Acta Odontologica Scandinavica. 2015; 73: 336–342

informa
healthcare

ORIGINAL ARTICLE

Apoptosis resistance-related ABCB5 and DNaseX (Apo10) expression in oral carcinogenesis

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Abstract

Background. Apoptosis resistance is a crucial factor for the carcinogenesis of oral squamous cell carcinoma (OSCC). ***Methods.*** Expression of apoptosis resistance-related ATP-binding cassette (ABC) transporter ABCB5 [subfamily B (MDR/TAP) member 5] and DNaseX (Apo10) were analyzed in normal oral mucosa ($n = 5$), oral precursor lesions (simple hyperplasia, $n = 11$; squamous intraepithelial neoplasia, SIN I–III, $n = 35$), and OSCC specimen ($n = 42$) by immunohistochemistry. ***Results.*** Expression of ABCB5 and Apo10 were significantly increased in the carcinogenesis of OSCC compared with normal tissue. Compared with SIN I–III, ABCB5 expression was significantly decreased in OSCC. Apo10 expression did not significantly differ from OSCC compared with SIN I–III. ***Conclusions.*** This study provides the first evidence of the expression of ABCB5 and Apo10 in the multi-step carcinogenesis of OSCC. Overcoming drug resistance of ABCB5+ and Apo10+ cells in precursor lesions and tumors by natural compounds may act as sensitizers for apoptosis or could be useful for chemoprevention.

Key Words: *oral squamous cell carcinoma, apoptosis resistance, ATP-binding cassette (ABC) transporter, DNaseX, early detection and diagnosis*

Introduction

In general, cancer disease is regarded as an acquired genetic multi-step process. The rise of malignant tumors from a single transformed cell (monoclonal theory of carcinogenesis) and subsequent development through morphologically and clinically detectable pre-cancerous stages is described by the model of multi-step carcinogenesis [1]. In oral squamous cell carcinoma (OSCC) the carcinogenesis is regarded as a highly complex multifocal process that occurs when squamous epithelium is affected by several genetic alterations [2]. Focusing on the mechanistic basis harbors the availability of molecular tools to selectively and experimentally manipulate this multi-step process. Subsequent clinical implications for diagnosis and therapy of precursor lesions as well as OSCC are expected from this basic scientific research.

OSCC is an aggressive tumor with low response to chemotherapy and basic resistance to most standard of care anti-cancer drugs [3,4]. Apoptosis is the process of programmed cell death that may occur in multi-cellular organisms and is a genetically regulated cell death involved in the deletion of cells in normal as well as malignant tissues [5]. Apoptosis resistance [6] is regarded as a crucial factor for the carcinogenesis of OSCC and is associated with radio- and chemotherapy resistance, as well as tumor recurrence [7,8].

The effectiveness of cytostatic chemotherapy to induce apoptosis in OSCC might be restricted due to an inducible cellular mechanism called multi-drug resistance (MDR) [8,9] that can be mediated by elevated expression of ATP-binding cassette (ABC) transporters. The human ATP-binding cassette (ABC) transporter ABCB5 [sub-family B (MDR/TAP) member 5] acts as an energy-dependent cytostatic drug efflux transporter and marks tumor cells of a putative cancer stem cell (CSC) compartment [9,10].

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(Received 29 May 2014; accepted 24 August 2014)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2014.961029

The biochemical suicide molecule endonuclease DNaseX (DNaseI-like 1) has been used to identify the Apo10 protein epitope that marks tumor cells with abnormal apoptosis and proliferation [7].

Recently, we have demonstrated apoptosis resistance-related ABCB5 [9] and Apo10 [7] as adverse prognostic factors for the survival of patients with OSCC. However, the expression of ABCB5 and Apo10 during a multi-step carcinogenesis has not been described yet. Therefore, the purpose of this study was to examine the relationship between apoptosis resistance-related proteins ABCB5 and Apo10 with a multi-step carcinogenesis.

Materials and methods

Patients and tumor specimen

The records of healthy individuals (normal oral mucosal tissues, $n = 5$), patients with oral precursor lesions (simple hyperplasia, $n = 11$; squamous intraepithelial neoplasia SIN I, $n = 5$; SIN II, $n = 9$; SIN III, severe dysplasia, $n = 10$; SIN III, carcinoma *in situ*, $n = 11$) and patients with invasive OSCC ($n = 42$, Table I) were retrospectively assessed from January 2009 to December 2013. The diagnosis of normal oral mucosal tissues, precursor lesions and invasive squamous cell carcinoma was confirmed by the department of Pathology, University Hospital Tuebingen. The material was archival formalin-fixed, paraffin-embedded tissue from routine histopathological work-ups. The material has been stored with permission of the local ethics committee of the University Hospital Tuebingen (approval number: 562-2013BO2), after informed consent obtained from the patients prior to surgical resection. Tumor blocks of paraffin-embedded tissue were selected by experienced pathologists, evaluating the routine H&E stained sections. Sections from all available tissues underwent histopathological assessment, blinded to the prior histopathology report. Serial tissue sections (2 μm thickness) were cut from formalin-fixed paraffin-embedded (FFPE) blocks on a microtome and mounted from warm water onto adhesive microscope slides. First, we assessed H&E sections from each tissue section to differentiate between normal tissue,

Table I. Clinicopathological characteristics of 42 patients with OSCC.

Characteristics	Number of patients, total $n = 42$
Gender	
Male	24
Female	16
Histological grading	
G1	7
G2	25
G3	9
G4	1
Depth of invasion	
pT1	10
pT2	11
pT3	5
pT4	16
Cervical lymph node metastasis	
pN0	26
pN1	4
pN2	10
pN3	2
UICC stage	
UICC I	8
UICC II	4
UICC III	6
UICC IV	24
Distant metastasis	
Yes	2
No	40

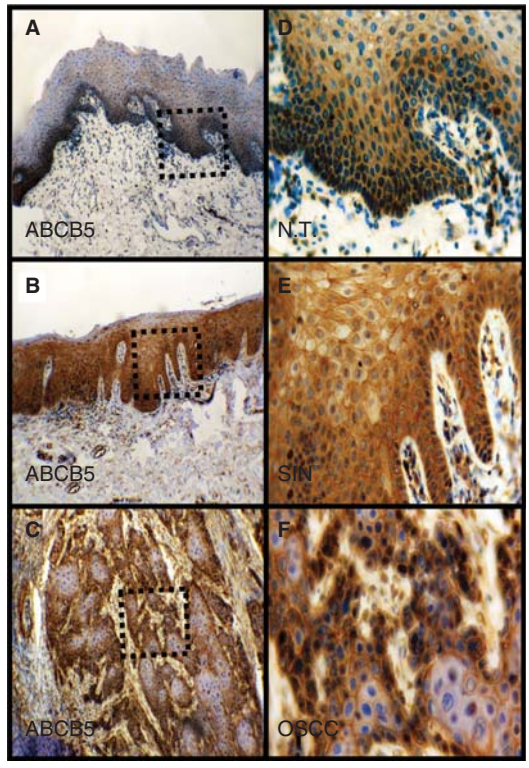


Figure 1. Immunohistochemical staining of ABCB5 in OSCC. Immunohistochemical staining shows representative images of ABCB5 in N.T. (A, D), SIN (B, E) and OSCC (C, F). Brown chromogen color (3,3′-Diaminobenzidine) indicates positive staining, the blue color shows the nuclear counterstaining by hematoxylin. The square box demonstrates the area of interest (original magnification: ×100-fold, left panel) which is also shown in larger magnification (×200-fold, right panel). ABCB5, ATP-binding cassette sub-family B member 5; SIN, squamous intraepithelial neoplasia; N.T., normal tissue.

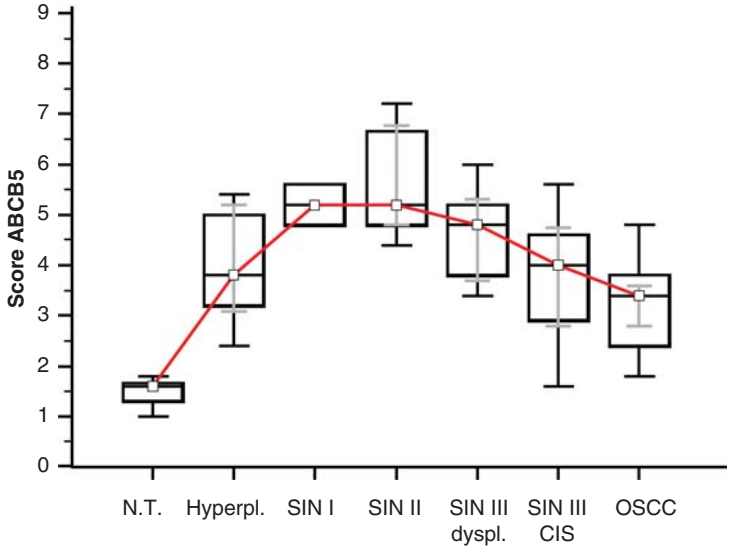
precursor lesions, tumor cell areas, stromal areas and infiltrating immune cells. Oral precursor lesions were classified according to WHO criteria [1]. Tumor staging was performed according to the 7th edition of the TNM staging system by the UICC/AJCC of 2010 [11]. Grading of OSCC was defined according to WHO criteria [12].

Staining procedure and quantification of immunohistochemistry

We stained for ABCB5 (Atlas Antibodies AB, Stockholm, Sweden, rabbit anti-human ABCB5 pAb, HPA026975, dilution: 1:100 [9]) and Apo10 (TAVARTIS GmbH, Hainburg, Germany, rat anti-human mAb, 5 µg/ml [7]) in tissue sections. Staining was performed on serial sections of 2 µm thickness, which were deparaffinized in xylene and ethanol and rehydrated in water. Heat induced epitope retrieval (HIER) was performed with citrate buffer pH 6.0 (Dako, Hamburg, Germany). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. Endogenous biotin activity was blocked using the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). After incubation with the primary or control antibody the Dako LSAB2 peroxidase System (Dako, Hamburg) or biotin-conjugated AffiniPure donkey-anti-rat IgG (Jackson ImmunoResearch Laboratories Inc., Suffolk, UK) was used. Slides were subsequently incubated for 3–5 min in DAB (3,3′-diaminobenzidine, Biogenex) counterstained with haemalaun and mounted with Glycergel (Dako).

Five representative high power fields (1 HPF = 0.237 mm^2, original magnification: ×200-fold) were analyzed for ABCB5 and Apo10 expression in

A



B

Factor	n	Different ($p < 0.05$) from factor nr
(1) N.T.	5	(2)(3)(4)(5)(6)(7)
(2) Hyperplasia	11	(1)(3)(4)(5)(7)
(3) SIN I	5	(1)(2)(6)(7)
(4) SIN II	9	(1)(2)(6)(7)
(5) SIN III (sev. dysplasia)	10	(1)(2)(6)(7)
(6) SIN III (CIS)	11	(1)(3)(4)(5)(7)
(7) OSCC	42	(1)(2)(3)(4)(5)(6)

Figure 2. Immunohistochemical analysis of ABCB5 in normal oral mucosal tissue, oral precursor lesions—hyperplasia, SIN and invasive OSCC. In comparison with normal tissue a significantly increased expression of ABCB5 is observed in hyperplasia, SIN I and SIN II lesions ($p < 0.05$, Kruskal-Wallis Test; A and B). In comparison with SIN I, SIN II and SIN III (sev. dysplasia, CIS), a significantly decreased expression of ABCB5 is observed in OSCC. ABCB5 expression is significantly decreased in OSCC compared with SIN I–III ($p < 0.0001$, Mann-Whitney U-test). Analysis refers to averaged scores. The red line indicates ABCB5 expression results during carcinogenesis. The grey lines show 95% confidence intervals. Analysis of significant statistically different single values is indicated in the table below (B). SIN III is subdivided in severe dysplasia and carcinoma *in situ* (CIS). ABCB5, ATP-binding cassette sub-family B member 5; SIN, squamous intraepithelial neoplasia; N.T., normal tissue.

normal tissue, oral precursor lesions, tumor tissue and averaged, respectively. The extent of the staining, defined as the percentage of positive staining areas of tumor cells in relation to the whole tissue area, was semi-quantitatively scored on a scale of 0–3 as the following: 0, <10%; 1, 10–30%; 2, 30–60%; 3, >60%. The intensities of the signals were scored as 1+, 2+ and 3+. Then, a combined score (0–9) for each specimen was calculated by multiplying the values of these two categories [13]. Cases were classified as negative, 0 points, positive, 1–9 points. Two observers blinded to the diagnosis performed scoring on identical sections marked by circling with a water-resistant pencil and finally with diamond-tipped pencil on the opposite side of the microscopic slide. Pictures were analyzed using a Canon camera (Krefeld, Germany). The photographed images were imported into the Microsoft Office Picture Manager.

Statistical analysis

Statistical analysis was performed with MedCalc Software, Version 13.1.1 (Mariakerke, Belgium). Data were analyzed using the non-parametric Mann-Whitney U-test or Kruskal-Wallis test when more than two groups were compared. All p-values presented were 2-sided and $p < 0.05$ was considered statistically significant.

Results

Expression of ABCB5 and Apo10 in normal mucosa, oral precursor lesions and OSCC

ABCB5 was found expressed in all tissue types (Figures 1 and 2), normal oral mucosa (n = 5/5), oral precursor lesions (simple hyperplasia, n = 11/11; squamous intraepithelial neoplasia, SIN I–III, n = 35/35) and OSCC specimen (n = 42/42). Apo10 was found expressed in all tissue types (Figures 3 and 4), normal oral mucosa (n = 2/5), oral precursor lesions (simple hyperplasia, n = 11/11; squamous intraepithelial neoplasia, SIN I–III, n = 35/35) and OSCC specimen (n = 42/42). In comparison to normal tissue and hyperplasia, a significantly ($p < 0.05$) increased expression of ABCB5 (Figures 1 and 2) and Apo10 (Figures 3 and 4) was observed in tumor cells of OSCC. Compared with SIN I–III, ABCB5 expression was significantly decreased in OSCC (Figures 1 and 2). Apo10 expression did not significantly differ from OSCC compared with SIN I–III (Figures 3 and 4).

Discussion

For the first time, we analyzed apoptosis resistance-related proteins ABCB5 and Apo10 in the carcinogenesis of OSCC. In this study, we analyzed

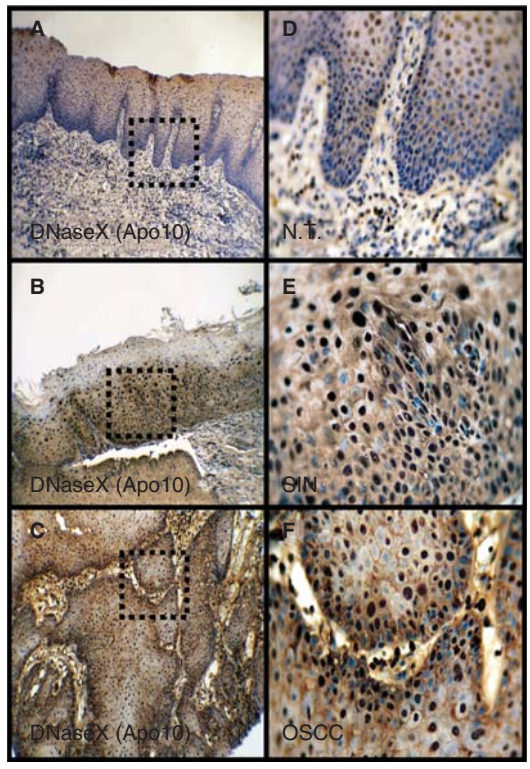
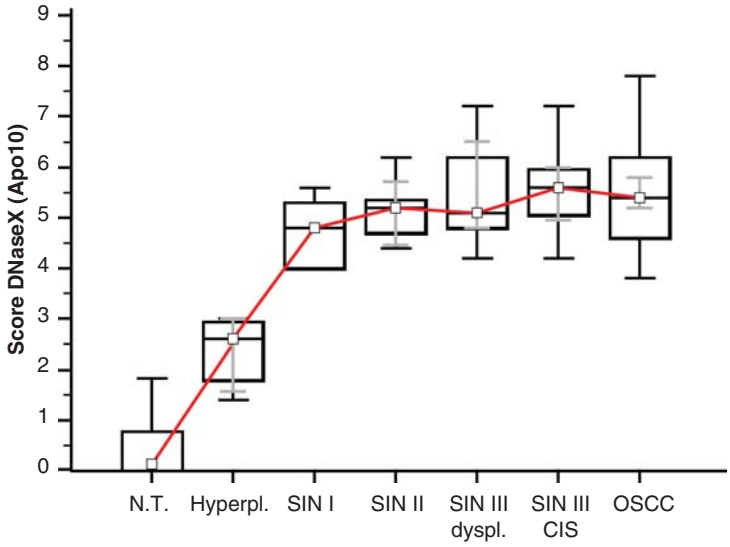


Figure 3. Immunohistochemical staining of Apo10 in OSCC. Immunohistochemical staining shows representative images of Apo10 in N.T. (A, D), SIN (B, E) and OSCC (C, F). Brown chromogen color (3,3′-Diaminobenzidine) indicates positive staining, the blue color shows the nuclear counterstaining by hematoxylin. The square box demonstrates the area of interest (original magnification: ×100-fold, left panel) which is also shown in larger magnification (×200-fold, right panel). SIN, squamous intraepithelial neoplasia; N.T., normal tissue.

increased expression of ABCB5 and Apo10 in OSCC compared with normal oral mucosa. However, compared with SIN I–III, ABCB5 expression was significantly decreased in OSCC.

To date there are two cancer models described to explain tumor heterogeneity and inherent differences of tumor-regenerating capacity [14]. The CSC hypothesis regards malignant transformation as a process, occurring in a sub-set of normal stem cells with pluripotent properties, which underlie deregulation of self-renewal pathways [15,16]. In contrast, the clonal selection model of multi-step carcinogenesis implies that a random solitary cell undergoes malignant transformation, accumulates multiple mutations and subsequently acquires a survival advantage, which leads to clonal selection [17,18]. Our results of significant up-regulation of ABCB5 in precursor lesions and down-regulation in OSCC are in concordance to results in other solid tumor entities investigating CSC markers during carcinogenesis in our previous study [19]. However, it is important to note that the two models are not mutually exclusive, as CSCs themselves may undergo clonal evolution, as already shown for leukemia cells [20,21]. Our results of decreased ABCB5 expression in OSCC might be explained with

A



B

Factor	n	Different ($p < 0.05$) from factor nr
(1) N.T.	5	(3)(4)(5)(6)(7)
(2) Hyperplasia	11	(3)(4)(5)(6)(7)
(3) SIN I	5	(1)(2)
(4) SIN II	9	(1)(2)
(5) SIN III (sev. dysplasia)	10	(1)(2)
(6) SIN III (CIS)	11	(1)(2)
(7) OSCC	42	(1)(2)

Figure 4. Immunohistochemical analysis of Apo10 in normal oral mucosal tissue, oral precursor lesions—hyperplasia, SIN and invasive OSCC. In comparison to normal tissue/hyperplasia, a significantly ($p < 0.05$, Kruskal-Wallis Test; A and B) increased expression of Apo10 is observed in SIN I, SIN II, SIN III and invasive OSCC. Apo10 expression does not significantly differ from OSCC compared with SIN I–III ($p = 0.4821$, Mann-Whitney U-test). Analysis refers to averaged scores. The red line indicates ATP synthase expression results during carcinogenesis. The grey lines show 95% confidence intervals. Analysis of significant statistically different single values is indicated in the table below (B). Apo10 is sub-divided in severe dysplasia (sev. dysplasia) and carcinoma *in situ* (CIS). SIN, squamous intraepithelial neoplasia; N.T., normal tissue.

the clonal selection model of carcinogenesis, which proposes that there is a subsequent clonal selection of putative stem cells [14,19]. Furthermore, these cancer stem cells may be inherently resistant to our current therapeutic approaches. Overcoming drug resistance by enhancing apoptosis of precursor and tumor cells is a key event for cancer prevention and therapy [22]. In this context, bioactive food components [23–26] have been demonstrated for induction of apoptosis and play an emerging role in cancer therapy. One mechanism is the activation of endogenous reactive oxygen species (ROS) [27,28] in OSCC [29–33], with subsequent apoptosis in cancer cells.

With specific regard to ABCB5, natural compounds like nanaomycin A, streptonigrin, toyocamycin, bryostatin 1, siomycin A, illudin M, michellamine B and pentoxifylline were shown to induce apoptosis in melanoma cells [34]. Authors assume that overcoming drug resistance of ABCB5+ precursor lesions and tumors by these natural compounds could be useful for chemoprevention or may act as sensitizers for apoptosis in OSCC. However, this hypothesis has to be evaluated in further studies.

A previous study by Taper [35] demonstrated that vitamin C and K3 administration produced *in vitro* and *in vivo* tumor growth inhibition, potentiation and sensitization of chemo- and/or radiotherapy and a decrease in the number of metastases in animals with experimental tumors by reactivation of DNase activity. Therefore, vitamin C and K3 application may be considered as a possible new, non-toxic, adjuvant cancer therapy, which can be easily introduced into the classic protocols of clinical cancer therapy without any supplementary risk for Apo10+ patients.

Moreover, our results show that Apo10 expression did not significantly differ from OSCC compared

with SIN I–III. The development of multi-focal pre-cancerous lesions or tumors in the upper aerodigestive track is not surprising. Based on the hypothesis of the field cancerization [36,37] the tissue is exposed to the two main exogenous carcinogenic factors, alcohol and tobacco [38]. If the carcinogenic stimuli continue, second metachronous tumors in related sites may occur [38]. Therefore, persisting increased levels of Apo10 post-operatively detected in blood samples using the epitope detection in monocytes (EDIM) technique [7] can be a marker for the early detection of apoptosis resistance in pre-cancerous lesions or invasive carcinomas. However, tissue-specific markers giving an indication for a topographical anatomic region using the EDIM blood test are still outstanding, but they are currently under development.

As suggested for multi-step carcinogenesis [2] it remains unclear whether phytochemicals are standardized effective for chemoprevention [2,25,26,39–47] in the treatment of precursor lesions or OSCC development, but they provide a clear rational for further studies in the carcinogenesis of OSCC [2,41–48].

Conclusions

This study provides the first evidence of the expression of ABCB5 and Apo10 in the multi-step carcinogenesis of OSCC. Overcoming drug resistance of ABCB5+ [34] and Apo10+ [35] cells in precursor lesions and tumors by natural compounds may act as sensitizers for apoptosis or could be useful for chemoprevention.

Acknowledgment

We thank Julia Grimm for her technical assistance.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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