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Advances in Brief

Tissue Microarray Analysis Reveals Site-specific Prevalence of Oncogene Amplifications in Head and Neck Squamous Cell Carcinoma[1]

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Abstract

Fluorescence *in situ* hybridization was applied on a collection of 609 squamous cell carcinomas of the head and neck (HNSCCs), including 511 primary carcinomas of different clinical stage and anatomical localization and 98 recurrent carcinomas, second primary carcinomas, and regional metastases on a tissue microarray. The overall prevalence of amplifications of five oncogenes analyzed was 34.5% for *CCND1*, 12.7% for *EGFR*, 8.8% for *MYC*, 6.2% for *ZNF217*, and 3.6% for *ERBB2*. *CCND1* amplifications were associated with the pharyngeal site in primary carcinomas ($P < 0.001$), whereas amplifications of *ZNF217* were less frequent in pharyngeal carcinomas as compared with primary oral and laryngeal carcinomas ($P = 0.02$). The amplification pattern of these oncogenes suggests that different molecular pathways are involved in HNSCCs of different localizations.

Introduction

HNSCC[3] is characterized by local tumor aggressiveness, high rate of early recurrences, and development of second primary carcinomas. Despite modern therapeutic strategies like adjuvant and neoadjuvant radio- and chemotherapy additional to operational management of the tumor, the overall 5-year survival rate does not exceed 55% (1). Furthermore, HNSCCs are a heterogeneous group presenting as tumors of different anatomical regions such as the oral cavity, the oro-/hypopharynx, and the larynx. These subentities show different, often contrary, biological and clinical behavior. For example, the potential of forming metastases is very low in laryngeal carcinoma, whereas it is quite common in pharyngeal carcinomas. It could be shown that the various HNSCC subentities exhibit different genetic alterations, which could explain the different biological behavior of the tumors (2).

It is widely accepted that HNSCCs develop in a multistep pathway of genetic aberrations (3). Among others, amplifications of several oncogenes were previously detected (4). To determine the role of oncogene amplification for the clinical course of HNSCC, we applied the approach of TMA, a novel high-throughput technology that allows screening of tumor specimens on a large scale for molecular genetic alterations and changes in protein expression (5). We generated a tissue array of more than 600 clinically well-defined HNSCCs and detected genomic amplification of proto-oncogenes *CCND1*, *MYC*, *EGFR*, *ERBB2*, and *ZNF217* using FISH. A possible association of amplification of these oncogenes with stage, anatomical site, or long-term survival of the patients was assessed.

Materials and Methods

HNSCCs. Altogether 609 HNSCCs from 584 patients were analyzed in this study. Five hundred eleven of the tumors were untreated primary carcinomas; 98 of the tumors were scored as advanced disease tumors, *i.e.*, recurrent tumors, second primary tumors, and regional metastases. The primary carcinomas were divided into three groups corresponding to their anatomical site (oral cavity, pharynx, larynx) and graded according to the Tumor-Node-Metastasis (TNM) system and the Unio Internationale Contra Cancrum (UICC) stage. Data about tumor localization and stage are summarized in Table 1. Advanced-disease tumors were not subdivided according to their different localization and TNM grade. Formalin-fixed, paraffin-embedded tumor samples were obtained from the archives of the Institute of Pathology of the University of Heidelberg and several centers connected to it. For control experiments, uvula mucosa tissue from healthy donors was available. The evaluation of the clinical follow-up of the patients was performed using data from the Department of Oto-Rhino-Laryngology and of the Department of Oral and Maxillofacial Surgery of the University of Heidelberg. The approval for this study was given by the Medical Ethics Commission of the Universitätsklinikum Heidelberg.

Generation of TMAs. H&E-stained sections were made from each paraffin block to define representative tumor regions. From these regions, tissue cylinders with a diameter of 0.6 mm were obtained and arrayed into a recipient block using the tissue chip microarrayer (Beecher Instruments, Silver Spring, MD). The recipient block was subsequently cut into 5-μm sections on silanized glass slides that were treated with 3-aminopropylemethoxysilane before cutting to support adhesion of the tissue samples.

FISH Analysis. Two-color FISH to the sections of the tissue microarray was performed using Spectrum Orange-labeled probes for *CCND1, MYC, ERBB2, ZNF217,* and *EGFR* and Spectrum Green-labeled centromeric probes of the chromosomes 7, 8, 11, and 17 (Vysis, Downers Grove, IL). Pretreatment of slides, hybridization, posthybridization processing, and signal detection were performed as described previously (6). Tumor signals were scored as amplified with 10% of cells showing eight or more signals or tight clusters of signals of the oncogene probe. Less than eight signals per cell were considered as low-level gains and not scored as gene amplification. The signals of the centromeric probes were used to control adequate hybridization and to exclude artifacts.

Statistical Analysis. Fisher's exact test was used to compare the prevalence of gene amplification in primary carcinomas with that of advanced disease carcinomas. For primary carcinomas, the distributions of amplifications according to anatomical site, tumor classification, and stage were compared using Fisher's exact test. An estimation was made about the distribution of amplifications in different localization and clinical stages. Every tumor site was analyzed individually, T_1-T_2 tumors were compared with T_3-T_4 tumors and stage I-III carcinomas with stage IV carcinomas. Estimation of the survival time distribution of patients with primary carcinomas was done using the method proposed by Kaplan and Meier. Survival curves were compared by

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[3] The abbreviations used are: HNSCC, squamous cell carcinoma of the head and neck; TMA, tissue microarray analysis; FISH, fluorescence *in situ* hybridization; C64, comparative genomic hybridization.

Table 1 *Number of amplifications for all of the analyzed oncogenes with respect to localization and stage*

	CCND1	*MYC*	*EGFR*	*ERBB2*	*ZNF217*
All tumors (n = 609)	34.5% (181/524)	8.8% (42/473)	12.7% (63/496)	3.6% (18/502)	6.2% (27/436)
adv.dis.[a] carcinomas (n = 98)	30.9% (25/81)	10.5% (8/76)	7.7% (6/78)	1.2% (1/83)	5.2% (4/76)
versus					
Primary carcinomas (n = 511)	36.0% (156/433)	8.6% (34/397)	13.7% (57/418)	4.2% (17/402)	6.4% (23/360)
*P*s	P = 0.15	P = 0.66	P = 0.19	P = 0.33	P = 1.0
Oral cavity (n = 245)	30.3% (61/201)	10.1% (21/207)	11.5% (24/209)	3.8% (8/213)	9.6% (19/197)
versus					
Pharynx (n = 160)	49.7% (73/147)	7.3% (9/114)	15.9% (20/126)	5.5% (7/127)	1.9% (2/107)
versus					
Larynx (n = 106)	25.9% (22/85)	6.0% (4/67)	15.7% (13/83)	2.5% (2/79)	3.6% (2/56)
*P*s	$P < 0.001$	P = 0.54	P = 0.41	P = 0.64	P = 0.02
T_{1-2} carcinomas (n = 199)	32.7% (54/165)	7.2% (11/152)	9.2% (15/163)	2.4% (4/167)	6.4% (9/141)
versus					
T_{3-4} carcinomas (n = 267)	39.0% (90/231)	8.5% (18/211)	14.3% (32/223)	4.2% (9/213)	6.7% (13/194)
*P*s	P = 0.24	P = 0.7	P = 0.16	P = 0.4	P = 1.0
Stage I–III carcinomas (n = 164)	23.1% (31/134)	7.6% (9/118)	10.0% (13/130)	3.1% (4/130)	5.5% (6/109)
versus					
Stage IV carcinomas (n = 300)	43.1% (112/260)	7.8% (19/244)	13.4% (34/254)	3.6% (9/249)	7.1% (16/224)
*P*s	$P < 0.001$	P = 1.0	P = 0.41	P = 1.0	P = 0.65

[a] adv.dis., advanced disease. The numbers in parentheses represent number of amplifications versus number of analyzed tumors.

log-rank tests. A result was considered as statistically significant if the corresponding *P*s were ≤0.05. The median follow-up time was 48.5 months.

Results

FISH analysis was performed on the HNSCC microarray with oncogenes *CCND1, MYC, EGFR, ERBB2,* and *ZNF217* (Fig. 1). The number of gene amplifications according to the different subsets of tumors is summarized in Table 1. There was no statistically significant difference between the samples of primary tumors and advanced disease tumors concerning the prevalence of individual oncogene amplification (*CCND1*, P = 0.15; *MYC*, P = 0.66; *ERBB2*, P = 0.33; *EGFR*, P = 0.19; *ZNF217*, P = 1.0). Regarding amplification of *CCND1,* a highly significant difference between the sites of the primary carcinomas was revealed. Prevalence of *CCND1* amplification was significantly higher in pharyngeal tumors than in oral and laryngeal carcinomas ($P < 0.001$). *ZNF217* amplifications were significantly less common in pharyngeal tumors than in tumors of the oral cavity and the larynx (P = 0.02; Fig. 2). With respect to the remaining oncogenes, there was no significant effect on the prevalence of amplification in different tumor sites. (*MYC*, P = 0.54; *ERBB2*, P = 0.64; *EGFR*, P = 0.41).

We further analyzed the impact of oncogene amplification on clinical stage and tumor localization in primary carcinoma. No association was found between the number of amplifications and any of the two groups of tumor classifications (*CCND1*, P = 0.24; *MYC*, P = 0.7; *ERBB2*, P = 0.4; *EGFR*, P = 0.16; *ZNF217*, P = 1.0). However, there were significantly more *CCND1* amplifications in stage IV carcinomas than in the group of carcinomas in stage I-III ($P < 0.001$; Fig. 3). Because stage IV is more common in pharyngeal carcinomas (81.75% of all primary carcinomas with pharyngeal site) than in carcinomas of the oral cavity (62.8%) and the larynx (40%) in our collective, this effect can mainly be explained by the high prevalence of *CCND1* amplifications in pharyngeal carcinomas. For the other four oncogenes, no statistically significant difference was detected between stage I-III and stage IV (*MYC*, P = 1.0; *ERBB2*, P = 1.0; *EGFR*, P = 0.41; *ZNF217*, P = 0.65).

An analysis of the survival curves revealed no association between the amplification of any of the five oncogene probes and overall survival (*CCND1*, P = 0.24; *MYC*, P = 0.71, *ERBB2*, P = 0.76; *EGFR*, P = 0.57; *ZNF217*, P = 0.78).

Discussion

The combination of TMA and FISH is a powerful approach to detecting gene amplification in large series of tumor specimens. In this study, the TMA technology was used to study a large series of HNSCCs. This allowed us to extend previous results (7, 8) concerning the prevalence of oncogene amplification in HNSCC. Notably, our data showed that *CCND1* amplifications were more common in pharyngeal carcinomas than in carcinomas of the larynx and of the oral cavity. This difference was highly significant and corresponds with elevated cyclin D1 protein expression levels that were observed previously in pharyngeal carcinomas (9). Additionally, we observed a significantly higher number of amplifications in stage IV tumors than in stage I-III tumors. One might, therefore, speculate that *CCND1* is directly involved in the formation of regional metastasis. However, the association between *CCND1* amplification and metastasis can be explained as well by the higher frequency of *CCND1* amplifications in tumors of the pharynx, for which other molecular changes could also lead to an increased risk for metastasis. Interestingly, the distribution of amplifications did not differ between small and large tumors (T_{1-2} *versus* T_{3-4}), suggesting a specific role of *CCND1* in the early carcinogenesis of HNSCC (Fig. 3). This is also supported by previous

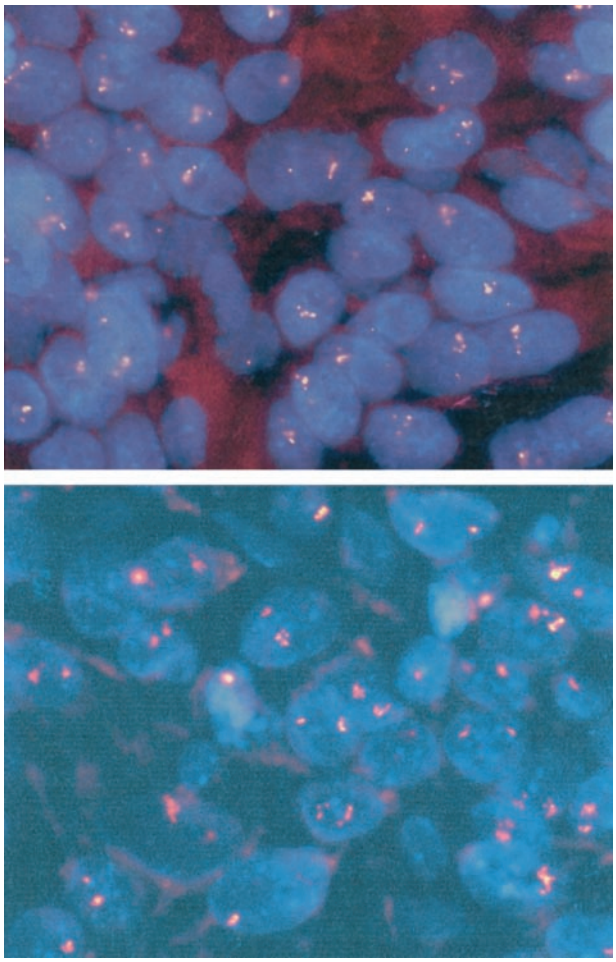

Fig. 1 Amplification of *CCND1* (*top*) and *ERBB2* (*bottom*) in primary HNSCCs.

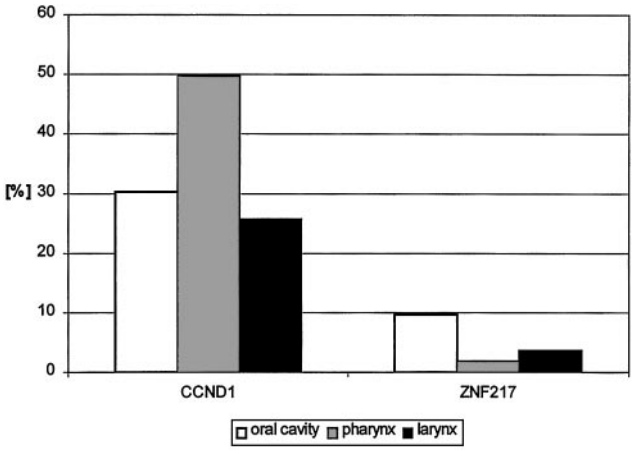

Fig. 2. Frequency of *CCND1* ($P = 0.001$) and *ZNF217* ($P = 0.02$) amplification in different anatomic sites of primary HNSCC.

findings of *CCND1* amplifications in preneoplastic oral lesions (10). In conclusion, our data strongly indicate that *CCND1* amplification is characteristic for pharyngeal HNSCC compared with carcinomas of the larynx and the oral cavity and, therefore, has the function of a site-specific molecular alteration.

The impact of *CCND1* amplification on long-term prognosis is the subject of ongoing discussion (6, 7). No such correlation was found in our patient collective. On the basis of a CGH-analysis on 113 primary HNSCCs by Bockmühl *et al.* (11), amplification of chromosomal subregion 11q13 was described as of prognostic relevance. It should be noted, however, that a relatively large number of parameters were analyzed in this study, and, therefore, the data obtained from this tumor collection might not be representative of the HNSCC entity as a whole. Alternatively, if the amplification of 11q13 is of prognostic relevance, *CCND1* might not be the only relevant gene for HNSCC progression in this subregion, but, instead, other candidate genes should be taken into account. For example, *EMS1* amplification has been found to predict for patients survival (8). Other potential candidate genes found to be amplified on 11q13 in HNSCC include *INT2*, *FGF4*, and *HST1* (12). Certainly, the TMA presented in the present study will be highly valuable to further delineate the prognostic role of candidate genes located at 11q13 in HNSCC.

The epidermal growth factor *ERBB2*(*HER2/NEU*) has become a subject of interest in HNSCCs because the amplification of *ERBB2*(*HER2/NEU*) demonstrated its power to predict patient outcome in breast cancer (13). Furthermore the *ERBB2* gene product represents a target for specific therapy with a monoclonal antibody (Herceptin). In HNSCC, data about overall prevalence of *ERBB2* amplifications are very rare. Recently, amplifications of *ERBB2* were detected in four cases of oropharyngeal carcinomas for the first time (14). On the protein level, ERBB2 overexpression has been observed in HNSCC, but its influence on survival rates remained open (15). According to the present study, 4.2% of primary HNSCCs were found to be harboring amplification of *ERBB2* without significant difference concerning tumor site and stage. Furthermore, there was no impact on patients' survival. Although *ERBB2* amplifications are generally infrequent in HNSCC and, therefore, unlikely to serve as potent clinical markers, these findings suggest that Herceptin therapy may be a therapeutic option in a small fraction of HNSCC. Concerning the oncogenes *ERBB1* and *MYC*, no correlation with site, stage, or clinical outcome was assessed.

Copy number gains of chromosomal subregion 20q13 has been frequently found in CGH-studies of HNSCCs (11, 16). One of the candidate genes in this region is *ZNF217*, which is predicted to encode two alternately spliced, Krüppel-like transcription factors and has been shown to be amplified in breast cancer (17). In this study, the overall prevalence of *ZNF217* amplification was revealed for the first time in HNSCC, but no influence on the clinical follow-up was observed. This is in accordance with the finding that 20q13 is not considered as a critical region with prognostic significance in HNSCCs (11). However, we detected a significantly lower rate of *ZNF217* amplifications in pharyngeal as compared with oral and

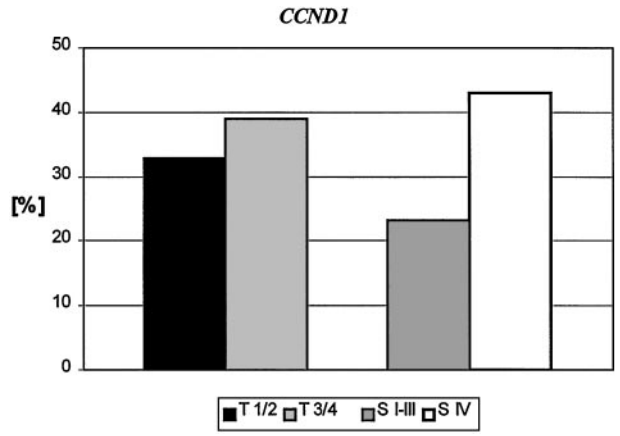

Fig. 3. Frequency of CCND1 amplification in HNSCC of different tumor classifications ($P = 0.24$) and stages ($P < 0.001$). *T 1/2*, T_{1-2}; *T 3/4*, T_{3-4}; *S I-III*, stage I-III; *S IV*, stage IV.

laryngeal carcinomas. This further indicates that HNSCCs of different anatomical sites are characterized by a different pattern of genetic aberrations and, therefore, might represent different subentities of this tumor type. Our data are supported by recent analyses that also revealed different genetic alteration patterns depending on the anatomical site in HNSCC (2, 18, 19). In conclusion, we demonstrate that molecular alterations and their relevance in the etiology and progression of HNSCC can be efficiently assessed by combining FISH and TMA technology. We showed in a large collection of HNSCCs that different amplification patterns of two oncogenes, *CCND1* and *ZNF217*, are found in HNSCC of different anatomical sites, which strongly suggests that different molecular pathways may be involved. This may contribute to a multistep pathway model of pathogenesis, eventually allowing individual clinical risk assessment and therapy decision in the future.

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