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ARTICLE

Multiple Chromosomal Underrepresentations Detected by Interphase Cytogenetics – Possible Prognostic Markers in Head and Neck Tumors?

Britta KLEIST,[1] Alexander BANKAU,[2] Gerd LORENZ,[1] Micaela POETSCH[3]

[1]Institute of Pathology, [2]Department of Otorhinolaryngology and [3]Institute of Forensic Medicine, University of Greifswald, Greifswald, Germany

Relevant prognostic factors for head and neck squamous cell carcinoma are tumor extension (pT), occurrence of lymph node metastases (pN) and grade of differentiation (G). We tried to correlate these histological characteristics with numerical aberrations of whole chromosomes as demonstrated by fluorescence *in situ* hybridization techniques (FISH). Therefore, we investigated isolated interphase cells from paraffin sections of squamous cell carcinomas of the head and neck region from 46 patients with centromeric DNA probes for chromosomes 1, 3, 4, 6, 7, 9, 10, 11, 12, 15, 17, 18, X and Y. The majority of tumor samples showed aneuploidy for most chromosomes analyzed. The main chromosomal abnormality was loss of chromosomal material, predominantly of chromosomes 3 (28%), 6 (20%), 9 (26%), 10 (24%) and 18 (33%). Multiple deletions could be demonstrated more frequently in poorly differentiated carcinomas (88% G3-tumors with more than one deletion in contrast to 66% G2-tumors). The occurrence of multiple deletions may also correlate with progression in lymph node metastasis (66% in pN0-tumors vs. 85% in pN2-tumors), whereas the differences between the stages of primary tumor extension were not so obvious. Despite of a somewhat disproportionate distribution of tumors in the different pT- and pN-stages and the rather low number of cases, our results suggest a relationship between the quantity of chromosomal underrepresentation, grade of differentiation and higher lymph node stage. Therefore, they underline the importance of chromosomal deletions as a possible additional prognostic marker in head and neck squamous cell carcinoma. (Pathology Oncology Research Vol 7, No 1, 28–32, 2001)

Keywords: head and neck carcinoma, FISH, numerical chromosomal aberrations, TNM status, grading

Introduction

Head and neck carcinomas represent 6% of all human cancers with squamous cell carcinoma (SCC) being the most important histological group. More than 500,000 new cases of SCC of the upper aerodigestive tract occur worldwide annually.[19] One of the major etiologic agent in these malignancies is tobacco. SCC of the upper aerodigestive tract are characterized by marked heterogeneity in their biological behavior.[7] For this reason, clinicians and pathologists long have sought parameters to determine grade of malignancy, predict individual prognosis and thereby indicate suitable adjuvant therapy. Many studies were performed in this field and identified tumor extension at time of diagnosis,[22,25] depth of invasion,[1] the burden of lymph node metastasis[10,25] and grade of tumor cell differentiation[16,31] as clinical and pathomorphological factors predictive for course and outcome of disease, whereas the influence of tumor localization is controversially discussed.[22,30] But the TNM-classification system can not foresee clinical outcome in all cases of HNSCC and a multifactorial predictive formula is demanded.[3,17] In order to optimize treatment of patients, attempts are being made to establish new prognostic indicators. In recent times, cytogenetic techniques could demonstrate the contribution of many alterations to prognostic information in HNSCC. These abnormalities comprise chromosome locations

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Correspondence: Dr. Britta KLEIST, Institute of Pathology, Ernst-Moritz-Arndt-University, F.-Loeffler-Strasse 23e, D-17489 Greifswald, Germany, Tel. +49-3834-865718, Fax +49-3834-865704

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7p13-p12, 8q24, 9p21-p22, 11q13, 11pter-p13, 12p12, 17p13, 17q23-ter and 18q21.[7]

All these studies analyzed single events in tumor development and progression, but they could not reflect the multistep nature of cancer, involving the activation and inactivation of more than one gene locus. Studies on other solid tumors as colorectal carcinomas,[29] breast cancer,[8] ovarian tumors[24] and urinary bladder cancer[23] were designed to investigate how multiple genetic alterations contribute to tumor behavior or course of disease and focused on the search for a possible additional tool in therapeutic management.

Here we tried to determine, whether the multiplicity of chromosomal deletions correlate with the clinical and morphological predictive factors tumor extension (pT), lymph node metastasis (pN), grade of differentiation (G) and tumor localization in HNSCC.

Material and Methods

Patients

Forty six patients with SCC in the head and neck region (41 male and five female) were studied. Specimens were obtained from surgical resections of non-selected patients which have been partially included in another study.[21] They were divided into groups with regard to tumor extension (pT1-4), occurrence of lymph node metastasis (pN0-2) and grade of differentiation (G1-G3) and tumor localization (oropharynx, hypopharynx, larynx). Grading was performed according to the criteria defined by Anneroth et al.[2] Histological diagnosis of SCC was documented for all cases. All specimens underwent additional independent histopathological review (B. K.).

FISH analysis of isolated cells from paraffin embedded sections

Microscopic examination of tissue sections and comparison with the corresponding paraffin-embedded material assured isolation of tumor tissue without adherent non-neoplastic structures. Isolation of cells from paraffin-embedded material for FISH analysis was done as described before.[20] *In situ* hybridization was performed with commercially available biotinylated or digoxigenin-labeled centromeric probes (chromosomes 7 (D7Z1) and 15 (D15Z2) from Boehringer (Mannheim, Germany) and chromosomes 1 (D1Z5), 3 (D3Z1), 4 (D4Z1), 6 (D6Z1), 9 (D9Z1), 10 (D10Z1), 11 (D11Z1), 12 (D12Z3), 17 (D17Z1), 18 (D18Z1), X (DXZ1) or Y (DYZ3) from Appligene Oncor (Heidelberg, Germany)) as described before.[20] Normal blood lymphocytes, fibroblasts and isolated cells from paraffin sections of normal tonsils and thyroid glands were used as controls. The FISH results were verified independently by a second observer without any striking differences to the first observer (data not shown).

Results

Control studies

All DNA probes used showed an expected number of signals in peripheral blood lymphocytes from healthy donors, e.g. the frequency of a trisomy or higher ploidy was lower than 1% and the frequency of a monosomy was lower than 10% for all DNA probes applied. At least 500 cells were analyzed for each donor and probe combination.

The frequency of a trisomy or higher ploidy was lower than 1% and the frequency of a monosomy was lower than 15% in isolated cells from paraffin sections of normal tonsils or thyroid glands. Approximately 200-500 cells were analyzed for each sample and probe combination.

FISH on isolated cells of paraffin sections from head and neck squamous cell carcinomas

All tumor samples from the 46 patients showed strong signals for all applied DNA probes after optimizing the proteinase K treatment for each sample. The majority of carcinomas displayed an aneuploid number of signals for most chromosomes analyzed. Only in 22% of all tumor samples did we find predominantly two signals for the

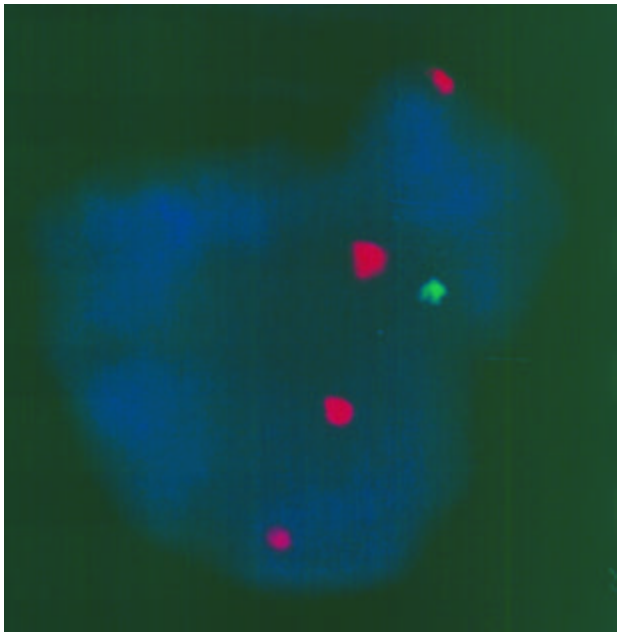

Figure 1. Interphase cell of a poorly differentiated laryngeal tumor after hybridization with a DNA probe for the centromeric region of chromosome 17 (red signals) and the centromeric region of chromosome 18 (green signal). The cell shows four red signals, but only one green signal, indicating a loss of chromosomes 18.

Table 1. **Correlation between the pTN status and the number of chromosomal deletions in squamous cell carcinoma of 46 patients**

Tumor extension and lymph node metastasis	*number of chromosomal deletions*			
	none	*one*	*two*	*three*
pT1 (n = 3)	–	1	1	1
pT2 (n = 9)	1	3	2	3
pT3 (n = 12)	1	3	4	4
pT4 (n = 22)	1	4	12	5
pN0 (n = 29)	2	8	12	7
pN1 (n = 4)	1	1	1	1
pN2 (n = 13)	–	2	6	5

Table 2. **Correlation between the grade of differentiation and the number of chromosomal deletions in squamous cell carcinoma of 46 patients**

Grade of differentiation	*number of chromosomal deletions*			
	none	*one*	*two*	*three*
G2 (n = 38)	3	10	17	8
G3 (n = 8)	–	1	2	5

DNA probes, whereas in 50% mainly three signals, in 13% four signals and in 15% more than four signals were detectable. Carcinomas in higher malignant stages (poorly differentiated carcinomas) always displayed at least a triploid set of chromosomes. The main numerical chromosomal aberration was loss of chromosomal material (*Figure 1*), predominantly of chromosomes 3 (28%), 6 (20%), 9 (26%), 10 (24%) and 18 (33%).

The number of chromosomal underrepresentations in correlation to tumor extension (pT), lymph node metastasis (pN) and grade of differentiation (G) are summarized in *Tables 1 and 2.*

Multiple deletions could be demonstrated more frequently in poorly differentiated carcinomas (88% G3 tumors with more than one deletion in contrast to 66% G2 tumors). Well differentiated (G1) tumors have not been diagnosed. The number of deletions was also higher in tumors with advanced lymph node metastasis (66% in pN0 tumors vs. 85% in pN2 tumors). The differences between the stages of primary tumor extension were not so obvious.

Table 3. **Correlation between tumor localization and number of chromosomal deletions in squamous cell carcinoma of 46 patients**

Tumor localization	*number of chromosomal deletions*			
	none	*one*	*two*	*three*
Oropharynx (n = 10)	2	2	4	2
Hypopharynx (n = 9)	–	3	5	1
Larynx (n = 27)	1	6	10	10

In contrast, seven tumors with three chromosomal underrepresentations were found among the 29 samples diagnosed free of lymph node metastasis; similarly, four of twelve pT1 and pT2 stage tumors and eight of 38 moderately differentiated tumors had three chromosomal deletions. Cases with more than one deletion tend to slightly accumulate from pharyngeal sites (oropharynx 60%, hypopharynx 67%) to laryngeal localization (74%) (*Table 3*).

Discussion

Human cancers now are considered to occur through the accumulation of multiple genetic alterations within a tumor cell population. Relevant changes include activation of protooncogenes because of point mutations or gene amplifications and inactivation of tumor suppressor genes because of chromosomal losses or point mutations.[9,14] Cytogenetic studies of SCC of the upper aerodigestive tract demonstrated deletions in chromosomal regions 1p, 3p, 7q, 9p, 11q, 17p,[5,11] and also gain of chromosomal material in 11q13,[4] but there are still few data about a possible correlation between a greater number of chromosomal abnormalities and clinical or pathomorphological prognostic parameters. Here, we propose a connection of multiple chromosomal deletions detected by FISH with grade of tumor cell differentiation and tumor progression, in particular advanced lymph node metastasis.

We observed chromosomal deletions more often in tumors of more undifferentiated histologic types (G3) and in patients with higher lymph node stage (pN2). In this study, only four pN1 tumors could be analyzed and no G1 tumors could be obtained. Therefore, conclusions with regard to step by step development and progression of tumors have carefully to be done on this small population. However, our findings support the view that multiple genetic alterations are required for the development and progression of HNSCC. This concept has been well illustrated for colon carcinogenesis[6,28] and has been confirmed by studies on other solid tumors. Multiple chromosomal alterations were shown to be associated with lymph node metastasis**,** advanced tumor stages and poorly differentiated histologic types in breast carcinomas,[8] with tumor progression in early invasive urinary bladder cancer,[23] with tumor progression and loss of differentiation in ovarian tumors[24] and with advanced and poorly differentiated hepatocellular carcinomas.[18] In addition, LOH studies and karyotyping analysis on HNSCC could demonstrate the significance of multiple chromosomal deletions for short survival[12,27] and for early recurrence.[13]

Another finding in this study was the slightly increased number of cases with multiple deletions in laryngeal carcinomas compared with tumors of oropharynx and hypopharynx. For a given T stage, tumors of the oropharynx and hypopharynx are known to do worse than those of the larynx whereas

overwhelming evidence for intrinsic biological differences between these different sites seems to be lacking.[7] In contrast to this opinion, immunohistologic and molecular genetic studies of Takes et al,[26] Matthias et al[15] and our investigations[21] indicate the different tumor biology of cancers originating from different sites in the head and neck. The data of this study tend to support this view, but because of the relatively low number of pharyngeal tumors in this study, the significance of this finding could be questioned.

Hypothetical explanations for the physiologic basis of genetic alterations, such as deletions, are given by Vogelstein et al.[29] First, genetic changes may include tumor suppressor genes with incremental effects on the regulation of cell growth leading to the formation of a tumor. On the other hand, many alleles could be deleted simultaneously in an abnormal mitosis in which the majority of chromosomes segregate aberrantly. Because allelic losses are irreversible, cells with such alterations will eventually become the predominant clone in the tumor population. Whatever the role of these deletions, their multiplicity must be taken into consideration in any model for the genetic origin of human tumorigenesis and in the search for useful molecular correlates of tumor behavior.

As many of the genetic alterations studied here are proposed to be potentially predictive for poor prognosis by oneself,[7,12] tumors with multiple of these deletions may be considered at risk for recurrence and/or progression. In this context, the occurrence of multiple deletions in lymph node negative (pN0) and moderately differentiated (G2) tumors in this HNSCC tumor population is also a remarkable finding. In accordance to observations of Harada et al[8] on breast carcinomas, we suspect here that these tumors, though proportionally few in number, may actually have poorer prognoses than would be predicted by conventional clinicopathologic staging.

These facts imply possible predictive power of genetic diagnosis for patients with poor prognosis**,** especially, if this poor prognosis escaped the clinicopathologic diagnosis. Despite of the low number of cases in this study, we propose to treat patients whose tumors show multiple genetic alterations as a new risk group for purposes of postoperative management. Further studies with long term follow-up and higher number of cases are required to prove the usefulness of these genetic markers as routine prognostic indicators in HNSCC clinics.

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