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CK8 correlates with malignancy in leukoplakia and carcinomas of the head and neck

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Abstract

Screening of head and neck carcinoma patients with the proteomics-based AMIDA technology yielded a set of tumour-associated antigens, including the intermediate filament protein cytokeratin 8 (CK8). The expression pattern and specificity of CK8 was compared with those of the established markers pan-cytokeratins and CK13, and with that of the proliferation marker Ki67. Expression of CK8 correlated positively with malignancies of the head and neck areas. CK8 was not expressed in healthy epithelium, except for some rare cases of cells of the basal layer and laryngeal tissue. In contrast, the vast majority of head and neck squamous cell carcinomas and metastases strongly expressed CK8. Interestingly, CK8 de novo expression correlated with dysplastic areas of oral leukoplakic lesions, while hyperplastic leukoplakia remained CK8-negative but strongly panCK and CK13 positive. Thus, CK8 is an attractive marker molecule for a differentiated diagnosis of leukoplakia and head and neck carcinomas, which possesses notedly improved specificity as compared with panCK and CK13.

Keywords: CK8; Leukoplakia; Carcinoma; AMIDA; Ki67

The knowledge of tumour-specific molecular markers is of paramount importance with respect to a more particularized, earlier diagnosis, and individual therapy of malignant diseases, which may result in an improved prognosis eventually. We have recently reported on a novel technology termed AMIDA for autoantibody mediated identification of antigens, designed to isolate and identify such tumour-associated antigens (TAAs) [1,2]. Screening of primary head and neck carcinoma samples and cell lines yielded a set of proteins with immunogenic potential in patients. These proteins included cytokeratins (6d, 8, mutant 9, and 16), signalling molecules (Grb2, ZNF-70), chaperones (Grp78, Hsp27, and Hsc70), metabolism-associated proteins (E-FABP, cytidine deaminase, and eIF3S2), and nuclear proteins (hnRNP H, PSP-1). Subsequent experimental validation demonstrated that cancer patients have significantly increased antibody titres against CK8, E-FABP, and Grp78 [1,2]. Additionally, CK8 revealed to be present at the external cell surface of tumour cells, while absent on healthy tissue with the exception of hepatocytes [3]. Biochemical approaches demonstrated the capacity of cell surface-associated CK8 to bind component of the urokinase plasminogen activator system and eventually lead to the activation of plasmin [4–6]. Thus, several lines of evidence pointed towards CK8 as a promising candidate TAA for further detailed studies.

In the present report, the specificity and sensitivity of the AMIDA antigen CK8 was compared with the reactivity of established markers for carcinomas, i.e., panCK and CK13. Serial sections of healthy mucosal specimens and squamous cell carcinomas of the head and neck area were stained with antibodies specific for the selected antigens to enable a direct comparison of these marker molecules. In

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healthy mucosa, CK8 was expressed only in cells of the basal membrane layer, if at all. This is in sharp contrast to panCK and CK13, which were both present to high expression level throughout the entire healthy epithelium. Noteworthy, CK8 antibodies stained strongly carcinoma areas without cross-reacting with surrounding stromal tissue or immune cells. Furthermore, the expression of CK8 was studied in oral leukoplakia, which is regarded as a potential precursor of oral malignancies [7]. The expression of CK8 allowed to distinguish between dysplastic tissue, carcinoma in situ, and asymptomatic or hyperplastic leukoplakia. Thus, CK8 has great potential with respect to a differentiated diagnosis and may further help to improve the identification and staging of pre-malignancies, recurrent, and residual tumour cells.

Materials and methods

Tissue samples. Twenty-six cases of squamous cell carcinomas, two lymph node metastases thereof, which had been confirmed by clinical diagnosis and histopathologic examination, 9 healthy oral mucosa specimens, and 10 oral leukoplakia were included in the investigation. Briefly, tissue specimens were shock-frozen in liquid nitrogen and thereafter embedded in tissue-tek (Sakura, Fintek, NL) to generate 4 μm serial sections (5 carcinomas, 5 healthy specimens, and 10 leukoplakia) and non-consecutive sections (21 carcinomas, 4 healthy specimens, and 2 lymph node metastases), respectively.

Immunohistochemistry. The following primary antibodies were used: mouse anti-human pan-cytokeratin KL1 (Immunotech, Marseille, France) (dilution 1:5 in 0,1 M phosphate-buffered saline (PBS), pH 7.4), mouse anti-human CK13, clone KS-1A3 (Sigma, Deisenhofen, Germany) (dilution 1:500), and mouse anti-human CK8, clone 35βH11 (Dako, Glostrup, DK), (diluted 1:100). Immunostaining was performed using the avidin–biotin–peroxidase complex method (Vectastain, Vector laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. Briefly, after fixation in acetone (10 min), endogenous peroxidase activity was inhibited upon treatment with 0.03% H_2O_2/PBS (10 min). Before specific staining, unspecific antigenic sites were blocked with normal goat serum or normal horse serum. Sections were then incubated with the respective primary antibody for 1 h at room temperature (RT) followed by incubation with biotinylated anti-rabbit or anti-mouse immunoglobulins and then with avidin–biotin–peroxidase complex (30 min at RT for each step). After each step, sections were washed with PBS. Specific peroxidase activity was visualized with 0.05% 3-amino-9-ethylcarbazol as a substrate (Sigma, Deisenhofen, Germany) and 0.02% H_2O_2/0,1 M Na-acetate buffer, pH 5.5). Counterstaining was performed with Mayer's haematoxylin. Control staining was performed in the absence of primary antibody. Immunostained sections were evaluated upon two independent investigators by light microscopy. Double immunostainings were performed with a monoclonal anti-Ki67 antibody (Dako, Glostrup, DK) using the avidin–biotin–peroxidase method (ABC, red-brown staining) [8], together with the monoclonal anti-human CK8 antibody (Dako, Glostrup, Denmark) using alkaline phosphatase–anti-alkaline phosphatase method [9] and fast Blue BB salt (Sigma, Deisenhofen, Germany) as a chromogenic substrate (deep blue staining).

Results

Expression of CK8 in healthy and transformed epithelium of the head and neck area

CK8 was identified as a potential tumour-associated antigen using the target identification technology AMIDA. In the present study, the expression of CK8 was analysed in serial sections (4 μm) of cryosamples from healthy epithelium of the head and neck area and compared with those of the established markers panCK and CK13. Microscopically healthy squamous epithelium was devoid of CK8, except for some rare cases of epithelial cells of the basal membrane layer (stratum basale). Nevertheless, in these cases CK8 was entirely lacking in suprabasal squamous epithelial layer. In contrast, antibodies specific for a large subset of cytokeratins (panCK: CK1, 2, 5, 6, 7, 8, 11, 14, 16, 17, and 18) and for a single keratin (CK13) stained strongly healthy epithelial cells, including suprabasal cell layers. These comparative results were confirmed in five independent specimens of healthy epithelium and are exemplified in Fig. 1A. Additionally, Fig. 1A depicts weak lymphocytic infiltrate within the lamina propria.

Next, we studied the expression of the above-mentioned molecules in serial sections of squamous cell carcinoma specimens originating from the head and neck area (HNSCCs). Twenty-six HNSCC specimens were analysed with respect to their CK8 expression, five of which were processed as serial sections and stained with panCK and CK13 specific antibodies in parallel. Only 7.6% of the HNSCCs analysed revealed negative for CK8, 15% showed a weak expression and 77% a moderate to strong CK8 expression in carcinoma cells (Table 1). For easier orientation, CK8-negative cells as well as a weak, moderate, and strong staining for CK8 are exemplified in Fig. 1B. It is of note that stromal and immune cells surrounding the carcinoma did not show any cross-reactivity with CK8 antibodies. Furthermore, single tumour cells, which were detached from the tumour collective, were thoroughly detected upon CK8 staining (Fig. 2A, arrow heads). Microscopically hyperplastic, healthy tissue stained faintly for CK8 in cells of the stratum basale, while adjacent invasive carcinoma cells in the same specimen expressed CK8 very strongly (Fig. 2B). PanCK staining resulted in a comparable detection of carcinoma cells, however occasionally with a reduced intensity (Fig. 2). A differentiation between carcinoma cells and hyperplastic epithelium, as seen for the CK8 marker, was neither achieved with panCK nor with CK13 specific staining, because both tissue types were strongly and equally well stained with panCK- and CK13-specific antibodies (Fig. 2B). Furthermore, CK13 was not expressed homogeneously across the carcinoma areas as depicted in Fig. 2A. Thus, CK8 has the potential to differentiate healthy and transformed tissue, while panCK and CK13 clearly lacked these characteristics.

In a further step, we compared the expression of CK8 with the proliferation marker Ki67 in serial sections of primary carcinomas. As shown in Fig. 3A, CK8 and Ki67 showed a robust correlation. However, tumour-infiltrating lymphocytic cells were occasionally Ki67 positive. This issue was further corroborated upon double-staining of primary carcinomas and lymph node metastases thereof. Although CK8 and Ki67 showed high correlation in distinct tumour areas (Fig. 3B, left upper panel), CK8

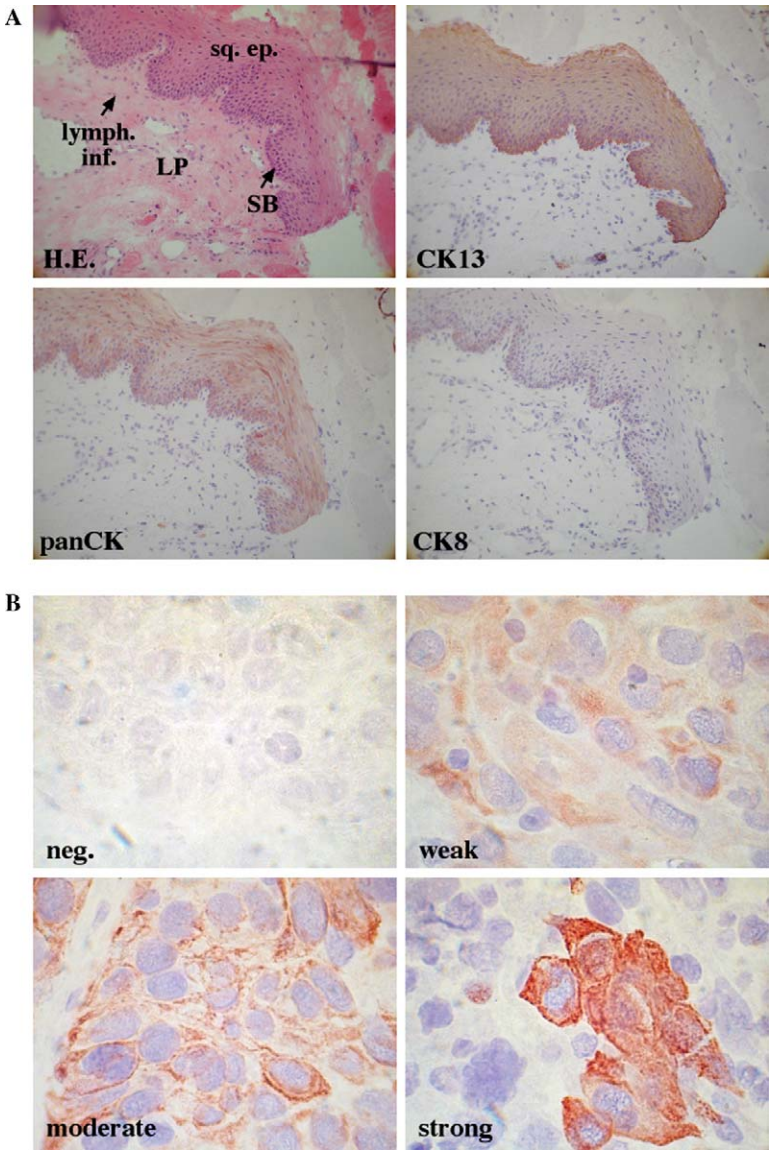

Fig. 1. (A) PanCK, CK13, and CK8 expression in healthy oral mucosa. Macroscopically healthy oral mucosa was processed to generate 4 μm serial sections and stained with antibodies specific for the indicated markers. Additionally, specimens were stained with hematoxylin/eosin (HE). Shown is one representative specimen from five. Lymph. infil., lymphocytic infiltrate; LP, lamina propria; SB, stratum basale; sq. ep., squamous epithelium (multilayered). (B) CK8 expression in primary carcinomas. Representative examples of CK8-negative, weak, moderate, and strong CK8 expression in primary head and neck carcinomas are shown.

Table 1
CK8 expression in head and neck squamous cell carcinomas, metastases thereoff, and healthy mucosa

	CK8 expression level			
	−	+	++	+++
HNSCCs	2/26 (7.6%)	4/26 (15%)	8/26 (31%)	12/26 (46%)
Metastases	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)
Mucosa	6/9 (67%)	3/9 (33%)	0/9 (0%)	0/9 (0%)

−, no expression; +, weak expression; ++, moderate expression; +++, strong expression.

was far more reliable for the detection of carcinoma cells and the definition of tumour borders (Fig. 3B). Furthermore, CK8 was expressed in all cells of larger tumour areas, which was in clear contrast to Ki67. With respect to lymph node metastases, the advantage of CK8 detection was even more pronounced: CK8 stained exclusively carcinoma cells, including disseminated cells. Ki67 on the other hand was expressed in carcinoma and lymphocytic cells (Fig. 3C). Thus, the determination of carcinoma and metastatic areas was not feasible based on the single staining with Ki67-specific antibodies, but upon staining of CK8.

CK8 expression in oral leukoplakia

As shown above, the AMIDA antigen CK8 has the capacity to differentiate between healthy and transformed tissue. Therefore, we next investigated whether the expres-

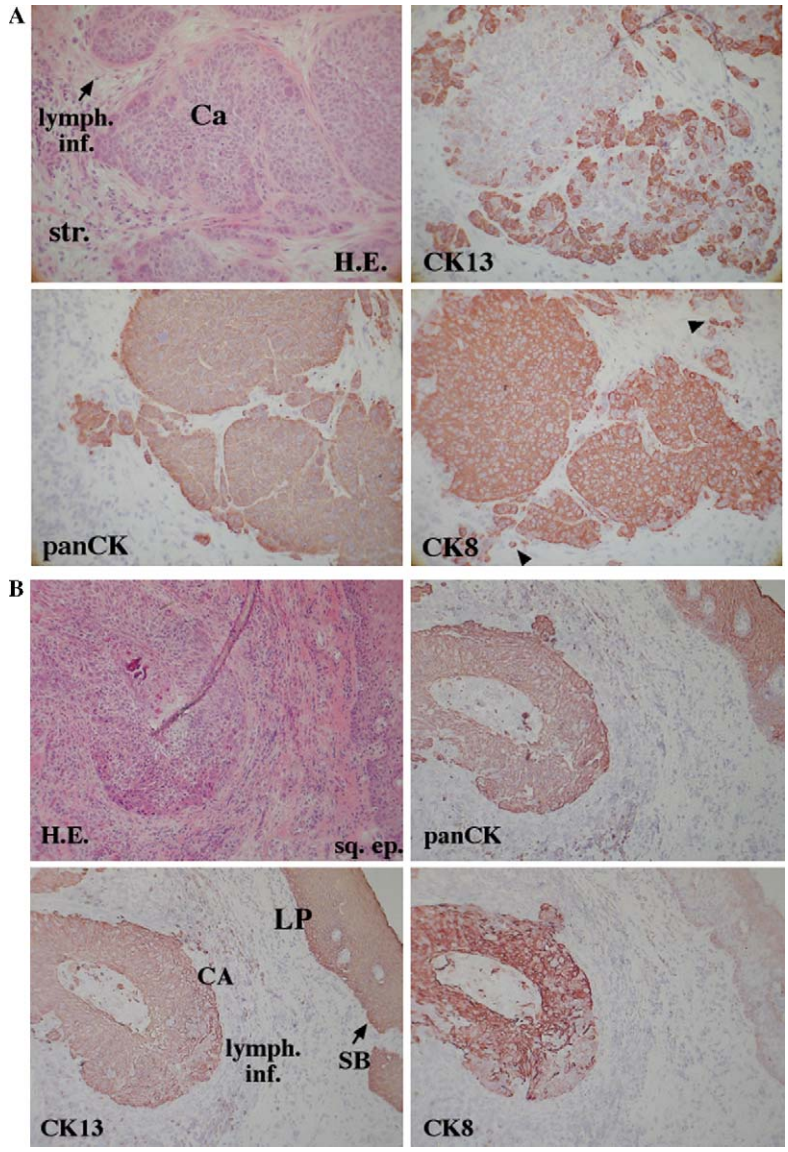

Fig. 2. PanCK, CK13, and CK8 expression in head and neck squamous cell carcinomas. Carcinoma samples, which had been confirmed by clinical diagnosis and histopathologic examination, were processed to generate 4 μm serial sections and stained with antibodies specific for the indicated markers. Additionally, specimens were stained with hematoxylin/eosin (HE). Shown are two independent and representative specimens from five. Single disseminated carcinoma cells are marked with arrowheads (A). Invasive carcinoma cells and microscopically non-transformed cells are visualized in one specimen (B). Lymph. infil., lymphocytic infiltrate; LP, lamina propria; SB, stratum basale; sq. ep., squamous epithelium (multilayered); Str., stroma; CA, carcinoma.

sion of CK8 also correlated with early steps in malignant transformation of epithelial cells. Towards this end, oral leukoplakic lesions were analysed with respect to the expression of panCK, CK13, and CK8. Oral leukoplakic lesions ($n = 10$) were processed to generate 4 μm serial sections and stained with antibodies specific for panCK, CK13, and CK8. In all cases, panCK and CK13 were moderately or strongly expressed in leukoplakic epithelium, including the stratum basale and suprabasal squamous epithelium. Cells of the lamina propria and lymphocytic infiltrates remained negative for panCK, CK13 (Fig. 4 and Table 2). Noteworthy, CK8 was absent in the majority of leukoplakia, but strongly expressed in a proportion of specimens (Fig. 4). The comparison of CK8 expression pattern with the diagnosis of the confirmed leukoplakia demonstrated a positive correlation between CK8 and the level of transformation of the tissue. CK8 expression was detectable only in dysplastic lesions of leukoplakic tissue, except for epithelium of laryngeal origin, where CK8 was always expressed (Table 2). CK8 expression was restricted to epithelial cells, while stromal and immune cells surrounding the lesions were omitted. In the case of hyperplastic epithelium and orthokeratosis, tissue sections did not reveal detectable CK8 expression (Fig. 4 and Table 2). In accordance with carcinoma samples, CK13 was heterogeneously expressed across dysplastic leukoplakic samples. Because CK8 correlated with the transformation status of oral epithelium, we next assessed the proliferation of

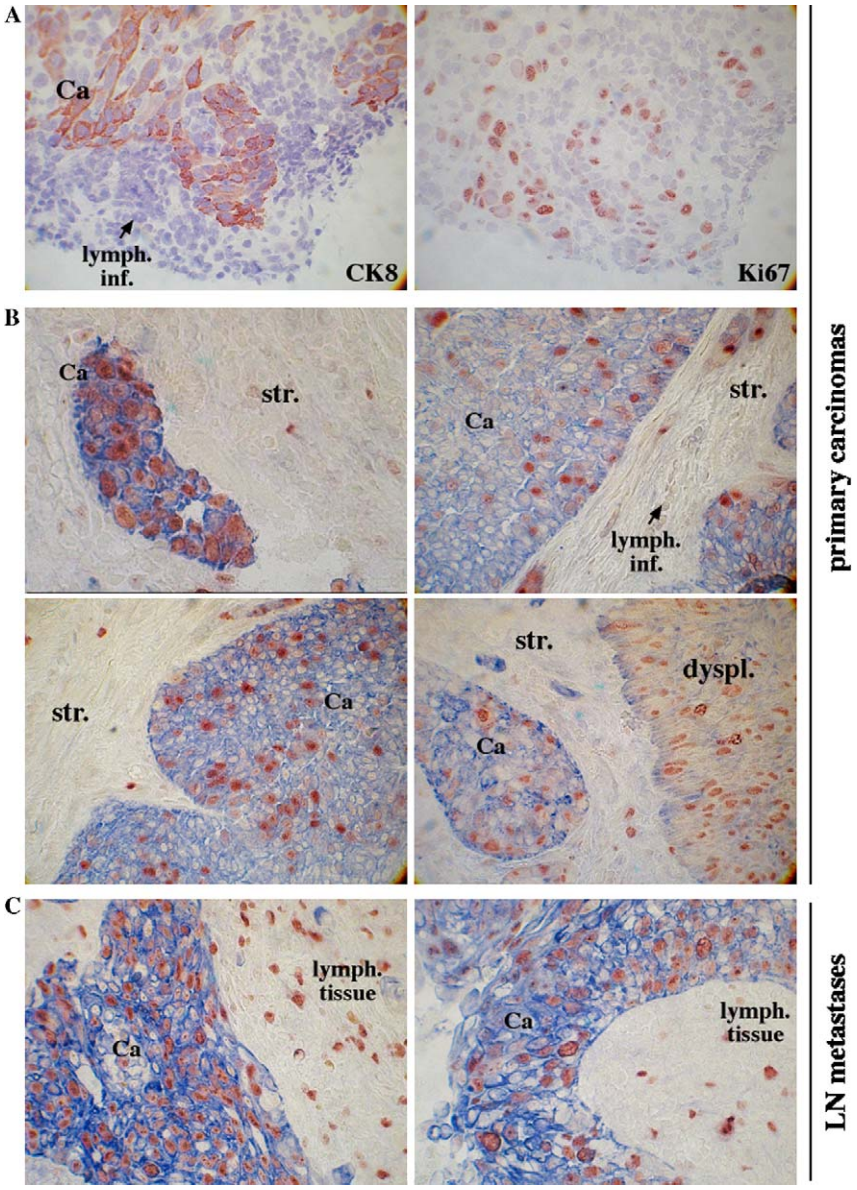

Fig. 3. CK8 and Ki67 expression in primary head and neck carcinomas and lymph node metastases. (A) Consecutive sections from a primary head and neck carcinomas were stained with CK8- and Ki67-specific antibodies. Shown are the comparable areas of both sections. (B) Primary carcinomas and (C) lymph node metastases were stained in parallel with CK8- and Ki67-specific antibodies (CK8, blue; Ki67, brown). Shown are different areas of one carcinoma in (B) and of two metastases in (C). Ca, carcinoma; str., stroma; dyspl., dysplasia; lymph. inf., lymphocytic infiltrate; lymph. tissue, lymphatic tissue.

CK8-positive cells in oral leukoplakia. In samples subjected to a double-staining, CK8 expression was characterized by frequent co-expression of the proliferation marker Ki67 (Fig. 5). However, CK8 expression expanded across the entire dysplastic lesion, while Ki67 staining did not, as shown by counter-staining with hematoxylin. Although Ki67 expression was significantly more frequent in cells of lesion areas compared with healthy epithelium, a substantial amount of cells within lesions did not express Ki67. Furthermore, non-lesion cells including stromal and immune cells were occasionally positive for Ki67 but not CK8 (Fig. 5 and data not shown). The smaller size of the Ki67-positive non-lesion cells pointed towards a lymphocytic origin.

Discussion

The recently published AMIDA technology yielded a catalogue of proteins, which have the potential to serve as diagnostic and/or therapeutic markers [1,2]. Amongst these, CK8 is an interesting molecule because it was over-expressed in head and neck carcinoma cells, in which it ectopically localised at the external leaflet of the plasma membrane [3]. Membrane-associated CK8 binds components of the plasminogen activator system, eventually supporting the generation of plasmin upon proteolytic cleavage of its precursor plasminogen [4–6]. Furthermore, CK8 is de novo or over-expressed in a variety of malignan-

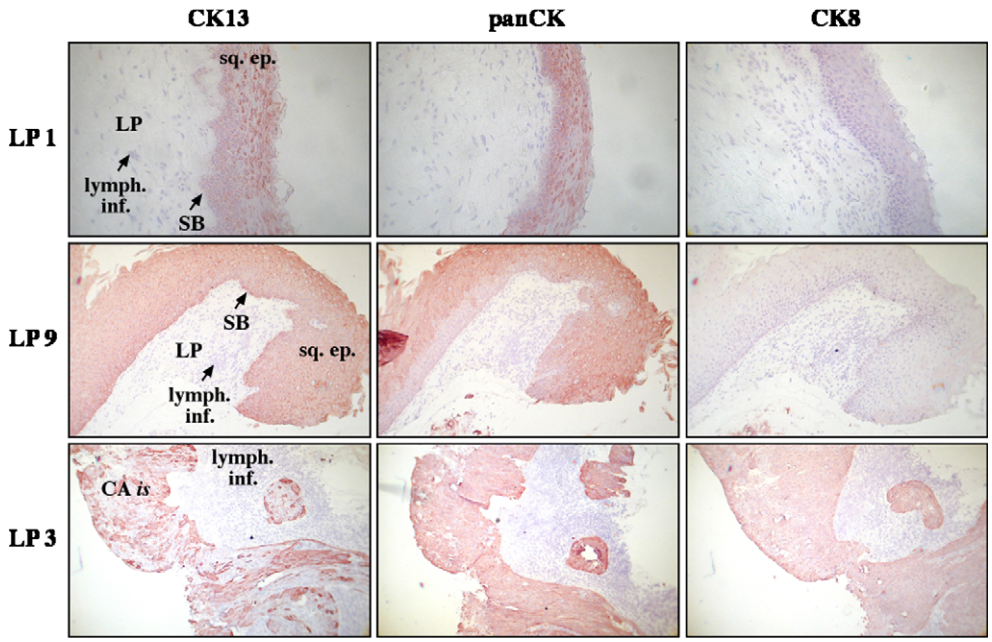

Fig. 4. PanCK, CK13, and CK8 expression in oral leukoplakia. Oral leukoplakia (LP), which had been confirmed by clinical diagnosis and histopathologic examination, were processed to generate 4 μm serial sections and stained with antibodies specific for the indicated markers. Shown are three independent and representative sections out of 10 specimens. Lymph. infil., lymphocytic infiltrate; LP, lamina propria; SB, stratum basale; sq. ep., squamous epithelium (multilayered); CAis, carcinoma in situ.

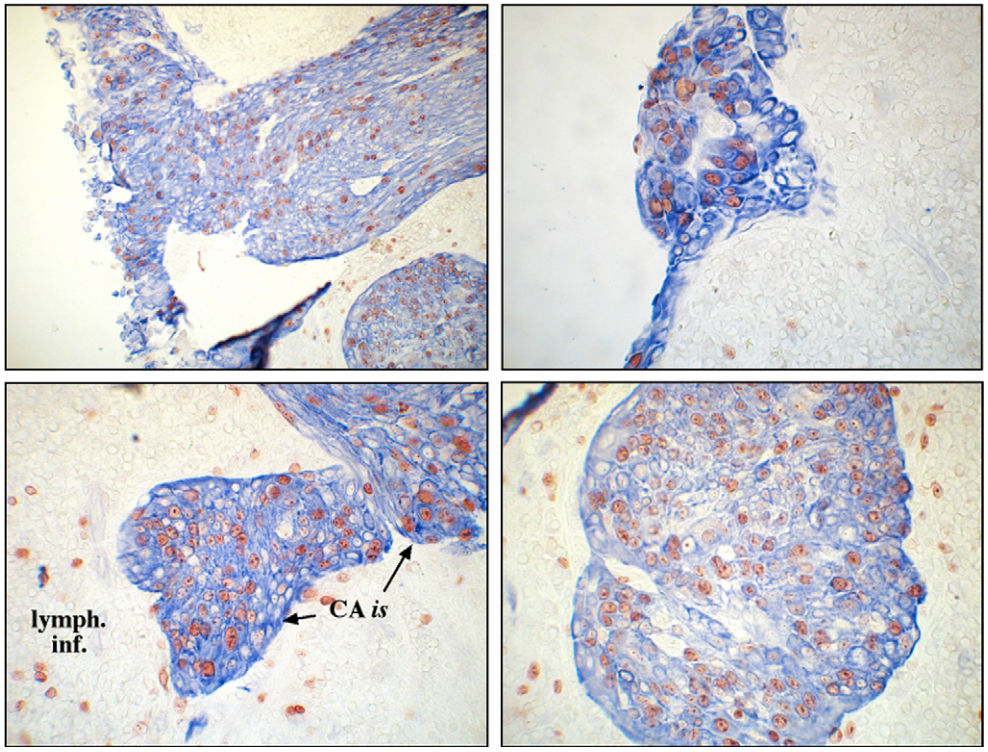

Fig. 5. CK8 and Ki67 expression in oral leukoplakia. Leukoplakia 3, which displayed dysplastic lesions, was stained with antibodies specific for CK8 (blue staining) and the proliferation marker Ki67 (brown staining). Shown are representative sections including dysplastic and microscopically healthy areas. Lymph. infil., lymphocytic infiltrate; CAis, carcinoma in situ.

cies such as sinonasal undifferentiated carcinomas [10], gastric carcinomas [11], and in oesophageal squamous cancers [12], amongst others.

In the present study, we investigated the specificity and sensitivity of the AMIDA antigen CK8 in a cohort of samples including healthy oral epithelium, oral leukoplakia,

Table 2
CK8, panCK, and CK13 expression in oral leukoplakia

Sample	Diagnosis	CK8	panCK	CK13
LP1	Fibrosis, weak inflammation, no dysplasia	–	++	++
LP2	Hyperplasia, keratosis, weak dysplasia	–/+ (++ dysplastic areas)	+++	+++
LP3	Chronic inflammation, strong dysplasia/carcinoma in situ	+++	+++	+++
LP4	Hyperplasia, no dysplasia	–	+++	++
LP5	Orthokeratosis, no dysplasia	–	+++	+++
LP6	Hyperplasia, no dysplasia, larynx	+++	+++	+++
LP7	Hyperplasia, inflammation, no dysplasia, laryngeal areas	– (++ laryngeal areas)	+++	+++
LP8	Weak hyperplasia, no dysplasia, larynx	+++	+++	+++
LP9	Hyperplasia, no dysplasia within section	–	+++	+++
LP10	Hyperplasia, no dysplasia	–	++	++

–, no expression; +, weak expression; ++, moderate expression; +++, strong expression.

head and neck squamous cell carcinomas, and metastases thereof. Oral leukoplakia were chosen since they include pre-malignant stages of oral cancer [7], and thus appeared suitable for the assessment of the potential of CK8 for early diagnosis, which is of paramount importance [13,14]. Prospective clinical studies of subjects with oral leukoplakia revealed rates of malignant transformation close to 18% following eight years, which were even twice as high (36%) for those lesions harbouring dysplastic epithelial cells [15]. Staining with antibodies specific for a cytokeratin panel (panCK), CK13, and CK8 was performed in parallel on serial sections of the above-mentioned tissues. This allowed the direct comparison of CK8 with markers conventionally used for immunohistopathological purposes. CK8 displayed a high specificity and sensitivity for transformed tissue, i.e., carcinoma cells, metastases, but importantly also for dysplastic lesions in oral leukoplakia. As an exception from this observation CK8 expression was detected in healthy epithelial cells of the larynx. This finding is in accordance with previous studies describing a comparably high CK8 messenger RNA expression in healthy and malignant larynx samples [16]. In sharp contrast to CK8, panCK, and CK13 were expressed in all tissues independently of their transformation status and morphology. Even more disadvantageous, CK13 was not homogeneously expressed across the tumour mass, which may result from de-differentiation of tumour cells and concomitant loss of CK13 expression. Thus, CK8 staining allowed for a notedly increased, specific, and sensitive detection of transformed cells at an early stage of tissue transformation, namely dysplasia and carcinoma in situ.

Expression of CK8 further correlated with Ki67-positive areas, i.e., cell proliferation, in dysplastic areas of leukoplakic lesions, carcinomas, and metastases. Noteworthy, CK8 expression characterized those areas in a more confined and accurate manner as did Ki67 expression, as judged by hematoxylin/eosin staining. Especially with respect to lymph node metastases, CK8 staining was far more appropriate to determine malignant tissue, since lymphocytic cells also displayed frequent Ki67 positivity. This is of interest with respect to leading edges of dysplasia, malignancies including tumour borders, and metastases, and is further corroborated by the finding that single detached tumour cells were stained efficiently with CK8-specific antibodies. Thus, CK8 might have prognostic value as has Ki67 in diverse neoplasias including cervical intraepithelial neoplasia [17], whilst being somewhat more sensitive and precise. In addition to Ki67/p53 co-expression and the aneuploidy grade, which are thorough prognostic markers [18–21], CK8 expression represents an interesting target for the evaluation and prognosis of oral leukoplakia. Further studies should therefore aim at the assessment of the prognostic value of CK8 in leukoplakia and carcinomas.

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