Cytokeratins 6 and 16 are Frequently Expressed in Head and Neck Squamous Cell Carcinoma Cell Lines and Fresh Biopsies

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Abstract. *Background: Cytokeratins (CK) are members of intermediate filaments, which are predominantly found in epithelial cells. Different types of epithelia are characterized by a distinct composition of CK. Recently immunohistochemical investigations demonstrated that, among others, CKs 6, 14, 16 and 17 are regularly expressed in benign stratified squamous epithelium of the head and neck as well as in squamous cell carcinoma of the head and neck (HNSCC) in contrast to CKs 1, 10 and 11, that were only rarely expressed in these tissues. Materials and Methods: Total RNA was isolated from 15 primary cell lines derived from HNSCC and from 15 tissue samples of oro- and hypopharyngeal carcinomas obtained from surgery specimens. CK expression was evaluated by RT-PCR, Western blot analysis and immunohistochemistry. Results: CK6 and 16 were found to be expressed in both groups at almost 100%. The expression level of CK14 remained constant (73%) in both groups, at the RNA and protein level. CK17 was more frequently present in tumour specimens than in HNSCC cell lines. The immunohistochemical results of the surgical tumour specimens confirmed the results of Western blot analysis. Conclusion: The presented results show high and stable expression rates for CK6 and CK16 in HNSCC. These results will serve as a basis for further investigations concerning the search for circulating tumour cells and micrometastases. In addition, we found that cytokeratin expression in HNSCC is different on the RNA level compared to the protein level.*

Cytokeratins (CK) are members of intermediate filaments, responsible for the stability of the epithelial cell (1-5). Together with actin-microfilaments and microtubules, keratin filaments form the cytoskeletons of vertebrate epithelial cells (1). Cytokeratins are subclassified into 20 different polypeptides (CKs 1-20) (3). CK's are expressed in various combinations depending on the epithelial cell type (3-5). They are subdivided into two sequence types (I and II) that are typically, but not necessarily, coexpressed as specific pairs with complex expression patterns (1, 5). Except for CK18, all genes of type-I-CKs are localized on chromosome 17, whereas the genes from type-II-CKs are localized on chromosome 12 (3). CKs were found to be specific in distinct constellation for different types of epithelia (5). Antibodies against certain CKs are regularly utilized for the histological diagnosis of epithelial tumours (3-5). Recent investigations, utilizing immuno-histochemical and molecular biological techniques, demonstrated that, in contrast to some rarely expressed CKs (CKs 1, 10 and 11), there are, among others CKs (CKs 6, 14, 16 and 17) that were regularly found in stratified squamous epithelium (3-7). Also, the occurrence of simple epithelial CK like CKs 8, 18 and 19 are not regular but common (5). The fact that CK expression is stable in the event of malignant transformation of the epithelial cell (5, 8, 9), suggests that CKs 6, 14, 16 and 17 could also be found in squamous cell carcinomas. Therefore, it is of particular interest whether these predominantly immunohistochemical results could be confirmed at the RNA and protein level, and if there are differences in the CK expression between normal squamous epithelium and squamous cell carcinoma. Briefly, this study was designed to establish a CK expression model in HNSCC utilizing RT-PCR and Western blot analysis. The results of this study should help other investigations, especially those concerning the detection of lymph node micrometastasis and circulating tumour cells. Therefore, we examined the CK expression and composition of 15 HNSCC cell lines from the oro- and hypopharyngeal region and 15 surgical SCC specimens derived from tumours of the hypopharynx. In addition, we performed immunohistochemistry to verify the CK location in the tumour cells.

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Table I. *Primer sequences for RT-PCR amplification of CKs and β-actin.*

mRNA	Product size	Primers 5'-3'
β-actin sense	778 bp	5'-GATGATGATATCGCCGCG CTCGTCGTC-3'
β-actin antisense		5'-GTGCCTCAGGGCAGCGGAA CCGCTCA-3'
CK6 sense	583 bp	5'-GACTGTGAGGCAGAACCTG GAGCCG- 3'
CK6 antisense		5'-GACGTGGTCGATCT CAGAT CTCAG- 3'
CK14 sense	257 bp	5'-GCAGAGCCAGGACCC ACCT GAAGACC- 3'
CK14 antisense		5'-CAGGCCTGACCTG CCGTGC TGTG C- 3'
CK16 sense	585 bp	5'-GGATGAGCTGACCC TGGCC AGGACTGAC- 3'
CK16 antisense		5'-CTCCAGCAGGCGGCGGTAG GTGGCAATC- 3'
CK17 sense	409 bp	5'-GCACCCTCTAGCTGACTGTA AAAC- 3'
CK17 antisense		5'-GGATGTTGGCATTGTCCAC GGTGGCTGTGAG- 3'

Materials and Methods

Cell lines. The cell lines UM-SCC- 1, UM-SCC-3, UM-SCC-4, UM-SCC-9, UM-SCC-11B, UM-SCC-14A, UM-SCC-22B, UM-SCC-27, UT-SCC-8, UT-SCC-10, UT-SCC-12A, UT-SCC-16B, UT-SCC-19A, UT-SCC-19B and UT-SCC-24A were derived from HNSCC (10). Four keratinocyte cell lines were used as control. The cells were grown in DMEM media supplemented with 10% foetal calf serum (FCS) in the presence of penicillin and streptomycin.

Surgical primary tumour specimens. A total of 15 patients underwent surgery because of a SCC of the oro- and hypopharynx. None of the patients received preoperative radiation or chemotherapy. Fresh surgical specimens of primary tumours were stored deep-frozen at –28˚C until RNA extraction. Histologically all tumour specimens were graded moderately-differentiated (G2).

RNA extraction. Total cellular RNA was extracted with the RNeasy Total RNA kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. In brief, the cell lysates were mixed with an equal volume of 70% ethanol, transferred onto an RNeasy spin column and extracted RNA was eluted with 40 μl RNAse-free water and stored at –28˚C. The quantity and quality of isolated RNA was determined by absorbance at 260 and 280 nm, respectively.

RT-PCR. All CK-primers were designed according to the sequences derived from Genebank and were synthesized by Sigma-ARK (Darmstadt, Germany). The nucleotide-sequences of the primers are shown in Table I. RT-PCR was performed using the 1st Strand cDNA Synthesis Kit for RT-PCR (Boehringer, Mannheim, Germany). Samples containing 5 μl total RNA were resuspended in a mastermix containing 2 μl of 10x Reaction Buffer, 4 μl of 25 mM $MgCl_2$, 2 μl Deoxynucleotide Mix, 1 μl of Oligo-p$(dT)_{15}$ primer, 1 μl of RNAse Inhibitor, 0.8 μl of AMV Reverse Transcriptase and 4.2 μl of DNase-free water. Water instead of the template was used as a negative control. The mixture was incubated at 25˚C for 10 min to allow annealing of the primers to the RNA. Reverse transcription of RNA was performed at 42˚C for 60 min, stopped at 95˚C for 5 min and finally placed on ice. From this cDNA solution, 5 μl was removed for subsequent use in PCR amplification by adding each sample to 95 μl of PCR Master Mix solution (10x Reaction Buffer, 25 mM $MgCl_2$, deoxynuleotide mix, gelatin 0.1%, primers specific for different CKs and Taq DNA polymerase). β-actin amplification was used to demonstrate RNA integrity. The samples were briefly vortexed and covered with one drop of mineral oil. PCR was carried out under the following conditions: denaturation for 3 min at 94˚C, followed by 30 cycles of 1 min denaturation at 94˚C, 1.5 min annealing at 51˚C and 1 min primer extension at 72˚C. The last cycle was performed for 10 min at 72˚C and finally stopped at 4˚C. The different PCR products were identified by 2% agarose gel electrophoresis followed by ethidium bromide staining. A PCR size marker (New England, Biolabs) was used as a standard. The specificity of the amplified DNA fragments of interest was verified by sequencing of the product (MWG Biotech, Ebersberg, Germany).

Antibodies. The primary monoclonal-mouse-antibodies Actin (C-11), CK 6 (LHK6B), CK 14 (LL002), CK 16 (LL025) and CK 17 (E3) were used.

SDS-PAGE and Western blot analysis. SDS-PAGE electrophoresis was performed, as described by Laemmli (11), on samples derived from 15 HNSCC cell lines and from 15 surgical hypopharyngeal carcinoma tissue specimens. After electrophoresis, the proteins were transferred to nitrocellulose membranes as described by Towbin *et al.* (12). Protein immunoblotting was performed as described by Batteiger *et al.* (13), using a buffer containing 5%(w/v) non-fat dry milk and 0.05% (v/v) Tween 20 in PBS. The proteins were detected using a mouse anti-goat IgG-coupled horseradish peroxidase substrate (ECL, Amersham™).

Immunohistochemistry. Immunohistochemistry was performed with the Streptavidin Biotin Complex/HRP method to detect CK. The procedure was carried out according to the manufacturer's instructions (StreptABComplex/HRP-Kit, DAKO, Hamburg, Germany) and as previously reported (14). CK-antibodies are as described above. Positive staining of 75% of the target cells was defined as a homogeneous staining pattern.

Results

RT-PCR. The results (Table II) demonstrated that CKs 6, 14, 16 and 17, as well as actin, were expressed in all four tested keratinocyte cell lines. In HNSCC cell lines, the

Table II. *Cytokeratin expression results (RT-PCR; Western blot analysis; immunohistochemistry).*

	RT-PCR					Western Blot Analysis					Immunohistochemistry			
CELL LINES	Actin	CK6	CK14	CK16	CK17	Aktin	CK6	CK14	CK16	CK17	CK6	CK14	CK16	CK17
UM-SCC-1	+	+	+	+	–	+	+	+	+	–				
UM-SCC-3	+	+	+	+	–	+	+	+	+	–				
UM-SCC-4	+	+	+	+	–	+	+	+	+	–				
UM-SCC-9	+	+	+	+	+	+	+	+	+	+				
UM-SCC-11B	+	–	–	–	–	+	+	–	+	–				
UM-SCC-14A	+	+	–	+	–	+	+	–	+	–				
UM-SCC-22B	+	+	+	+	+	+	+	+	+	–				
UM-SCC-27	+	+	+	+	–	+	+	+	+	–				
UT-SCC-8	+	+	–	+	–	+	+	+	+	–				
UT-SCC-10	+	+	–	+	+	+	+	+	+	–				
UT-SCC-12A	+	+	+	+	–	+	+	–	+	–				
UT-SCC-16B	+	+	+	+	–	+	+	–	+	–				
UT-SCC-19A	+	+	+	+	–	+	+	–	+	–				
UT-SCC-19B	+	+	+	+	–	+	+	+	+	–				
UT-SCC-24A	+	+	+	+	–	+	+	+	+	–				
Positive	100%	93%	73%	93%	20%	100%	100%	66%	100%	6.6%				
TISSUE														
542	+	+	+	+	+	+	+	+	+	+	++	+	+	++
599	+	+	+	+	+	+	+	+	+	+	+	+	+	+
612	+	+	+	+	–	+	+	–	–	+	++	+	++	++
652	+	–	–	+	–	+	+	+	+	+	++	+	++	++
705	+	+	–	+	+	+	+	+	+	+	+	(+)	++	++
729	+	+	+	+	+	+	+	+	+	+	++	+	+	++
731	+	+	+	+	+	+	+	+	+	+	+	(+)	+	+
738	+	+	+	+	+	+	+	+	+	+	++	+	+	+
745	+	+	+	+	+	+	+	+	+	+	++	+	+	+
893	+	+	–	+	+	+	+	+	+	+	++	((+))	+	++
902	+	+	+	+	+	+	+	+	+	+	+	+	+	+
934	+	+	+	+	–	+	+	+	–	+	++	+	+	++
949	+	–	–	–	–	+	+	+	+	+	++	+	+	+
955	+	+	+	+	–	+	+	+	+	+	++	+	+	++
991	+	+	+	+	+	+	+	+	+	+	+	–	–	+
Positive	100%	80%	73%	93%	66%	100%	100%	93%	86%	100%	100%	73%	93%	100%

Legend: + positive; – negative; ++ intense staining; (+) up to 10% of target cells stained positive; ((+)) single cells stained positive

hyperproliferative CKs 6 and 16 were detectable by RT-PCR in all of the lines except for UM-SCC-11B. CK 14, as one of the basic CKs, was expressed in only 11/15 of the cell lines and CK 17 was detectable in only 3/15. The surgical tumour specimens had a different CK composition. CK 14 was also expressed in 11/15 of the cases, as in the HNSCC cell lines. The expression of CK 17 in the tumour specimens was more than three times as high as in the cell lines (10/15). The expression of CK 6 (13/15) and CK 16 (14/15) was relatively stable compared to the cell lines.

Western blot analysis. CKs 6, 14, 16 and 17 were expressed in all of the four keratinocyte cell lines. CK 6 was detectable in all (15/15) of the cases in both groups, cell lines and tumour specimens, almost like CK 16 which was absent just in the tumour specimen 934 (14/15). Ten out of 15 of the cell lines and 14/15 of the tumour specimens were positive for CK14, whereas the CK17 expression was very low (1/15) in the HNSCC cell lines, but was high (15/15) in the tumour specimens.

Immunohistochemistry. The immunohistochemical investigation of the surgical tumour specimens in general confirmed the results of Western blotting. The strongest staining signals were detectable for CK 6 (Figure 1) in all of the 15 tumour specimens. CK 14 was detectable in 14/15 cases. Out of those, in 2 cases staining was positive in up to 10% of the cases and in one case just single cells stained positive. CK 16 was detectable in 14/15 and CK 17 in 100% of the cases.

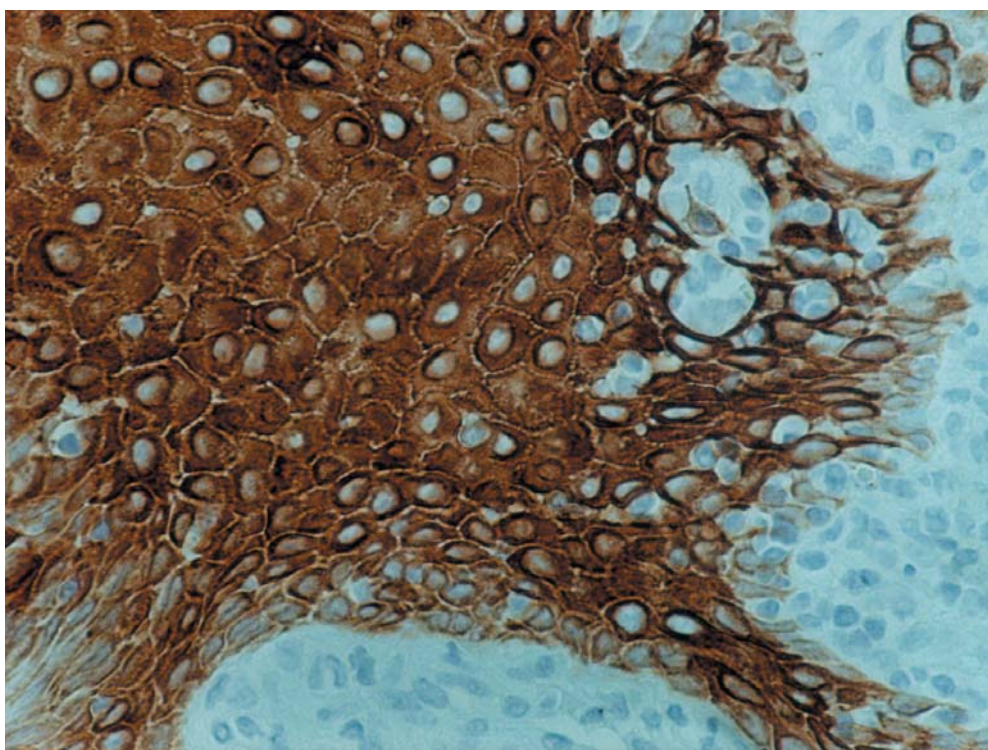

Figure 1. *Immunohistochemical detection of cytokeratin 6.*

Discussion

In the fields of cell biology and pathology, CKs as a group have gained widespread acceptance as very sensitive epithelial cell and tumour markers (2-9, 15-20). Analysis of CK expression patterns in carcinomas are important, since a specific CK pattern may facilitate histopathological tumour diagnosis when clinically and pathologically difficult cases such as carcinoma of unknown primary are encountered (5, 21). Another diagnostic application of CK antibodies involves their use in serological assays to detect soluble CK fragments released from carcinoma cells. This has already been demonstrated by the use of CYFRA 21-1, which represents soluble fragments of CK19. It has clinical value for assessing the prognosis in squamous cell carcinomas of the lung (5) and also for the detection of distant metastases of HNSCC.

CKs are regularly expressed in stratified squamous epithelia and are classified into two different categories. Basal cells express the primary stratified epithelial CKs 5, 14 and 17. CKs 6 and 16, which are particularly found in the epidermis, are constitutively expressed in mucosal stratified squamous epithelia and are also regarded as markers of hyperproliferation (2-5, 7-8, 12, 17-18). The pattern of CK expression in squamous cell carcinomas of the pharyngeal region is very complex. A certain variability is possible and probably correlates to several parameters such as degree of differentiation and tumour grade (5). The basic immunohistochemical observations by Moll (2-5) showed that CK14, which is a typical feature of basal cells, is predominantly detectable in the peripheral cell layers of well-differentiated areas. The hyperproliferative CK6 and CK16 were found to be the most prevalent components of maturating central tumour cells. CK6 may also be used as another marker of squamous differentiation in poorly-differentiated carcinomas. CK16 stains intensely in the central maturation regions of tumour nests. CK17 was detected in most cases too, however its distribution pattern seems to be rather irregular. The presented results demonstrate that the recent, predominantly immunohistochemically established CK expression pattern in stratified squamous epithelia could be confirmed on the RNA level using RT-PCR and on the protein level by Western blotting. It was somewhat surprising to observe different CK expression rates between HNSCC cell lines and tumour specimens. The CK detection by RT-PCR showed in the HNSCC cell lines a low expression rate of CK17 (3/15) compared to 10/15 in the tumour specimens. To our knowledge, this is the first time that the specific expression of CKs 6, 16 and 17 in HNSCC has been described at the

mRNA level. In the Western blot examination, CK17 was detectable in 1/15 of the HNSCC cell lines, while 15/15 tumour specimens were positive for CK17.

Van der Velden *et al.* (8) and others have pointed out that, during malignant transformation of epithelial cells, the CK expression patterns changed depending on the differentiation grade of the carcinomas. With increasing tumour grade, there is decreased expression of stratification-keratins and increased expression of basal cell-, simple cell- and hyperproliferation-related CKs. Our tested samples were all graded G2 (moderately-differentiated), so that we can not comment on the matter. The strong and stable expression of CK6 and CK16 in almost 100% of tested samples was verified by all three used detection methods. This observation is most significant and encourages further studies in the future. During the past two years, several groups have reported the detection of micrometastasis and circulating tumour cells, utilizing RT-PCR for CKs 5, 14, 19 and 20 and other tumour markers (6, 19-20, 22-31). However, none of them used CKs 6, 16 and 17, which are also typical for HNSCC.

The present study generally confirmed the CK expression pattern of squamous epithelia and their carcinomas. It could have been observed that CKs, which are characteristic for a certain epithelium, are not necessarily expressed in all of the tumour specimens or tumour cell lines. However, the highly stable expression patterns of CKs 6 and 16, which are characteristic for squamous cell carcinomas and squamous epithelia and are used as markers of hyperproliferation, seem to be a promising target for further investigations concerning the early detection of circulating tumour cells and in lymph nodes micrometastasis.

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