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ORIGINAL ARTICLE

Spin electron paramagnetic resonance of albumin for diagnosis of oral squamous cell carcinoma (OSCC)

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Abstract
Objectives Albumin has a known capability to modulate free serum concentrations of proteins produced by tumour cells. The technique of spin probe labelling of albumin followed by electron paramagnetic resonance (EPR) spectroscopy may allow identification of these structural and functional changes, which regularly occur as consequence of binding tumour metabolites as ligands. The aim of the present study was a proof of principle evaluation of EPR-analysis of peripheral blood samples as possible predictor for oral squamous cell carcinoma (OSCC).
Material and methods The present study is designed as gender-matched cohort. EPR was tested after retrieval of peripheral blood samples. The study group is represented by 32 patients with OSCC, and the control group consisted of 30 healthy patients.
Results Overall analysis exhibited a diagnostic sensitivity of 72% (23/32 OSCC group) and a specificity of 80% (24/30 control group). Subgroup analysis revealed ten patients with elevated leukocytes (>10,000/μl; $n=9$ [OSCC group] and $n=1$ [control group]). After exclusion of patients with elevated white blood cell count, sensitivity considerably increased to 87% and specificity to 83%.
Conclusion EPR analysis of peripheral blood samples might be appropriate to support the clinician in primary and follow-up diagnosis of potential tumours such as OSCC. Unfortunately, subgroup analysis characterises the method vulnerable to inflammation.
Clinical relevance Nevertheless, our preliminary results are intriguing, as diagnosis of OSCC appears possible by simple peripheral blood examination. Thus, further appraisal of this novel method with inclusion of different tumour entities, systemic conditions and inflammation in a larger study population appears highly valuable.

Keywords Albumin · EPR · Electron spin resonance · Oral cancer · Diagnosis

Abbreviations

SCC	Squamous cell carcinoma
OSCC	Oral squamous cell carcinoma
EPR	Electron paramagnetic resonance spectroscopy

Introduction

Oral squamous cell carcinoma (OSCC) represents a considerable worldwide problem as the overall survival rate is reported to be 54%. This outcome for survival is in most cases only achievable by invasive treatment with negative consequences for appearance and oral function. According to the report of the World Health Organization, oral malignancies represent the sixth most common tumour worldwide of which the most common subtype are squamous cell derived carcinoma (SCC) [1]. At present, therapeutic strategies are based on early detection of the primary lesion and are complemented by close recall concepts, which in

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combination allow appropriate adjustment of treatment goals [2]. Therefore, the clinician has to evaluate different mucosal lesions with respect to their biological dignity by inspection, palpation and pathological evaluation utilizing scalpel or brush biopsy in case of doubt [3]. Unfortunately, a lot of patients present recurring changes of the oral mucosa which macroscopically resemble a tumour-like condition. For these patients, repeated scalpel biopsies imply a burden and thus, a complementary approach of early detection in case of invasive carcinoma would be of high clinical value. A possible new approach for cancer diagnosis utilizes the vast array of tumour metabolites present in peripheral blood for identification of tumour derived metabolites. These substances possibly reflect the physiologic state of the tumour and may reveal the effect that the tumour has on the organism itself [4]. Bounding of these peptides by carrier proteins (e.g., albumin) protects them from catabolism and significantly amplifies their serum concentration [5]. Furthermore, these peptides may cause allosteric modifications of the albumin molecule (Fig. 1), consecutively leading to a change of binding capacity and transport properties, thus, rendering albumin a candidate for early tumour diagnosis in different tumour entities [6–8]. The core principle of this technique is the measurement of different albumin binding variables after addition of a fatty acid spin probe. The binding variables of the spin probe at different permutations of ethanol concentration, and the ratio of spin probe and albumin concentration is of importance. Non-covalent spin labelling of albumin in combination with electron paramagnetic resonance (EPR) spectroscopy describes an approach to evaluate the structural and functional changes that occur after binding of tumour related proteins as ligands (Fig. 2) [9]. The assessment of changes in albumin conformation, transport efficiency and binding characteristics by distribution of the fatty acid spin label on albumin in healthy individuals compared with changes observed in cancer patients has revealed unique alterations [6, 10]. These

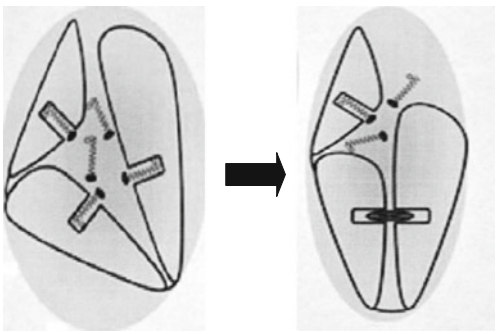

Fig. 1 Tumour cells secrete peptides and peptide fragments into the blood. Those are binding to albumin and may cause an alteration of fatty-acid binding capacity. Here, normal albumin becomes modified by vast tumour metabolites (modified from MedInnovation GmbH, Wildau, Germany)

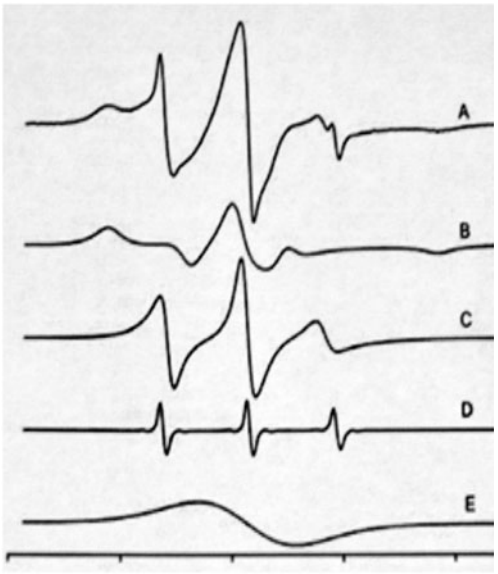

Fig. 2 The modified binding capacity of albumin for fatty acids can be measured by EPR. The composed analysis of the EPR spectrum (**a**) consists of the signals of the fatty acid binding sites 1 and 2 (**b** and **c**), the unaffiliated fatty acids (**d**) and the micelles of the fatty acids (**e**) (from MedInnovation GmbH, Wildau, Germany)

alterations can be assessed with EPR analysis and possibly offer distinct discrimination between healthy patients and those with cancer as well as with and without other pathologic conditions like sepsis or hepatic cirrhosis [9, 11, 12].

The benefit of EPR analysis for early OSCC diagnostic has not been investigated. Therefore, the aim of the present pilot study was to evaluate the EPR methodology as potential diagnostic tool for OSCC detection. The cases were proofed by histological evaluation as reported by the pathologist. The primary hypothesis was EPR measurement may allow diagnosis of OSCC by analysis of peripheral blood samples.

Materials and methods

Study design

The study was conducted in a gender-matched cohort design. EPR analysis was conducted blinded to the patients' clinical data.

Study population and sample handling

The study was approved by the local ethical committee (No. 837.233.08(6230)), and all individuals provided written informed consent for study participation. 7.5 ml of peripheral venous blood was obtained from 32 patients with histological confirmed OSCC in front of radical intended surgical

resection. The patients were treated between October 2007 and October 2008 at the Department of Oral and Maxillofacial Surgery, University Medical Centre Mainz. Blood samples were collected from 30 healthy individuals who comprised the control group. The clinical and pathological characteristics were obtained from medical records. Patient data are shown and summarized in Table 1. All peripheral venous blood samples were stored in serum tubes (Sarstedt, Nümbrecht, Germany) after serum isolation by centrifugation at 1,000 rpm for 15 min within 1 h after sampling. The serum was stored at −80°C until EPR analysis.

Electron paramagnetic resonance spectroscopy

All samples were blinded for the clinical data and sent to MedInnovation GmbH (Wildau, Germany) for further EPR spectroscopy. The samples were measured by EPR spin probe technique utilizing a commercially available EPR spectrometer (EPR-Analyzer/MMS, MedInnovation GmbH). Since the aim of the present study was the evaluation of the clinical effectiveness for OSCC diagnosis by EPR analysis, the highly sophisticated mathematical and methodological background of EPR can only be shortly summarized by the authors as described in-depth elsewhere before [9, 12] (see below).

Characterization of spin probe binding affinity to albumin is possible by incubation with varying ethanol concentrations and changes in the relationship of spin probe to albumin concentration at different hydrophobic conditions. Commercial 16-doxyl stearic acid (TCI Deutschland GmbH, Eschborn, Germany) was applied as spin probe on the basis of an extremely high binding affinity for albumin to this stearic acid (6.9×10^7 l/mol), generally leading to >99.9% binding of this spin probe to albumin. Modification in binding affinity was induced by extra pure Ethanol (Merck, Darmstadt, Germany). Time from thawing to analysis of the frozen serum samples was within 2 h. Each 50 µl of serum sample was analyzed at separate aliquot concentrations. Each aliquot received a defined concentration of ethanol and binding probe for further analysis on a microtiter plate covered by Parafilm. After incubation on a microtiter shaker for 10 min at 37°C, the aliquots were transferred into capillary glass tubes for analysis within the EPR-Analyzer/MMS (temperature: 37°C). Analysis of the aliquots in triplets took 1 min of reading performance. The EPR-Analyzer/MMS utilized a microwave power of 15 mW at a frequency of 9.5 GHz. The magnetic field strength with a scan range of 12 mT was 0.34 T embedding a modulation amplitude of 0.07 mT. Analysis of the EPR spectra followed a complex mathematical computer simulation of its components related to a Hamilton spin function with axial anisotropy also previously described in detail [13]. The EPR spectra of the experimental probe delivered a vast number of data points, which were approximated to an ideal spectral curve during simulation. The point estimates included g factors, hyperfine structure constants, and line widths that characterized the shape and intensity of each spectral component. These variables were utilized to approximate biophysical characteristics of the 16-doxyl stearic acid spin label including the "angle of the spin-labelled molecule axis' precession", "polarity of the environment surrounding the spin label", and the "rotation speed of the spin label". For differentiation of patients with cancer from healthy individuals, a discriminant variable (DR) was calculated using a squared discriminant analysis and calculated as a linear function as also previously described [12].

Table 1 Demographic data of control and OSCC group

Group	Median age (years)	Age range	Male/female
Healthy (n=30)	30	22–45	15:15
OSCC (n=32)	55	35–68	18:14

Statistics

Sensitivity and specificity as fundamental parameter for the clinical relevance of the EPR method were calculated via crosstabs. As the tumour patients were significant older as the control group, a Student's t-test was conducted to test, if age influenced the diagnosis by EPR. Chi-square tests were performed for possible correlations between EPR results and TMN category parameters as well as the localization of the primary tumour.

This study is characterized as an exploratory investigator initiated clinical study without further funding. Thus, the obtained data are limited, since only a relative small sample size was recruitable. As consequence, the authors suggest the reported p values to be descriptive, with $p\leq0.05$ considered significant. Statistical analyses were conducted using SPSS version 15.0 (SPSS, Chicago, IL, USA).

Results

The demographic data of the two groups are summarized in Table 1, comprising a summary of the OSCC characteristics for patients in group 2 (Tables 2 and 3). The threshold value of the DR was set to 1.0 for the differentiation of patients with and without cancer. The diagnostic sensitivity of the test for discriminating patients with invasive carcinoma from healthy patients was 72%. Diagnostic specificity was somewhat higher at 80%.

Inspection of the patient records revealed an elevated leukocyte count (>10,000/µl) compared to the normal

Table 2 TMN categorization of the OSCC group

TMN category	OSCC group (n=32)
T1	11
T2	10
T3	6
T4	5
M0	32
N0	26
N1	6

values (3,500–10,000/μl) of our laboratory in ten cases. For evaluation of a possible interference with respect to the EPR measurements, a subgroup analysis with exclusion of these patients was carried out. Within this subgroup sensitivity increased to 87% and diagnostic specificity to 83%. Within the subgroup presenting with leukocyte elevation, sensitivity and specificity were poor. Test results of the different groups are summarized in Table 4.

The patients which were correctly diagnosed for OSCC had a mean age of 42.9 years (standard deviation [SD], 14.61), while patients which were diagnosed incorrectly for OSCC had a mean age of 43.3 years (SD 14.1; p=0.915). Within the limits of this pilot study, no influence of patients' age on EPR analysis results was found within the present study (p=0.915). Nevertheless, a possible influence of age on EPR test results has to be ruled out in a prospective clinical study taking a larger patient population into account.

Further patient record analysis revealed no association between EPR spectra and TMN category parameters (T: p=0.801; N: p=0.677; Grading: p=0.181) as well as the localization of the primary tumour (p=0.272).

Discussion

Albumin represents approximately two thirds of total plasma proteins inheriting different metabolic functions. On the one hand, albumin plays a major role in plasma oncotic pressure maintenance, and on the other hand, it preserves amino acids as structural supplement for other proteins. It carries a mixture of low molecular weight molecules resulting in a prolonged half-life of 15–19 days, thus, binding to albumin extends the half-life of these substances that would otherwise be metabolized or eliminated [5]. Additionally, albumin functions as transporter for fatty acids and five different primary binding sites were identified in the crystal structure of fatty acid–albumin complexes. Within these, albumin carries one or two long-chain fatty acids at typical physiologic conditions [14, 15]. Furthermore, this general behavior allows spin labeling of albumin in combination with EPR spectroscopy as potential approach for detecting structural changes of this protein [9]. The shape of the EPR spectrum reflects the state of the spin probe molecules, characterized by the molecular motion as also the electric and magnetic fields in the surrounding environment [13]. Changes in the mobility and accessibility like the distances between the spin labels lead to variations in the secondary and tertiary structure of proteins to be discerned by EPR spectroscopy. Recent results of EPR spectroscopy applied in animal models and humans suggest that EPR may have diagnostic potential [16]. Although X-ray crystallography gives insight into the three-dimensional structure of a protein, spin labeling combined with EPR spectroscopy may offer a supplemental way for the detection of important functional changes in the protein structure. The high sensitivity of EPR spectrometer allows the investigation of small sample volumes in a range of 50–100 pmol, whereas no upper limit for the molecular weight of proteins exists. The varying nature of binding proteins and peptides as representatives of different tissues could also reflect important disease-related information, such as presence of invasive malignoma or inflammation. A recent study could demonstrate that carrier proteins like albumin represent a rich source of disease associated biomarkers, thus potentially supporting new models as base for the molecular composition of the circulation [8]. A previous investigation in patients with stomach cancer clearly exhibited a decrease in albumin binding reserve in patients with operable disease vs. those with non-operable cases [17]. Hence, our pilot study investigated the EPR spectral changes of albumin in

Table 3 Localization of the OSCC

OSCC localization	OSCC group (n=32)
Mandible	11
Mouth floor	10
Tongue	8
Lip	2
Buccal mucosa	1

Table 4 Diagnostic sensitivity and specificity of EPR spectroscopy of serum albumin for the differentiation of cancer patients from healthy volunteers

Group comparison	Sensitivity (%)	Specificity (%)
All patients		
OSCC vs. healthy	72 (23/32)	80 (24/30)
Patients with leukocytes <10,000/μl		
OSCC vs. healthy	87 (20/23)	83 (24/29)
Patients with leukocytes >10,000/μl		
OSCC vs. healthy	33 (3/9)	0 (0/1)

a group of OSCC patients compared to healthy controls to evaluate the effectiveness of this approach for OSCC detection. Our first results render this novel technique to be promising for the identification and monitoring of patients with invasive OSCC. Unfortunately, the outcome was negatively influenced, when clinical signs of inflammation were present, thus, limiting the clinical value of the method. Therefore, an influence on albumin binding characteristics by inflammation markers seems to be possible, although not evaluated yet. However, subgroup analysis of patients with normal leukocyte (3,500–10,000/μl) count revealed acceptable sensitivity and specificity for EPR analysis as complementary tool in early diagnosis of invasive oral malignancy. The results are intriguingly, due to detection of oral cancer appears to be possible solely by simple investigation of peripheral blood samples. Since EPR analysis turned out to become positive in other types of cancer [6, 10], systemic conditions [2, 9] and as this study indicates — inflammation — it cannot be stated as a distinct method for specific OSCC diagnosis. On the other hand, novel methods for fast and cost-effective tumour detection are of high clinical value. Thus, the present data is worth to be verified in a larger study population with inclusion of different tumour entities, systemic conditions and inflammation.

Conflict of interest statement The authors MM, PWK, MOK and BA declare that they have no conflict of interest. The study was conducted in cooperation with MedInnovation GmbH Berlin which performed the EPR analysis of the blood samples blinded to the clinical data as regular purchased order. The author KS works for MedInnovation GmbH Berlin as chief of the laboratory and supported the paper with respect to methodological background information. All authors have read and approved the final manuscript.

Additional note The study was approved by the local ethical committee (No. 837.233.08(6230)) and all individuals provided written informed consent for study participation. The study has been presented at the 60th annual meeting of the German Society of Oral and Maxillofacial Surgery, Munich, Germany 2010 and was awarded for the best poster presentation. The poster itself was published in *Int Poster J Dent Oral Med* 2011, Vol. 13, No. 1, Poster 522.

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