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S19-mRNA Expression in Squamous Cell Carcinomas of the Upper Aerodigestive Tract

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Abstract. *Background: The differential display method showed altered expression of ribosomal protein S19 gene in human head and neck squamous cell carcinoma (HNSCC) cell lines. Materials and Methods: To verify these results, RT-PCR analysis was carried out in 18 HNSCC and 17 benign epithelial cell lines as well as 30 HNSCC and 8 reference tissue samples. In the HNSCC cells S19 mRNA expression was significantly reduced as compared to benign epithelial cells. Results: Change of the S19 gene expression in surgical samples was detectable but not significant, although the histopathological grading of the HNSCC biopsies correlated significantly with the S19 mRNA expression levels. The expression of ribosomal protein S6 and S14 genes were additionally analyzed using the same methods. Conclusion: High correlation was found between the expression of S6/S14 and S19 suggesting that changes in S19 gene expression might be the result of loss of ribosomes in HNSCC cells.*

It is generally accepted that the generation of carcinoma involves a series of genetic events (3, 24). One possibility of assessing a cell's actual genetic state is to analyze its mRNA pattern, which represents genes that are transcribed at a certain stage in a cell's life. The differential display method allows easy identification of differentially expressed genes, and therefore is a promising tool for analyzing individual mRNA molecules after reverse transcription and amplification by PCR. Applying this method, considerable change of a messenger RNA expression was found between HNSCC cells and benign epithelial cells. This partial gene fragment was recovered, cloned and sequenced revealing an exact match with the S19 gene that encodes for a ribosomal protein (RP).

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S19 ribosomal protein synthesis has already been found to be altered in different malignancies (13, 19), but so far no systematic examination of S19 at a transcriptional or translational level has specifically been performed in carcinomas of the upper aerodigestive tract. RT-PCR analysis of a larger number of head and neck squamous cell carcinoma (HNSCC) cell lines and tissue samples should be performed to prove the results obtained by differential display.

Ribosomal protein S19 forms part of the small ribosomal subunit (14). To elucidate whether ribosomal variations between malignant and benign cells are the reason for altered S19 gene expression, the expression pattern of further genes that encode for ribosomal proteins should simultaneously be analyzed.

Materials and Methods

Cell lines and tissue samples. A total of 17 well characterized HNSCC cell lines (UMSCC: University of Michigan Squamous Cell Carcinoma, UTSCC: University of Turku Squamous Cell Carcinoma) and SCC-745, established by us, were studied. The HNSCC cell lines were derived from the oral cavity (n=9), the oropharynx (n=1), the larynx (n=3) and metastatic lymph nodes (n=5). The primary sites in these cases were the hypopharynx (n=1), the nose (n=1) and the oral cavity (n=3). Seventeen benign keratinocyte cell lines established from oropharyngeal mucosa, obtained during diagnostic or therapeutic surgery from patients of the Department of Otorhinolaryngology of the Philipps-University Marburg, Germany, served as controls.

Carcinoma tissue samples from 30 patients were additionally investigated, deriving from the lower lip (n=1), the oropharynx (n=9), the hypopharynx (n=5), the larynx (n=13) and metastatic lymph nodes (n=2). The primary sites in these cases were unknown (n=1) and the oropharynx (n=1). Eight benign tissue biopsies, all from oropharynx or larynx, were used for control experiments. Informed consent was obtained from all patients prior to operation. A closer characterization concerning patients' age and sex, specimen site, TNM stage of disease and histological grading is summarized in Table I.

Culture conditions. Gained tissue samples were transfered into 500 ml sterile keratinocyte-serum-free-medium (keraSFM, GibcoBRL Life Technology, Eggenstein, Germany) containing 250 μg/ml Amphotericin B (Squibb-von Heyden GmbH, Munich, Germany), 5ng/ml

Table I. *Clinical data and S19-, S6- and S14-mRNA expression levels in HNSCC and cell cultures.*

Number of biopsies and cell lines	Patient age (years/sex)	Location of carcinoma	Type of lesion	TNM at diagnosis	Grading	S19-mRNA expression	S6-mRNA expression	S14-mRNA expression
1	66/M	Oropharynx	Primary	T4N0M0	G4	+	+	+
2	52/M	CUP	Metastasis	TxN2bM0		-	+	-
3	61/M	Larynx	Primary	T4N0M0	G2	-	-	-
4	53/M	Oropharynx	Primary	T3N0M0	G2	++		
5	61/F	Oropharynx	Primary	T4N2aM0	G2	-	-	-
6	45/M	Larynx	Primary	T3N2cM0	G3	+		
7	50/M	Hypopharynx	Primary	T4N2cM0	G2	-	-	-
8	55/M	Larynx	Primary	T1N0M0	G2	++		
9	57/M	Oropharynx	Primary	T4N2bM0	G1	+	-	-
10	63/M	Larynx	Primary	T4N2cM0	G2	++	++	+
11	63/M	Larynx	Primary	T4N2cM0	G2	+		
12	50/F	Larynx	Primary	T4N2cM0	G2	-		
13	37/M	Larynx	Primary	T4N2cM0	G3	+	-	+
14	61/M	Oropharynx	Primary	T4N2cM0	G2	++	++	+
15	63/M	Larynx	Primary	T2N1M0	G3	++	+	+
16	63/M	Lower lip	Primary	T2N0M0	G1	++		
17	53/M	Oropharynx	Primary	T2N2cM0	G2	++		
18	52/M	Oropharynx	Primary	T2N2aM0	G3	++		
19	58/M	Hypopharynx	Primary	T3N0M0	G3	+	+	-
20	47/M	Oropharynx	Metastasis	T4N2cM0	G3	++		
21	48/M	Oropharynx	Primary	T4N2bM0	G2	-	-	-
22	51/M	Hypopharynx	Primary	T3N0M0	G3	-	-	-
23	71/M	Larynx	Primary	T2N1M0	G2	++	++	++
24	61/M	Larynx-Hypopharynx	Primary	T4N3M0	G3	++		
25	42/M	Larynx	Primary	T4N1M0	G2	++	++	+
26	66/M	Hypopharynx	Primary	T4N3M1	G3	-	-	-
27	64/F	Hypopharynx-Esophagus	Primary	T4NxM0	G2	-	-	-
28	79/F	Larynx	Primary	T2N2cM0	G2	-	-	+
29	59/M	Larynx	Primary	T3N0M0	G2	-	-	+
30	61/M	Oropharynx	Primary	T4N2aM0	G2	--	-	-
SCC 745	48/M	Oropharynx	Primary	T4N2bM0	G2	--	-	--
UM 1	73/M	Floor of the Mouth	Primary	T2N0M0	G2	--	--	--
UM 14a	58/F	Floor of the Mouth	Primary	T1N0M0	G3	--	-	--
UM 14b	59/F	Floor of the Mouth	Primary	T1N0M0	G3	--	-	--
UM 14c	59/F	Floor of the Mouth	Primary	T2N1M0	G3	--	-	-
UM 19	67/M	Tongue	Primary	T2N1M0	G2	--		
UM 22b	58/F	Hypopharynx	Metastasis	T2N1M0	G2	++	+	-
UM 27	missing	Tongue	Primary	T1N0M0	G2	+	-	+
UM 3	73/F	Nose sinus	Metastasis	T1N0M0	G2	--	-	-
UM 9	71/F	Tongue	Metastasis	T2N0M0	G2	+		
UT 10	62/M	Tongue	Primary	T1N0M0	G2	+		
UT 16a	77/F	Tongue	Primary	T3N0M0	G3	-	-	--
UT 16b	77/F	Tongue	Metastasis	T3N0M0	G3	--	-	--
UT 19a	44/M	Larynx	Primary	T4N0M0	G2	--	-	-
UT 19b	44/M	Larynx	Primary	T4N0M0	G2	++		
UT 24a	41/M	Tongue	Primary	T2N0M0	G2	-	+	-
UT 24b	41/M	Tongue	Metastasis	missing	G2	-		
UT 8	42/M	Larynx	Primary	T2N0M0	G1	-	--	-

The expression of S19-, S6- and S14 mRNA was determined by RT-PCR and estimated in range from - - (strongly reduced) to + + (strongly elevated). Comparison was made to the median of the examined reference samples.

mycoplasma removal agent (MRA, ICN Biomedicals, Meckenheim, Germany) and 250 U/ml penicillin/streptomycin (Grunenthal, Aachen, Germany), supplemented with 1.5 ng/ml epidermal growth factor and 50 mg/ml bovine pituitary extract (GibcoBRL Life Technology). Specimens were cut under sterile conditions, washed several times with phosphate-buffered saline (PBS) and incubated in 3 ml PBS containig 2.4 U/ml dispase (Roche, Mannheim, Germany) at 37˚C for 1 h. The removed epidermis was washed with 5 ml PBS again and incubated in 0.02%-0.05% (w/v) trypsin solution (Biochrom KG) at 37˚C for 20-30 min, to dissociate cells. After centrifugation, the cell pellet was washed with fetal calf serum to inactivate trypsin, centrifuged again and resuspended in fresh SFM following incubation at 37˚C in a 5% CO_2 humidified athmosphere.

The SCC cell lines were grown in minimum essential medium (MEM) containing L-glutamine, 20mM Hepes buffer, 10% (v/v) fetal calf serum (Bio Whittaker, Verviers, Belgium), 100 U/ml penicillin/streptomycin and 5ng/ml MRA under the same conditions as described for benign keratinocytes.

Isolation of total RNA. For preparation of total RNA, the QIAGEN Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. After photometric measurement at 260 nm, samples of total RNA were adjusted to 1 μg for subsequent first-strand cDNA synthesis. To judge the native character of the RNA samples, 10 μg of total RNA of each probe were separated on a 2% agarose gel to check the quality of the 28S- and 18S-rRNA units.

First-strand cDNA synthesis. Total RNA (1 μg) was heat denatured (65˚C, 10 min), chilled on ice and subjected to oligo (dT) primer reverse transcription using 2 μl 10 x PCR buffer, 4 μl 25 mM $MgCl_2$, 20 μM dNTP, 2.5 μM oligo(dT) anchored primer, 1 μl RNAse inhibitor and 300 units of MMLV reverse transcriptase. The resulting double-stranded RNA:cDNA heteroduplex was heat denatured at 95˚C for 5 min to provide cDNA as template for amplification.

mRNA differential display. Two carcinoma cell lines as well as two benign keratinocyte cell lines (see section 'cell lines and tissue samples') were applied to messenger RNA differential display. After heat denaturation, the cDNA was incubated with 0.5 μM of the 5'-GATCTGACAC-3' arbitrary primer (GenHunter, Nashville, TN, USA), 2.5 μM of the corresponding anchored primer 5'-AGTTTTTTTTTTT-3', 2.5 μM dNTPs, 1.5 μM $MgCl_2$, 2μCi [^{33}P]dATP (Amersham, Braunschweig, Germany) and 2.5 U Taq polymerase in a final volume of 50 μl. Low-stringency PCR was carried out for 40 cycles at 95˚C for 60 sec, 40˚C for 120 sec, 72˚C for 30 sec, with a final extension step at 72˚C for 10 min (Thermocycler 9600; Perkin-Elmer, Norwalk, CT, USA). After thermocycling, 3.5 μl of the amplicon plus 2μl loading dye (97% deionized formamide, 10 mM EDTA, 0.1% xylene cyanole, 0.1% bromphenol blue) were mixed, incubated at 80˚C for 2 min and loaded onto a 6% denaturing polyacrylamide sequencing gel. Electrophoresis was carried out for 2 h at 35 Watts constant power until the xylene dye was 1 cm from the bottom. After electrophoresis, the gel was dried without fixing with methanol/acetic acid, and autoradiographed. The orientation of the autoradiograph was marked with needle punches.

Analytical high-stringency RT-PCR and sequencing of cDNA. For amplification of the S19 gene fragment 200 ng cDNA product, 5 μl 10x PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.1 μM of upstream (5'- AGG CCG CAC GAT GCC TGG AGT TAC TGT AAA AGA - 3') and downstream primer (5'- GCC AAC AAG AAG CAT TAG AAC AAA CCA TGC - 3') and 2.5 U Taq-polymerase were subjected to PCR in a volume of 50 μl. PCR was run for 30 cycles at 94˚C for 60 s, 58˚C for 60 s and 72˚C for 90 s with a 5 min extension at 72˚C. After thermocycling 5 μl of the amplicon plus 2 μl loading dye (97% dionized formamide, 10 mM EDTA, 0.1% xylene-cyanol, 0.1% bromphenol blue) were mixed and loaded onto a 2% agarose gel containing 0.01% ethidium bromide. The 100 bp DNA ladder (323-1S, BioLabs Inc.) was used as DNA marker. The electrophoretic pattern was analyzed by means of the Gel Doc 2000 and Quantitation Software (Biorad, Germany).

PCR products for each of the employed primers of S6 (upstream 5' - CTG AAC ATC TCC TTC CCA GCC - 3', downstream 5' - CGC CGC CTG GGC CCC AAA AGA - 3'), S14 (upstream 5' - ATG GCA CCT CGA AAG GGG AAG - 3', downstream 5' - CTC CGG GCC ACA GGA GGA AAT - 3'), S19 and beta actin (upstream 5' - GAT GAT GAT ATC GCC GCG CTC GTC GTC - 3', downstream 5' - TGA GCG GTT CCG CTG CCC TGA GGC AC - 3') were purified using the GFX Kit (Pharmacia Biotech) and sequenced using the LI-COR 4200 system at MWG Biotech (Ebersberg, Germany).

Statistics. The levels of S19, S6 and S14 gene expression were put into relation with the constitutive expression of the housekeeping gene beta actin, which was used as an internal standard. These values were used for the following statistical operations. Data were analyzed for normal distribution using the Kolmogorov-Smirnov-test. To compare the HNSCC and the reference groups, the Whitney-Mann *U*-test was employed. Spearman-Rho correlation was performed to examine RP expression levels in the context of patient and disease parameters. All statistical operations were performed using EXCEL and SPSS software.

Results

mRNA differential display. Following the electrophoretic separation of PCR products, differential display fingerprints were detected by autoradiography. Analyzing the migration pattern, a remarkable change of a 109 bp fragment was detected in benign keratinocytes compared to laryngeal SCC cells (Figure 1). The expression of this fragment in each repeated experiment was judged by the same quantitative difference occurring in the autoradiogram of both cell types.

For sequence determination, the differentially expressed fragment was ligated into a pGEM-T vector. Using both forward and reverse M13 oligonucleotides, three clones were sequenced on both strands and examined for potential overlaps. A databank search indicated complete homology with a region (position: from 388 to 598) of the human ribosomal S19 mRNA (data not shown).

RT-PCR analysis. To evaluate the extent and frequency of S19, S6 and S14 mRNA expression in HNSCC, RT-PCR was performed using gene-specific primers to amplify a 460 bp fragment of S19 mRNA, a 405 bp fragment of S6 mRNA, a 309 bp fragment of S14 mRNA as well as a 778 bp fragment

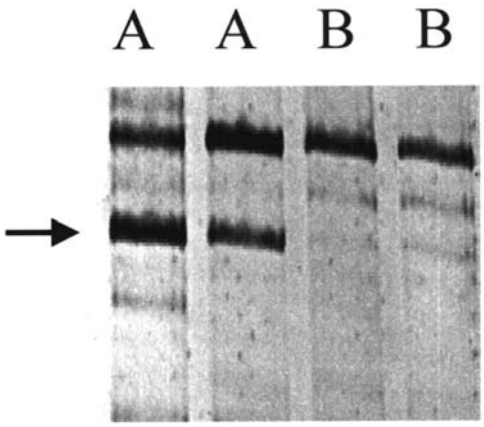

Figure 1. *Part of the mRNA expression profile after differential display. Total RNA from two independent normal upper aerodigestive tract mucosal keratinocytes (A) and SCC cell lines (B) were reverse transcribed using HT11G anchored primer. After RT-PCR, the amplicons were subjected to denaturing polyacrylamide gel electrophoresis. The gel was dried and fingerprints were visualized by autoradiography. The arrow indicates the differentially-expressed fragment.*

of human beta actin (Figure 2). The RNA quality was proved for every single probe by obtaining distinct bands for 28S and 18S rRNA in electrophoresis. After sequencing the samples, database search was performed revealing an exact homology match with S19, S6 and S14 as well as with human beta actin gene sequences.

Analysis of the data obtained. The Kolmogorov-Smirnov test showed that the results are not normally distributed: range, median, percentiles Q_{25} and Q_{75} for S19, S6 and S14 in the reference and the HNSCC groups are therefore chosen to present the results (Figure 3). The median values for mRNA expression levels of all three ribosomal proteins S19, S6 and S14 were significantly reduced in the carcinoma cell lines compared to the reference cell lines (S19 Z=-2.295 (p=0.022), S6 Z=-3.118 (p=0.002) and S14 Z=-2.597 (p=0.009)). The results for the carcinoma tissues compared to the reference tissues did not show any significant differences (S19 Z=-0.859 (p=0.390), S6 Z=-1.119 (p=0.263) and S14 Z=-0.509 (p=0.611)).

mRNA expression levels of S19, S6 and S14 in carcinoma cells and tissues. Carcinoma samples were classified by means of the difference between the value of the mRNA expression level of each malignant sample and the median value of the mRNA expression levels of the reference samples and estimated as strongly reduced (x < -0.5 relative expression units (REU)), moderately reduced (-0.5 REU=x < 0 REU), moderatly elevated (0 REU=x < 0.5 REU) and strongly elevated (x=0.5 REU) (Table I).

Regarding the cell lines, two lines presented strongly elevated levels of S19 mRNA, three moderatly elevated levels of S19 mRNA, four moderately reduced levels of S19 mRNA and nine lines strongly reduced levels of S19 mRNA. Concerning the S6 mRNA, two cell lines exhibited moderately elevated levels, nine moderately reduced levels and two strongly reduced levels. Regarding the S14 mRNA, for one cell line moderately elevated levels could be measured, for six moderately reduced levels and for another six strongly reduced levels.

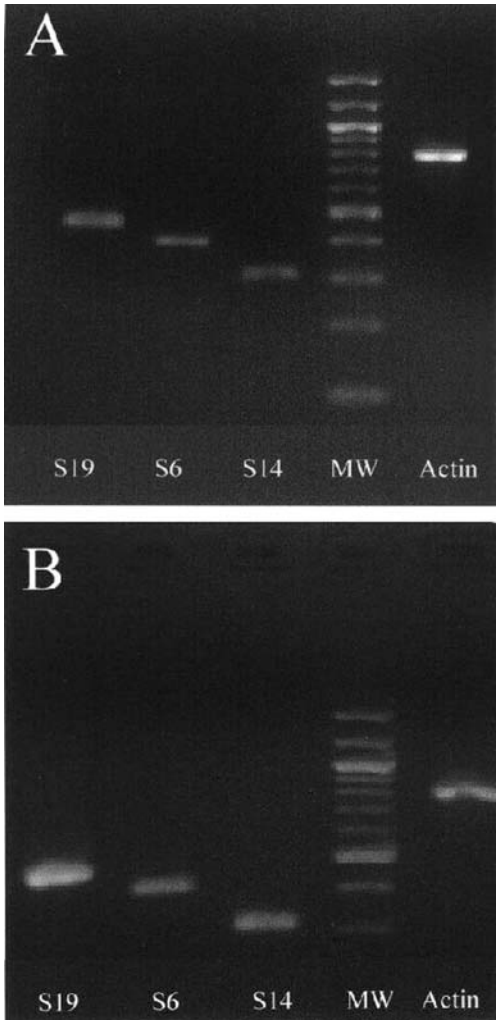

Figure 2. *Representative electrophoresis of S19, S6, S14 and beta actin RT-PCR products for cell lines (A) and tissue samples (B). The amplicons obtained were separated on a 2% agarose gel.*

With regard to the tissue samples, twelve showed strongly elevated S19 mRNA levels, six samples moderately elevated S19 mRNA levels, eleven moderately reduced S19 mRNA levels and one strongly reduced S19 mRNA levels. Four samples revealed strongly elevated S6 mRNA levels, four moderately elevated S6 mRNA levels and twelve moderately reduced S6 mRNA levels. Strongly elevated S14 mRNA levels could be measured in one case, moderately elevated S14 mRNA levels in eight cases and moderately reduced S14 mRNA levels in eleven cases.

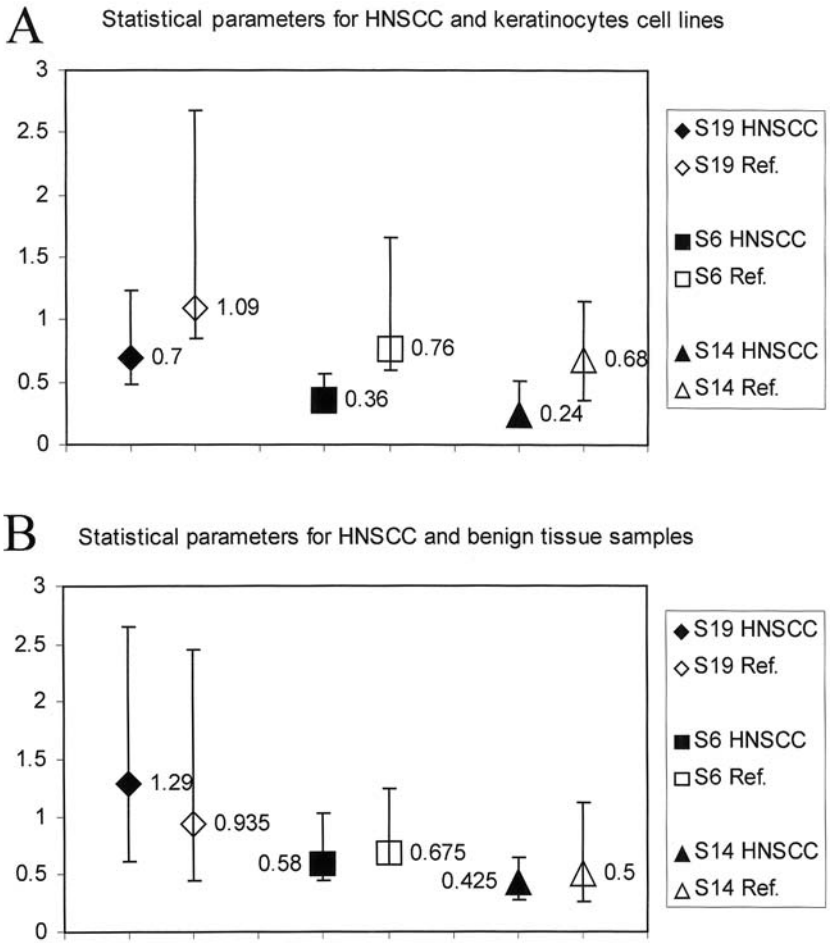

Figure 3. *Comparison of S19, S6 and S14 expression levels in HNSCC and reference cell lines (A) and carcinoma and reference tissue samples (B). Median, Q25 and Q75 are shown for the malignantly transformed and reference samples as the Kolmogorov-Smirnov test showed that the obtained results were not normally distributed. Data are presented in relative expression units (REU).*

Correlation for HNSCC and reference samples. Spearman-Rho correlation was performed between the results obtained for S19, S6 and S14 and the disease-describing parameters stage, TNM classification and histopathological grading based on a two-sided 5% significance. Significant correlation was found between S19-, S6- and S14 mRNA expression levels by $p=0.000$. Furthermore, in the HNSCC cell line group a significant correlation between S19 ($p=0.003$) and S14 ($p=0.012$) expression levels and the histopathological grading could be shown: decreasing S19 and S14 levels correlated with higher grading levels. All other results could not be proved to be significant.

Discussion

Differential display examination showed a remarkable change of S19 gene expression in HNSCC cell lines compared to benign cell lines. The following RT-PCR proved significantly reduced expression levels of the S19 gene in HNSCC cell lines compared to benign cell lines, while the same examination did not give significant results for the examined tissue samples. Gene expression of two further ribosomal proteins was examined simultaneously by the same method and revealed a significant correlation with the S19 mRNA expression levels.

The results obtained in this work may contribute to better characterize S19 mRNA expression in HNSCC. Therefore, the regulation of RPs in normal cells should be considered in the context of the significant correlation between S19 and S6/S14 mRNA expression levels found in this study. Knowing the function of RPs is the first step to understanding the pathomechanism leading to an altered S19 expression. Ribosomal as well as extraribosomal functions of RP have to be thought of. Due to their various and important functions during cellular processes in a cell's life, RP expression is often changed in malignancies. The possible pathomechanisms of S19 underexpression in HNSCC will be discussed.

S19 expression pattern in different malignancies. The primary structure of S19, part of the small ribosomal subunit, was determined for different organisms. A strong sequence homology among those was found. This conservation argues that selection against mutations in the S19 sequence takes place and that a correct production of S19 protein is essential for normal cell function (2, 4, 18). Supporting these findings, S19 was shown to be altered in different malignancies: Rhodes and van Beneden identified S19 in an expression screen that compared normal to neoplastic gonads of the marine bivalve *Mya arenaria* (19). Kondoh *et al.* found significantly increased S19 levels in twelve colorectal carcinoma cell lines and seven benign/malignant tissue samples from colon and duodenum by Northern hybridisation analysis (13). Colorectal tissue is very similar to the tissue of the upper aerodigestive tract due to common development from the embryonic yolk sac. However, in this study it could be shown that S19 was significantly less expressed in 18 HNSCC cell lines compared to 17 keratinocyte cell lines. The highly significant negative correlation of S19 expression and histopathological grading well supports the general finding that S19 mRNA is less expressed in HNSCC cells.

RP regulation in normal cells. As S19 is only one component of the ribosome, the question arises as to whether S19 expression in HNSCC changes merely because of changed ribosome levels. To investigate this issue, RT-PCR was performed with two further RPs – S6 and S14. In benign cells, RPs are required in equimolar amounts. The 5' untranslated region shows a homologous sequence in most RPs, which is believed to play a critical role in the regulation of RP expression (1). S6 and S14 were chosen for this study, because they are already known to change their expression quotient in leukaemic blast cells (8). In contrast to Ferrari's findings for leukaemic cells, Spearman-Rho correlation for the 3 RPs shows a highly significant high correlation for all three pairs (S19-S6, S19-S14, S6-S14) in HNSCC. A comparison of S6 and S14 expression levels in HNSCC and reference cell lines shows similar results as for S19: S6 and S14 are significantly decreased in the HNSCC cell lines. For S14 as for S19, a significant negative correlation between histopathological grading and S14 expression level was found. These results strongly suggest that S19 is not completely freed from the strict ribosomal coordination in HNSCC cells. Furthermore, it seems more likely that the decrease is connected to the protein's ribosomal function and not to one of its various extraribosomal functions as similar changes were found for the two other RPs examined in this work.

Possible functions of S19 explaining the altered mRNA expression levels in HNSCC. The function of S19 in the cell might give some hints as to how carcinogenesis interacts with regulation of S19 and whether it is caused or followed by the changed expression levels. Mainly RPs are concerned with establishing the complex ribosomal structure to bring the various active sites into the right relationship (27). Referring to S19, different ribosomal functions were described, *e.g.* binding of the 16S rRNA during assembly of the small subunit (25), binding of initiation factors IF 1, IF 2 and IF 3 during initiation of translation (15) as well as binding of initiator tRNA (20). In the context of malignant degeneration, it is interesting to know that S19 is responsible for the accuracy control of translation in eucaryotes (5). Decreased S19 expression might lead to an increased mistake rate resulting in functionally or quantitatively altered proteins. Malignant transformation might be triggered by changed expression of proto-oncogene products or tumor suppressor gene products.

As for other RPs, further – extraribosomal – functions are described for S19. After polymerization, S19 gains a new function completely different from the S19 monomer. The homodimer is a very strong chemotactic agent specific to monocytes and takes part in the acquired immune response (21-23). S19 dimer is released from apoptotic cells (11, 17). Apoptosis is thought to play a crucial role in remodeling and repairing of tissues and, thus, in malignant transformation. Loss of apoptosis ability is one step in malignant transformation of cells. There is an inverse relationship between metastatic potential and apoptosis rate in cancers (12). This fact would well agree with the measured decreasing S19 expression levels in this study.

Different findings indicate that S19 plays a crucial role during embryogenesis. Mutations in the S19 gene cause malfunctions as is known from Diamond Blackfan anaemia, which is often combined with somatic dysmorphism (6, 9, 10, 16, 26). Another argument for S19's importance during embryogenesis comes from the nematode *Ascaris lumbricoides*, which needs S19 for chromatin condensation (7). S19 expression declines during gametogenesis of the marine bivalve *Mya arenaria*, but is increased again in neoplastic gonads (19). During embryogenesis tissue differentiation is achieved by a delicate balance between cell differentiation, apoptosis and cell division. This balance is lost in carcinoma tissues, which could lead to an explanation for the lowered S19 mRNA expression in HNSCC found in this study.

The different S19 mRNA expression levels in benign and malignant cells represent one more piece in the puzzle of molecular-biological changes in HNSCC cells. Correlation of S19 expression levels and histopathological grading could be helpful in determining a patient's prognosis or judging the effect of a certain therapy. In case of S19, it would be interesting to see whether its dimer is decreased in HNSCC in the same way. This would argue that S19's apoptosis-related function might be responsible for its underexpression in HNSCC, which was shown in the present work for the first time.

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