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Additional use of DNA-image cytometry improves the assessment of resection margins

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BACKGROUND: Despite the histopathologic findings of tumor-free margins, patients with oral squamous cell carcinoma (SCC) often suffer from local tumor relapse. The purpose of this study was to determine the prognostic value of DNA-image cytometry in the assessment of resection margins.

METHODS: DNA-image cytometry was performed in 40 SCC patients with histologically tumor-free resection margins. The follow-up period since the tumor resection was at least 3 years.

RESULTS: Twenty patients showed a locoregional relapse of the SCC. Fourteen of these patients had aneuploid cells in DNA-image cytometry. Two patients who were relapse-free revealed aneuploid cells too. The sensitivity of the adjuvant use of DNA-image cytometry was 70% and the positive predictive value was 87.5%.

CONCLUSIONS: The additional use of DNA-image cytometry is a reasonable tool for the assessment of the resection margins of SCCs. DNA-image cytometry could help to find the appropriate treatment option for the patients and thus might improve their prognosis.

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Introduction

Patients with squamous cell carcinomas (SCC) of the oral cavity have a fair prognosis with an overall 5-year survival rate of about 45% (1). Unfortunately, this figure has not substantially improved during the past 30 years (2). Locoregional failure after surgery or even after combined surgery and irradiation is the main cause of death in patients with SCCs of the mandibular region. Several authors have evaluated the relationship between locoregional recurrence of the tumor and the status of the resection margins (3, 4). The prevalence of tumoral infiltration at the resection margins varies from 3.5% to 60% (3) and is usually an indicator for additional excision, post-operative irradiation, and strict follow up (5). The recurrence rate in patients with positive margins of resection treated only by surgery ranges from 36% (4) to 64% (3), when post-operative irradiation is used, the recurrence rate decreases to 31% (3). Because of the fact that it can be difficult to distinguish between SCCs and other lesions of the oral mucosa using only hematoxylin and eosin-stained sections (6), the resection margins are routinely examined by immunohistology. Nevertheless, the histologic diagnose of oral mucosa lesions fail sometimes (7, 8). These days, an alternative method for the examination of oral lesions is exfoliative cytology. It is based on the technique of Papanicolaou, which is accepted worldwide, as a successful method in order to screen for epithelial dysplasias *in situ* or invasive carcinomas of the uteri cervix. Moreover, DNA-image cytometry has been introduced for diagnosis of malignant transformation of squamous epithelial cells as an adjuvant tool to the cytologic examination (9, 10). This is used to detect the cytometric equivalent of chromosomal or DNA-aneuploidy (11), which is accepted as a marker for the neoplastic transformation of cells. However, it is unclear whether DNA-image cytometry has additional value for the examination of resection margins.

Therefore, the purpose of this study was to determine the prognostic value of DNA-image cytometry in the assessment of resection margins.

Materials and method

DNA-image cytometry was performed in 40 patients with tumor-free margins (frozen section) after resection of an oral SCC (located at the tongue and floor of the mouth and involving the lower jaw in nine cases) according to the consensus reports of the European Society of Analytical Cellular Pathology (ESACP). The

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resection margins from 40 patients that had been investigated by representative intraoperative frozen sections. The resection margins (width 3–5 mm) were excised after the clinically distinguishable tumor was resected. Therefore, the resection margins represent the clinically unaltered mucosa/tissue adjacent to the resected tumor block. If any tumorous cells were found anywhere in the thin margin, the margin was considered as positive. After the intraoperative diagnostic was done, the frozen resection margins were unfreezed and paraffin-embedded. Definitive paraffin-histology was subjected to an enzymative cell separation as described in our previous publication (12). In most cases four sections were available (mode = 4, Table 1). Smears from the obtained unclear suspension were prepared and stained according to Feulgen with pararosanilin as described previously and recommended by the ESACP (12).

DNA-image cytometry was performed as mentioned earlier (13). Briefly, the AutoCyte QUIC DNA-workstation (AutoCyte, Burlington, NC, USA/Zeiss, Jena, Germany) was used for the measurement of the DNA-content in the Feulgen-stained slides. This system was standardized according to the guidelines of the ESACP for diagnostic DNA-image cytometry (12, 14). Internal calibration was performed with at least 30 phototypically unsuspicious intermediate squamous epithelial cells. Additionally, at least 300 morphologically suspicious cells were measured for analysis. A resection margin has been classified as DNA-euploid, if there was only one DNA stemline (STL) between 1.80c and 2.20c or an additional one between 3.60c and 4.40c. DNA-aneuploidy was assumed if there were abnormal STLs <1.80c and >2.20c or <3.60c and >4.40c and/or exceeding 5c.

All patients had a SCC of the oral cavity. Standardized treatment was performed by radical surgery (resection of the tumor with a resection margin of at least 1 cm to the clinically apparent tumor; neck dissection if necessary) without additional radiotherapy except patients with tumor-infiltrated resection margins in the paraffin sections. All patients presented tumor-free resection margins after histologic examination of the frozen sections. The post-operative observation period was at least 3 years. During this follow-up period the patients were examined for local relapse or metastasis at monthly intervals. If suspicious lesions were observed the diagnosis was confirmed by a biopsy. Locoregional relapse was defined as local recurrence only (T-site).

Sensitivity, specificity, positive and negative predictive value were calculated according to the four-field table (15).

Table 1 Number of blocks from resection margins that are available for each patient after tumor resection

Number of blocks from resection margins	*Number of patients*
2	5
3	6
4	13
5	9
6	1
7	3
8	0
9	2
10	0
11	0
12	0
13	1

Results

Within the 3-year follow-up period, 20 patients showed a locoregional relapse (T-site only) of the SCC. The tumor size of patients with relapse was comparable with that of patients showing no recurrence (Table 2). Most of the tumor recurrences occurred in the first and second year after the initial surgical treatment (Table 3). Fourteen of these patients had aneuploid cells in the DNA-image cytometry. Single cell aneuploidy was found to be the predominant type of aneuploidy followed by a combination of single cell and stemline aneuploidy. Stemline aneuploidy alone was observed only in one case (Table 4). In addition, two patients who were tumor-free during the follow-up period showed aneuploid cells as well. The sensitivity of the adjuvant use of DNA-image cytometry was 70% and the positive predictive value was calculated as 87.5%. Moreover, the additional DNA-image cytometry had a high specificity (90%) regarding the detection of local recurrences of SCCs (Table 5). The assessment of the paraffin sections approved the results of the frozen sections in 90% of all patients. Only in four cases the diagnosis was changed

Table 2 Initial tumor size of patients with vs. without local tumor relapse

	Local recurrence	*No recurrence*
T1	6	8
T2	8	8
T3	1	0
T4	5	4

Table 3 Type of DNA-aneuploidy that was detected in the patients

Type of DNA-aneuploidy	*Number of patients*
Stemline aneuploidy alone	1
Single cell aneuploidy alone	10
Stemline and single cell aneuploidy	5

Table 4 Interval from surgical resection until locoregional tumor recurrence

Interval until recurrence	*Number of patients*
<1 year after surgery	9
1–2 years after surgery	6
2–3 years after surgery	2
3–4 years after surgery	2
4–5 years after surgery	1

Table 5 Four-field table of the clinical outcome data vs. DNA-cytometry

	Local recurrence	*No recurrence*
DNA-aneuploidy	14 (2 years)	2
DNA-euploidy	6 (1 year)	18

Shown are the numbers of patients. The median interval (in years) until the local recurrence is in parentheses.

into tumor-infiltrated resection margins, because of tumor invasion in deeper layers. One of these four cases showed DNA-aneuploidy too and in three of them a recurrence was determined.

Discussion

Aneuploidy is defined by losses or gains of intact chromosomes or of their segments (16). Its net-effect on unclear DNA content can be detected by DNA-image cytometry (DNA-aneuploidy), whether it is larger than the error of measurement (currently below 5%). Exact associations between cancer and aneuploidy have been reported by many authors (17, 18). Duesberg and co-workers recently proposed a new theory regarding the chromosomal basis of cancer (19). They described that chromosomal alterations, alias aneuploidy, are ubiquitous in cancer and that aneuploidy is necessary for carcinogenesis. Chromosomal aneuploidy represents the crucial cellular alteration that causes cancer. Moreover, pre-neoplastic aneuploidy of initiated cells was found to be associated with subsequent malignant transformation (20, 21). That means that pre-neoplastic aneuploidy could be the precursor of cancer-specific aneuploidy. Recently, DNA-image cytometry has been introduced as an adjuvant tool for the detection of these cell transformations in oral mucosa (9, 10). The detection of DNA-aneuploidy has recently been described as a diagnostic aid for the identification of prospective malignancy in various organs, e.g. in dysplasias of the uterine cervix (22), suspicious cystic lesions of the neck (23), or bile duct brushings (24). The positive predictive value of DNA-aneuploidy for the subsequent deletion of histologically confirmed cancer was 100% in cells of these tissues.

In this study, the additional value of DNA-image cytometry regarding the occurrence of a locoregional relapse was assessed. The investigators of the histologic examination as well as of DNA-image cytometry were blinded to the clinical outcome of the patients. Therefore, the results of this investigation are found to be reliable. The high positive predictive value (87.5%) for the later reappearance of the resected tumor means that there is a clear coincidence of locoregional recurrence of oral cancer and the presence of aneuploid cells in the resection margins detected by DNA-image cytometry. In most of the relapse cases even the paraffin-embedded histologic examinations revealed no tumor infiltration. However, in dysplasia, which was histologically detected in paraffin sections, there could be aneuploid cells causing tumor relapse afterwards. DNA-image cytometry has repeatedly been used as an adjuvant tool in order to detect DNA-aneuploidy in oral epithelial lesions. DNA-image cytometry is indicated to clarify the prospective biologic behavior of squamous dysplasias and to verify the diagnosis in tumor cell-positive smears (10, 13, 25).

Taking into account that the diagnosis of tumor infiltration in the resection margins has often serious consequences (follow-up resection and/or post-operative irradiation), the presence of aneuploid cells could also change the treatment. However, it is unclear if these aneuploid cells cause the locoregional tumor relapse. Thus, it has to be investigated in a consecutive clinical trial, whether the additional or modified treatment leads to a longer relapse-free period. Within the limits of the present study, the adjuvant use of DNA-image cytometry improved the accuracy of the forecast for locoregional recurrence of SCCs. It is important to note that DNA-image cytometry was not used instead but as a supplement to the histologic examination.

In conclusion, the additional use of DNA-image cytometry is a reasonable tool for the assessment of the resection margins of oral SCCs. DNA-image cytometry could help to find the appropriate treatment option for the patients and thus might improve their prognosis.

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