding 60th months, no death event was detected in this group.

Figure 5. Cytokinetic parameters as related to the risk categories of patients (R1–3).
Summary statistics expressed as arithmetic means (points) with 95% confidence intervals (whiskers).
A/AB/B: marks of statistical significance of differences among G/T categories (ANOVA). Categories G/T marked by the same letter are not significantly different ($p<0.05$).

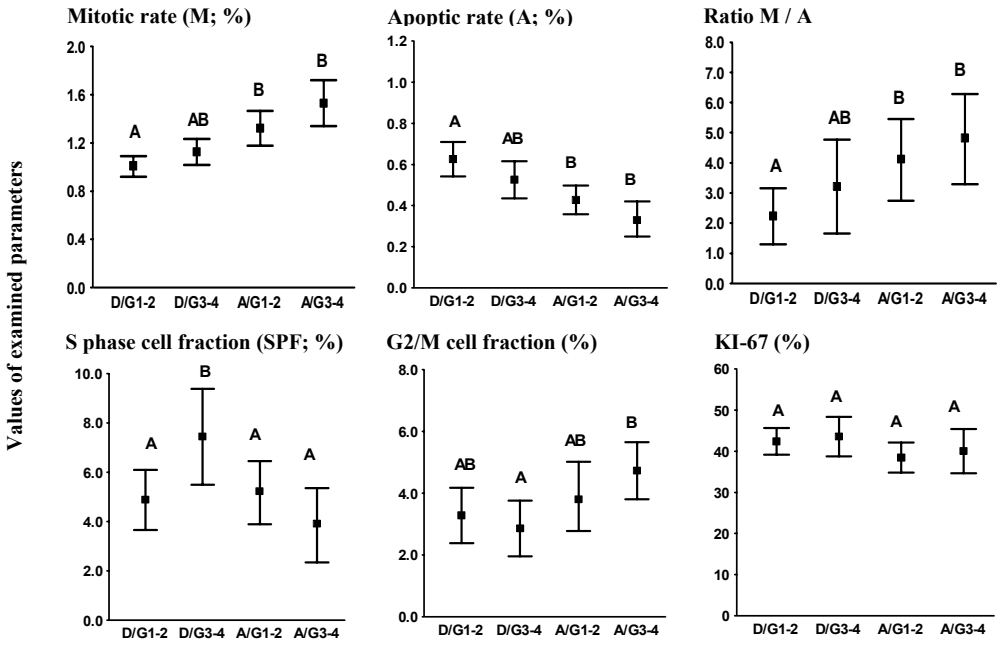


Figure 6. Cytokinetic parameters as related to the categories given by tumor grade (G1–4) and ploidy (diploid, aneuploid).
Summary statistics expressed as arithmetic means (points) with 95% confidence intervals (whiskers).
A/AB/B: marks of statistical significance of differences among G/T categories (ANOVA). Categories G/T marked by the same letter are not significantly different ($p<0.05$).

5–70% of the cases [10]. The disadvantages of flow cytometric analysis of DNA are that one cannot control for the morphologic variation of the analyzed cells and that single aneuploid cells may be hidden in a wide C0–G1 peak. It was demonstrated that the tumor biologic factors Ki67, PCNA, tumor front grading, and quantitative DNA analysis have a strong correlation to prognosis in patients with laryngeal carcinomas, stronger than any other clinical or histologic parameter [31].

The discussion about prognostic markers still continues [15–17, 22]. Recently OZDEK et al [21], found out, that neither c-myc nor bcl-2 were shown to be prognostic factors for laryngeal carcinoma in their study, but co-expression of these two genes may contribute to carcinogenesis.

The sufficient amount of evidence justifying its routine application in HNSCC has not been found for any of the biomarkers mentioned. In our continuing study we intend to include also evaluation of CD34, bcl-2, c-erbB2, EGFR and MMP9, because they were also found as markers of possible prognostic significance [8, 12, 17, 19].

Conclusions

Molecular and cytokinetic markers were proven very important prognostic factors in HNSCC, namely for early risk development of the disease. These factors were found to contribute to the prognosis of early risk stages that cannot be simply separated by common clinical risk factors (stage, tumor localization, grade). Molecular markers were significantly associated with tumor grade and ploidy and their profiles allowed to explain influence of grade degree on survival rates.

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