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Detection of Squamous Cell Carcinoma of the Oral Cavity by Imaging 5-Aminolevulinic Acid–Induced Protoporphyrin IX Fluorescence

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Objectives: **Early cancer detection is the best way to improve the prognosis of patients with oral cancer. Therefore this study presents quantitative fluorescence measurements and results in the visualization of cancerous oral mucosa with 5-aminolevulinic acid (5-ALA)–induced protoporphyrin IX (PPIX).** ***Methods:*** **Time progression and type of porphyrin accumulation were analyzed in neoplastic and surrounding healthy tissue of 58 patients with a suspected cancer of the oral cavity by measuring emission spectra of 5-ALA–induced PPIX fluorescence. Fluorescence images in the red and green spectral range from the tumor tissue were recorded with a charge-coupled device camera.** ***Results:*** **After topical application of 0.4% 5-ALA and incubation for 1 to 2.5 hours, all patients revealed higher intensities of red fluorescence in neoplastic tissue compared with the surrounding normal tissue. Maximum contrast was reached after 1.5 hours of incubation. In 13.8% (n = 8) of the patients, additional findings like dysplasia, carcinoma in situ, primary tumor, secondary carcinomas, and tumor branches were found by means of fluorescence marking in contrast to white light examination. An evaluation of the biopsy specimens resulted in a specificity of 60% and a sensitivity of 99%.** ***Conclusions:*** **As a fluorescent marker, PPIX could represent a possible new diagnostic tool to detect early malignant and secondary lesions in the oral cavity. In addition, 5-ALA–induced PPIX fluorescence is promising as a useful intraoperative tool for determining adequate surgical margins of resection. Further investigations aim to assess this diagnostic procedure as a sensitive and clinically reliable method for patients with oral cancer.** ***Key Words:*** **Cancer, oral cavity, 5-aminolevulinic acid, protoporphyrin IX, fluorescence, diagnosis.**

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INTRODUCTION

Head and neck cancer presents a major problem in modern health care. Within the German population a rise in both incidence and mortality rates has been reported over the past two decades. From 1978 to 1993, the estimated incidence of malignant lesions of the oral cavity within the male German population has risen from 8.4 to 15.1 per 100,000, thus showing a twofold increase. In the same period, the estimated mortality of this disease rose from 2.7 to 7.0 per 100,000 male Germans.[1] An increasing use of alcohol and tobacco, which play a synergistic role in the pathogenesis of oral cancer, is at least partly responsible for this unfavorable development,[2] resulting in diffuse changes within the entire lining mucosa of the upper aerodigestive tract. This phenomenon has been explained by the concept of field cancerization.[3,4] Thus this epithelial area as a whole is considered to be at high risk for subsequent neoplastic progression.

Prognosis is highly dependent on early detection and fast, radical, and complete surgery. According to Silverman,[5] this could double the cure rates of oral cancer from 39% to 78%. However, those small, apparently harmless areas of induration or localized morphological changes representing early lesions of the oral mucosa are often difficult to detect within innocuous tissue by normal examination. Because of the variability of the findings, those early tumors are sometimes missed even by clinicians with a high level of experience. Furthermore, complete resection of a previously diagnosed tumor of the oral cavity is mostly based on the knowledge of the superficial size of the malignant tissue. In clinical reality, however, the

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precise tumor margins are often poorly or even inaccurately defined; for example, tonguelike expansions of tumors are easily missed by the naked eye. Especially for high-risk patients, those addicted to alcohol or heavy smokers (or both) and patients with field cancerization of the oral cavity, highly sensitive tumor screening methods have to be found and evaluated for the clinical routine.

A recently introduced fluorescent marker with tumor-localizing properties is 5-aminolevulinic acid–induced protoporphyrin IX (5-ALA–induced PPIX). Topical or systemic administration of 5-ALA results in a selective accumulation of PPIX in neoplastic tissue, which is probably due to altered activity levels of the enzymes of the heme biosynthetic pathway within transformed cells.[6–9] Kennedy et al.[10] were the first to propose 5-ALA–induced PPIX for the detection of premalignant and malignant oral mucosa.

The aim of the present investigation was to evaluate fluorescence-aided detection of oral squamous cell carcinoma after topical application of 5-ALA as a minimally invasive tumor screening method that is, at the same time, free of side effects and easily reproducible.

MATERIALS AND METHODS

Patients

Fifty-eight patients (mean age, 57.7 y; range, 34–74 y) with a suspected squamous cell carcinoma of the oral cavity were investigated. All patients had a history of smoking and of drinking alcohol. Informed consent was obtained from all patients who underwent examination.

Experimental Procedure

5-Aminolevulinic acid (Medac, Hamburg, Germany), a physiological precursor of heme, was used in a 0.4% rinsing solution. The patients performed a 15-minute continuous rinsing of the oral cavity using the 5-ALA solution. After an incubation period of 1 to 2.5 hours, fluorescence investigation was performed with the patient either awake (n = 42) or under general anesthesia during surgery (n = 16).

Biopsy specimens were taken from tumor, tumor boundaries, and normal tissue under fluorescence illumination. To allow correlation with histological examination, the subjective intensity of red fluorescence at the biopsied site, which was depicted on a screen, was classified as strong, weak, or negative by the surgeon (A.L.) in all 58 patients. For the assessment of sensitivity and specificity, histological diagnoses of squamous cell carcinoma, carcinoma in situ, and severe and moderate dysplasia were classified as malignant, whereas light dysplasia and normal tissue were classified as benign.

To quantify fluorescence intensity, contrast spectral measurements were performed on 29 patients. A subgroup of 22 patients was examined repeatedly using fluorescence spectroscopy to determine the optimum incubation time. In addition, autofluorescence examinations (i.e., fluorescence examinations before 5-ALA application) were carried out in 30 patients. The time expenditure for fluorescence examinations varied from 5 to 10 minutes, depending on how many different procedures were performed (i.e., only imaging or imaging and spectroscopy).

Fluorescence Imaging and Spectroscopy

In vivo tissue excitation was carried out by a modified xenon short-arc lamp (D-Light, Storz, Tuttlingen, Germany). A foot-controlled switch allowed changing between common white light examination and fluorescence excitation at wavelengths from 375 to 440 nm. A modified 0° endoscope was used for both illumination and detection of the examined sites in the oral cavity. It could be optionally equipped with a filter (OG515, Schott, Mainz, Germany) that blocks all of the back-scattered excitation light. This leads to high-contrast pictures with PPIX-stained tissue displayed in red color and normal tissue autofluorescence in green color. Pictures were gathered by a target-integrating charge-coupled device camera (Telecam SL PAL, Storz, Germany) and depicted on a screen.

For spectral measurements, part of the remitted light was collected by a silicon fiber, passed on to an optical multichannel analyzer (S2000, Ocean Optics, Dunedin, FL) and interpreted for its spectral components. To achieve reproducible and comparable spectral data, the measurements were made in a fixed geometrical setup using a resection loop protruding exactly 12 mm from the front end of the endoscope. All spectra were taken from a spot in the center of the fluorescence image that was approximately 2 mm in diameter. A first description of our technique with preliminary results has been reported elsewhere.[11] The scheme of the complete fluorescence detection device is given in Figure 1.

RESULTS

Fluorescence Imaging and Spectroscopy

In only 33% (10 of 30) of tumors from a subgroup of patients investigated before 5-ALA application malignant lesions' surfaces disposed of a red fluorescence on the acquired images. However, the distribution of red fluorescent areas on the tumorous lesions was varying in size and appearance: only a part of the evident tumor surface showed this endogenous red fluorescence. Spectra taken from these fluorescent spots revealed no difference from spectra taken from PPIX-loaded tissue.

After 5-ALA application and 1 to 2.5 hours of incubation, PPIX fluorescence was detected in the oral cavity of 58 patients with a squamous cell carcinoma. Tumor tissue showed a much higher accumulation of PPIX than adjacent healthy tissue (Figs. 2–5), represented by differences in peak heights at 635 and 700 nm (Fig. 6). At the same time, tissue autofluorescence in the green spectral range (around 500 nm) was noticeably higher in healthy oral mucosa compared with cancerous lesions.

Repeated spectral measurements showed that the ratio of PPIX fluorescence between tumor and healthy tissue reached its maximum value of 12.5 after 1.5 hours of incubation (Fig. 7). In 13.8% (n = 8) of the patients, additional findings such as dysplasia (n = 2), carcinoma in situ (n = 1), primary tumor (n = 2), secondary carcinomas (n = 1), and tumor branches (n = 2) were obtained only with fluorescence staining in addition to white light examination.

Histopathological Evaluation

The overall evaluation of biopsy specimens obtained in the course of this study indicates that positive macroscopic PPIX fluorescence findings, which were classified as strong (F++), weak (F+), or negative (F−), were closely correlated to malignant histological findings (Table I). Of 92 biopsy specimens with a strong, macroscopically visible red fluorescence (F++), 82 were diagnosed to be squamous cell carcinoma, carcinoma in situ, or severe or moderate dysplasias. Twenty-seven biopsy specimens

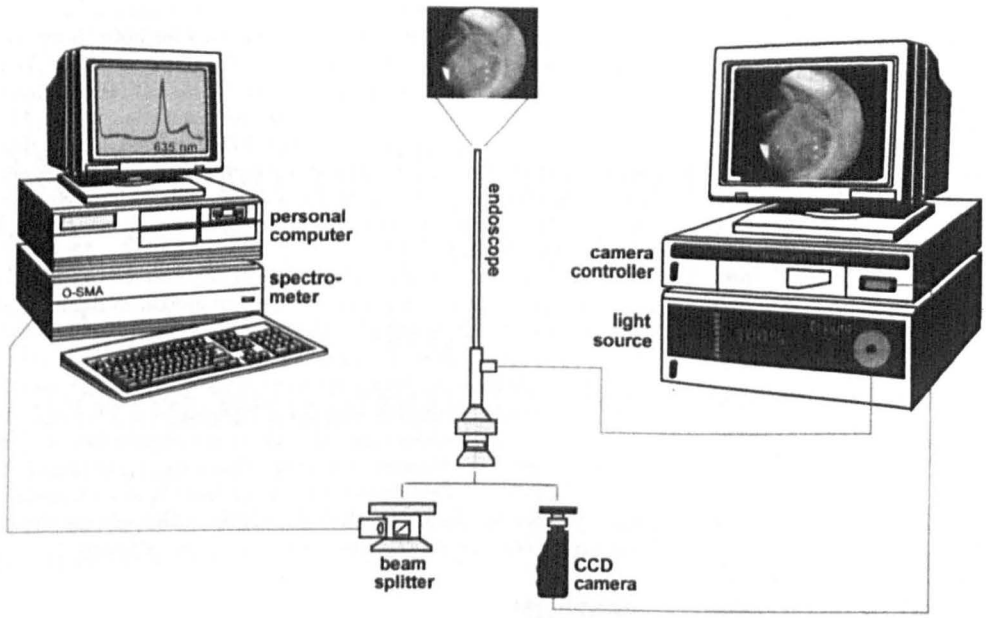

Fig. 1. Experimental setup used for protoporphyrin IX fluorescence measurements of normal and malignant tissue in the oral cavity.

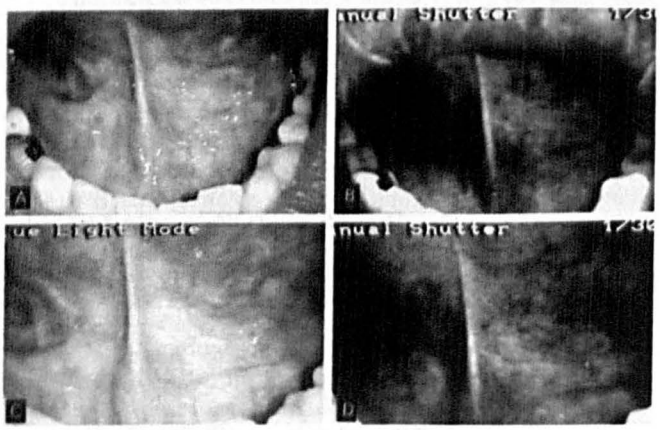

Fig. 2. Macroscopically visible contrasts in protoporphyrin IX fluorescence (fluorescence excitation [λ_{ex}] = 375–440 nm) between tumor and healthy tissues. **A.** Carcinoma of the right floor of the mouth (T1N0M0) in a 42-year-old patient under white light. **B.** Autofluorescence imaging under excitation with blue-violet light at the same localization. **C.** Malignant lesion and its surrounding tissue, again under ordinary white light 1.5 hours after application of 5-aminolevulinic acid (5-ALA). **D.** Protoporphyrin IX red fluorescence findings of the same tumor under fluorescence excitation.

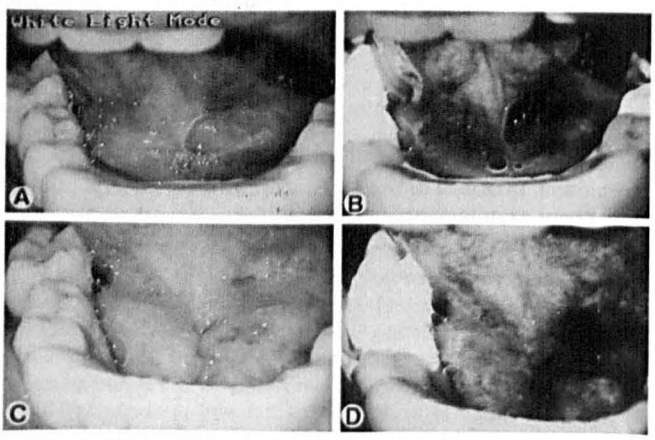

Fig. 3. **A.** Carcinoma in situ of the right side of the floor of the mouth in a 49-year-old patient under white light who was referred to us for treatment of a carcinoma of the left tonsil (T4N0M0). **B.** Appearance after excitation with blue-violet light fluorescence (λ_{ex} = 375–440 nm) at the same localization with no obvious pathological findings. **C.** Malignant lesion and its surrounding tissue, again under ordinary white light 1.5 hours after 5-ALA application. **D.** Protoporphyrin IX fluorescence findings of the same tumor under fluorescence excitation. The performed screening with 5-ALA revealed a suspected red fluorescence area in the right side of the floor of the mouth. The histological diagnosis of the biopsy was carcinoma in situ. Thus we were able to detect an early secondary cancer in this patient.

taken from normal and mildly dysplastic epithelium showed weak (F+) or strong (F++) fluorescence and were therefore counted as false-positive findings. All histopathological diagnoses of the 41 biopsy specimens taken from fluorescence-negative areas close to the tumors in the oral cavity were nonmalignant except for one carcinoma in situ. According to those results, the present study using the topical application of 5-ALA indicated a specificity of 60%, a sensitivity of 99%, a positive predictive value of 0.773, and a negative predictive value of 0.975.

No side effects could be observed during the course of this study. In particular, patients did not reveal any photosensitivity or signs for systemic absorption.

DISCUSSION

The prognosis for patients with oral cancer significantly improves with early detection of the malignant lesion. Thus several new methods for an improved diagnosis of early head and neck malignancies have been described over the past few decades. Dunn and Devine[12] described the fluorescence-aided marking of head and neck tumors using tetracycline agents in 1972. Epstein et al.[13] evaluated the imaging of cancerous tissue by double-staining the lesions with toluidine blue and Lugol's iodine.

In 1963, Richart[14] presented first experiments with toluidine blue in the field of gynecology. Toluidine blue is

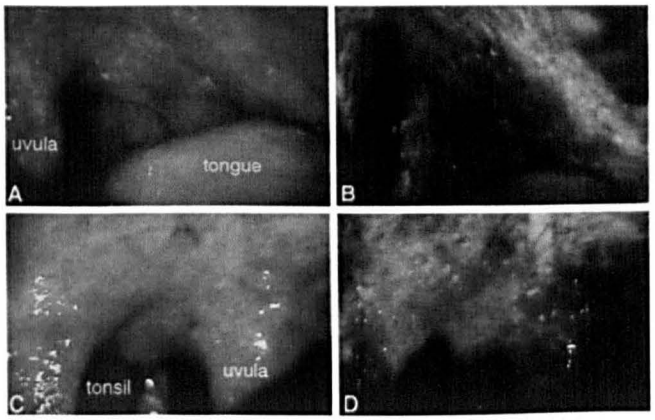

Fig. 4. Other interesting findings were observed in a 48-year-old patient **(A** and **B)** and a 55-year-old patient **(C** and **D)** with a squamous cell carcinoma of the left (T2N0M0) and right sides of the soft palate (T2N0M0). In both cases, under white light **(A** and **C)**, an exact definition of the extent of the tumor for adequate surgical margins of resection was not possible. After 5-ALA application **(B** and **D)**, the red fluorescence (λ_{ex} = 375–40 m) indicated a defined area, which showed tumor in histological diagnosis. Thus a more effective tumor resection could be performed.

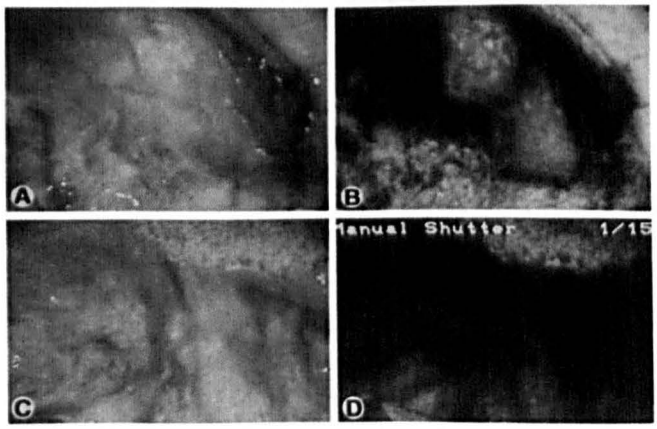

Fig. 5. **A** and **B.** A 66-year old patient with a squamous cell carcinoma (T1N0M0) of the left side of the tongue, before (white light illumination) and 1.5 hours after 5-ALA application (λ_{ex} = 375–440 nm). Four months after surgical treatment **(C** and **D)**, the tumor area is seen before (white light illumination) and 1.5 hours after 5-ALA application (λ_{ex} = 375–440 nm) with no sign of tumor.

a metachromatic dye of the thiazine group that binds to intracellular DNA and RNA. Selective staining properties of toluidine blue for neoplastic tissue might result from either higher levels of nucleic acids within neoplastic cells or wider and more numerous intracellular canals, which enhance penetration of the dye into the tissue.[13] Using this stain, he was able to accurately outline the surface margins of cervical carcinomas in situ. In the following decade, toluidine blue was subject to numerous trials and controversy in the field of otorhinolaryngology.[15–17] Generally this diagnostic method seems to provide the physician with considerable assistance in both detection and delineation of precancerous and cancerous lesions of the oral cavity. However, even though most promising results have been reported, general weaknesses of the method—

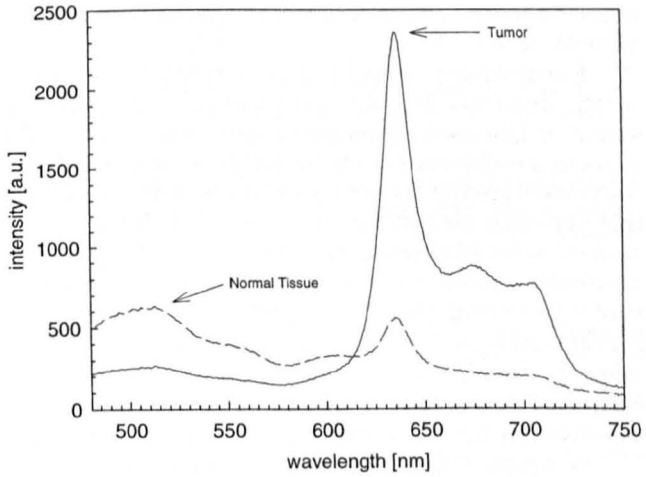

Fig. 6. Mean spectra of tumor and adjacent normal oral mucosa after application of 5-ALA in 29 patients (λ_{ex} = 375–440 nm).

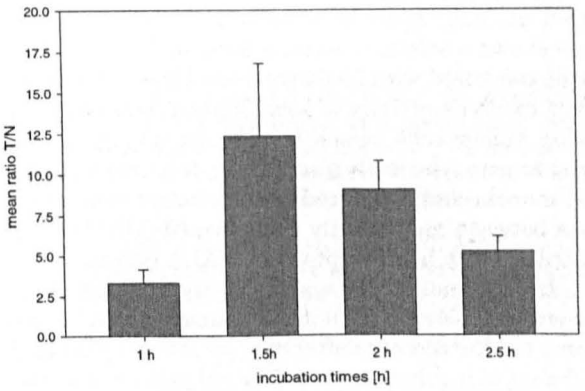

Fig. 7. The bars represent the ratios of protoporphyrin IX fluorescence between tumor and healthy tissue for 22 patients. Maximum red fluorescence intensity in the tumor tissue occurred 1.5 hours after 5-ALA application with a ratio of 12.5:1 between tumor and the adjacent healthy tissue.

TABLE I.
Histological Findings.

Histological Diagnosis	F++	F+	F−	Total
Squamous cell carcinoma	71	7	0	78
Carcinoma in situ	2	0	1	3
Moderate/severe dysplasia	9	3	0	12
Mild dysplasia	3	2	2	7
Normal tissue	7	15	38	60
Total	92	27	41	160

Specificity: 60%; sensitivity: 99%.

most apparent in a study presented by Sabes et al.,[16] who found a high percentage of false-positive and false-negative results—seem to have prevented it from becoming more widely used.

After the study of Leonhard and Beck[18] about the use of hematoporphyrin derivative for the diagnosis of tumors

in the head and neck region, several groups have used the same method.[19,20]

Furthermore, several groups investigated the use of autofluorescence for the detection of malignant tissues.[21–24] Our own observations have shown that differences in autofluorescence between tumor and normal mucosa varied greatly between different patients.[25] Although this approach yielded excellent results in some cases, it seemed to be insufficient in others.

However, none of these methods have found their way into clinical routine, because either they were not sensitive enough, they entailed enormous cost and a large number of qualified personnel, or because they have not been used on a sufficient number of patients to draw any objective conclusion concerning the value of the method.

Our present investigation aimed to evaluate the possible benefits of a fluorescence-aided detection of oral squamous cell carcinomas after topical application of 5-ALA.

After exogenous application of 5-ALA, PPIX is produced at high levels in eucaryotic cells. Many authors have shown a selective accumulation of PPIX in tumorous tissue compared with healthy tissue. Here, a changed rate of the catalytic activity of some heme-producing enzymes within tumor cells seems to play an important role.[6–9] Furthermore, the destroyed barrier function of tumorous oral mucosa and a reduced number of intercellular junctions between malignantly transformed cells[10,26–28] could contribute to a higher uptake of 5-ALA into tumor cells.

In all patients who were investigated, tumorous tissue was demarcated from its surrounding host tissue by a strong red fluorescence after application of 5-ALA and 1 to 2.5 hours of incubation time. Normal oral mucosa showed a bright green autofluorescence. The fluorescence contrast between tumors and healthy mucosa was quantified by spectral analysis. High subjective contrasts of cancerous tissue (red) to healthy mucosa (green) after 5-ALA application may be due to both a higher accumulation of PPIX and a decrease of green autofluorescence within the tumor. After 1.5 hours of incubation we found a maximum ratio of the PPIX fluorescence between malignant lesions and normal tissue. Therefore 1.5 hours seems to be the optimal time for fluorescence investigation.

Endogenous porphyrins have been controversial in discussions in the literature concerning their tumor-localizing properties.[29–36] A most common theory is that the observed red fluorescence is a product of microbial porphyrin synthesis and therefore its distribution is limited to the necrotic surface of exulcerated tumors.[30,33] Our own examinations have shown a bright red fluorescence attributable to endogenous porphyrins on the dorsum of tongues, on gingival plaques, and on the bacterial coating of necrotic parts of some of the oral neoplasms we have investigated in our previous studies.[11,37]

Only one third of the tumors examined (33%) seemed to be partly covered by strongly red fluorescing material.[25] Yet tumor-discriminating abilities were fairly limited using red fluorescence from endogenous porphyrins, because the observed "red spots" that represented bacterial porphyrin accumulations were not distributed homogeneously over the lesion's surfaces. The fact that 66% of evident tumors did not show any endogenous fluorescence and 33% showed only a nonhomogenous and partial staining disqualifies endogenous PPIX fluorescence for the detection of flat lesions or tumor boundaries.

For a histopathological evaluation of the method, 160 biopsy specimens were taken from fluorescing and nonfluorescing areas in the oral cavity. The results of the correlation between macroscopically visible findings of PPIX fluorescence at the biopsy sites and their histopathological diagnoses indicate a sensitivity of 99% for 5-ALA–induced PPIX, a specificity of 60%, a positive predictive value of 0.773, and a negative predictive value of 0.975.

A review of the literature shows that there has not been a controlled study using local application of 5-ALA in patients with head and neck tumors. According to initial results, local application of a 5% solution induces a twofold increase in intensity of PPIX fluorescence in tumor tissue as compared with the surrounding healthy tissue.[38] In contrast to these results, we determined a fluorescence intensity ratio of 12.5 to 1 between tumor and healthy tissue using a lower concentration (0.4%). In a study by Rick et al.,[39] side effects that were due to 5-ALA–induced PPIX after inhalation, intravesical instillation, or the use of ointment could be excluded.

Local application of 5-ALA combines the advantages of using a naturally occurring substance and the rapid metabolism of photosensitizing products with a short half-life and hence, only minimal skin photosensitivity. At the same time, this procedure is reproducible and easy to use.

5-ALA is an interesting and potentially valuable drug. As well as fluorescing, when excited by light of a certain wavelength, it may also be cytotoxic; therefore it is used frequently for photodynamic therapy. There is a potential role for both detection and photodynamic treatment at the same time. Photosensitization obtained after 5-ALA application has been successfully used for photodynamic therapy in vitro,[40] in animal tumor models,[41,42] in humans after topical administration in the treatment of cutaneous basal cell carcinomas,[43] and after systemic administration for the treatment of tumors of the mouth.[44]

The development of a noninvasive and accurate method for the screening and diagnosis, as well as treatment (photodynamic therapy), of oral cancer and areas of dysplasia would have great potential for improving the early detection of neoplastic changes and hence for improving patient quality of life and survival times.

CONCLUSION

Fluorescence staining of oral squamous cell carcinoma after topical application of 5-ALA presents a promising new procedure in the early detection of malignant oral lesions. The method is noninvasive, free of side effects, reproducible, and easy to use on an outpatient basis. The objective of further investigations will be the evaluation of this diagnostic procedure as a sensitive and clinically reliable method, as well as fluorescence-guided laser resection of oral cancer.

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