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European Journal of Cancer 41 (2005) 445–452

European Journal of Cancer

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Preoperative detection of laryngeal cancer in mucosal swabs by slide-based cytometry

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Received 9 June 2004; received in revised form 20 September 2004; accepted 8 October 2004
Available online 19 December 2004

Abstract

The aim of our study was to evaluate slide-based cytometry in screening for laryngeal cancer using swabs as a minimally invasive approach. Laser scanning cytometry (LSC) was used for the multiparametric analysis of cells stained for cytokeratin and DNA to determine the DNA-index (DI) of the tumour cells. Histograms with DI < 0.95, 1.05 < DI < 1.9, and 2.1 < DI were defined as DNA aneuploid. After subsequent haematoxylin–eosin (HE)-staining, single cells were re-localised and an analysis by conventional cytology was performed. Additionally, routine histopathology of parallel biopsies was obtained in all cases. Fifty one swabs from 49 lesions were analysed. Seven and 17 swabs, were classified as insufficient for LSC and cytology, respectively. One and two benign lesions, were misclassified as malignant, respectively. Out of 34 malignant lesions, LSC detected 25 and cytology 14. LSC was superior to cytology in all of the statistical parameters tested. This pilot study demonstrates the validity of LSC for the preoperative detection of malignancy in laryngeal tumours using swabs.

Keywords: DNA; Ploidy; LSC; Liquid-based cytology; Predictive medicine

1. Introduction

The prevalence for laryngeal cancer in the general population is rather low (1% of malignancies in Germany) [1]. However, its diagnosis poses specific problems, even for the specialist: by visual inspection, malignant tumours are difficult to differentiate from benign lesions, such as chronic laryngitis. Therefore, biopsies are taken for microscopical evaluation. Although studies have evaluated the role of cytological and cytometric analysis of swabs at other sites [2], this is not included in the clinical routine for cases of laryngeal lesions; in general, excisional biopsies for histological analysis are obtained at this location, mostly during general anaesthesia for obvious reasons. If the biopsy shows malignancy and if the patient prefers surgical therapy, another general anaesthesia is necessary for (laser) surgery. Regardless of the kind of therapy, the follow-up of these patients presents the same problem: whenever there is a suspicious lesion, a biopsy under general anaesthesia is necessary for histological analysis.

This could be avoided if a more reliable cytological analysis was available. Slide-based cytometry could help to achieve this goal: with this technology, specimens

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doi:10.1016/j.ejca.2004.10.012

with low cell counts are mounted on a slide; prior to conventional cytology, cells are analysed by multiparametric fluorescence-based assays, like those established for flow cytometry. This approach combines the power of fluorescence labelling with the reliability of direct visualisation: unlike in flow cytometry, there is a microscopical correlate for every cytometric event [3,4]. At present, the laser scanning cytometer (LSC®; CompuCyte, Cambridge, MA, USA) is the most common slide-based instrument [5–7]. Working with clinical material, we and others have compared DNA ploidy analysis by LSC with image analysis after Feulgen staining or with flow cytometry and shown a good correlation of these methods [8,9].

We therefore investigated whether LSC can predict malignancy in laryngeal lesions preoperatively. Cellular DNA was stained using propidium iodide (PI) and cytokeratin by immunofluorescence [10]. Similar assays have already been described for specimens from voided urine [11] and fine-needle aspiration biopsies [12]. Based on these publications, the aim of our present pilot study was to evaluate the applicability of LSC for the analysis of laryngeal swabs and to compare LSC with conventional cytology and routine histopathology.

2. Materials and methods

2.1. Specimen preparation

2.1.1. Specimens

The design of this study was approved by the local ethics committee. After written informed consent, 51 samples from 49 patients were taken with a cotton swab in the operation theatre under general anaesthesia. This step was part of a diagnostic or therapeutic surgical procedure and was performed at the very beginning of the intervention; these interventions were indicated for diagnostic reasons in cases of suspicious laryngeal lesions or for therapeutic reasons in already histologically proven malignancies and in clinically benign lesions (cysts, Reinke-oedema). The tissue fragments obtained were suspended in a 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany) that had been pre-filled with 200 μl phosphate-buffered saline (PBS; Gibco BRL, Paisley, Scotland, UK) pH 7.4, supplemented with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), by repeated dipping, and stored at 4 °C for up to 24 h until further preparation. Following the swab, a histological sample (excisional biopsy or resected tumour, in diagnostic or therapeutic procedures, respectively) was obtained from the same lesion for definitive histopathological diagnosis. A data-sheet was prepared for all 51 samples (Table 1).

2.1.2. Slide preparation

Erythrocytes were destroyed by adding one ml FACSLysis solution (BD Biosciences, San Jose, CA, USA) to each tube. After 15 min incubation at room temperature, the suspension was spun down (250*g*, 5 min) and the pellet was resuspended in 50 μl PBS supplemented with 0.5% BSA. Slides were prepared similar to liquid-based cytological samples as described earlier in [10]: to maintain identical conditions for both specific staining and negative controls, two 10–20 μl aliquots of the cell suspension were placed onto one microscopic glass slide (SuperFrost™; Menzel Gläser, Braunschweig, Germany) with each sample covering a 1.2 cm square. In order to place the suspension at reproducible sites a template was marked on paper and placed underneath the slide. According to the template, the drawing was transferred to the slide using a fat pencil. The suspension was then placed in the two squares to such a volume that the cells on the slide were not too loose and not too close: as a 'rule of thumb', the distance between two cells was the same size as their diameter. Slides were then air-dried and stored in 70% ethanol.

2.1.3. Staining

Slides were washed with PBS and then 200 μl of PBS supplemented with 1% BSA were pipetted onto each cell spot. After incubation for 15 min, the supernatant was discarded. Corresponding cell spots were loaded with either 9 μl of anti-cytokeratin antibody solution (3 μl Clone MNF116, Code M0821; 3 μl Clone 34ß E12, Code M0630; 3 μl Clone AE1/AE3, Code M3515) diluted in 91 μl PBS containing 0.5% BSA or with 9 μl of unspecific control antibody solution (Code X0931; all: Dako, Carpinteria, CA, USA) diluted in 91 μl PBS containing 0.5% BSA. After 20 min incubation at room temperature, slides were washed twice with 0.5 ml PBS prior to a 20 min incubation with 9 μl biotinylated anti-mouse antibody (Caltag, Hamburg, Germany, Code M32015) in 91 μl PBS with 0.5% BSA. Slides were washed twice with 0.5 ml PBS and were then incubated for 20 min with 9 μl streptavidin conjugated to APC (Caltag, Hamburg, Germany, Code SA1005) in 91 μl PBS/0.5% BSA supplemented with 50 μg propidium iodide (PI)/ml (Sigma, St. Louis, MO, USA) and 100 μg RNase/ml (Sigma, St. Louis, MO, USA) at room temperature in the dark. Allophycocyanin (APC) was used instead of the far more common fluorescein isothiocyanate (FITC) since FITC binds to eosinophilic granulocytes and this leads to the false-positive detection of a cytokeratin-positive diploid population [9]; pure FITC can be used in combination with anti-cytokeratin conjugated to APC to separate eosinophiles from epithelial cells in paranasal polyps [4]. Slides were washed twice with 0.5ml PBS and covered with 40 μl of glycerol in PBS 75/25% with 25 μg PI/ml. Following microscopic analysis by LSC, the coverslip was removed and a con-

Table 1
Patient data clinical and surgical data are given for the 51 swabs in combination with the results of LSC, cytology, and histopathology

Pat-ID	Pathology	LSC	Cytology	LSC–Cytology	Cytology–LSC	Histology (final grading, TN)	Procedure	Localisation
#1	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, pT4 pN0)	LE+SND	Subglottis
#2	Benign	Benign	Benign	Benign	Benign	Parakeratosis	MLS	Glottis
#3	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, pT2 pN0)	PLR+SND	Supraglottis
#4	Benign	Benign	Suspicious	Insufficient	Benign	Hyperplasia	MLS	Supraglottis
#5	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, cT2 cN0)	Panendo	Glottis
#6	Benign	Benign	Benign	Benign	Benign	Leucoplakia with moderate dysplasia	MLS	Glottis
#7	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, pT3 pN2c)	LE+SND	Supraglottis/ Glottis
#8	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (G2, pT1 cN0)	Panendo	Glottis
#9	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (S2, pT2 cN0)	MLS	Glottis
#10	Benign	Insufficient	Benign	Benign	Insufficient	Fibroid polyp	MLS	Glottis
#11	Malignant	Malignant	**Benign**	Malignant	Malignant	SCC (G2, pT2pN0)	Panendo	Supraglottis
#12	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, cT3 cN0)	Panendo	Supraglottis
#13	Malignant	Malignant	**Benign**	Malignant	Malignant	Intraepithelial neoplasia	MLS	Glottis
#14	Benign	Benign	**Malignant**	**Malignant**	**Malignant**	Papilloma	MLS	Glottis
#15	Benign	Benign	Benign	Benign	Benign	Reinke oedema	MLS	Glottis
#16	Benign	Insufficient	Benign	Benign	Insufficient	Chronic inflammation with dysplasia	MLS	Glottis
#17	Benign	Benign	Benign	Benign	Benign	Papilloma	MLS	Gtottis
#18	Benign	Benign	Benign	Benign	Benign	Contact granutoma	MLS	Glottis
#19a	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (G3, pT4pN2b)	LE+SND	Larynx
#19b	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (G3, pT4 pN2b)	LE+SND	Larynx
#20	Benign	Benign	Benign	Benign	Benign	Polyp	MLS	Glottis
#21	Benign	**Malignant**	Benign	**Malignant**	**Malignant**	Hyperplasia	MLS	Glottis
#22	Malignant	Malignant	**Benign**	Malignant	Malignant	SCC (G2, pT2 pN0)	Panendo	Glottis
#23	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (G2, pT2 pN2b)	Panendo	Supraglottis
#24	Malignant	Malignant	**Benign**	Malignant	Malignant	SCC (G2, pT1 pN2a)	MLS	Supraglottis
#25	Benign	Benign	Benign	Benign	Benign	Chronic inflammation	MLS	Glottis
#26	Malignant	**Benign**	Suspicious	Insufficient	**Benign**	SCC (S3, cT4cN1)	Panendo	Larynx
#27	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, pT3 pN0)	LE+SND	Larynx
#28	Malignant	Malignant	**Benign**	Malignant	Malignant	SCC (G2, pT3 pN0)	Panendo	Glottis/ Subglottis
#29	Malignant	Insufficient	Insufficient	Insufficient	Insufficient	SCC (G2, pT3 pN0)	MLS	Glottis/ Subglottis
#30	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (G2, pT2 pN0)	PLR+SND	Supraglottis
#31	Malignant	**Benign**	**Benign**	**Benign**	**Benign**	SCC (G2, pT4 pN2c)	MLS	Larynx
#32	Benign	Benign	Suspicious	Insufficient	Benign	Cyst	MLS	Supraglottis
#33	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, pT2pN0)	Panendo	Glottis
#34	Malignant	**Benign**	Malignant	Malignant	Malignant	SCC (G2, cT1 cN0)	Panendo	Glottis
#35	Benign	Benign	Suspicious	Insufficient	Benign	Fibroid polyp	Panendo	Glottis
#36	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, pT4pN2)	LPE+SND	Larynx/ Hypopharynx
#37	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (G2, pT1 cN0)	MLS	Glottis
#38	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, pT3 pN0)	LE+SND	Supraglottis
#39	Malignant	Insufficient	Malignant	Malignant	Malignant	SCC (G2, cT3cN1)	MLS	Larynx
#40	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2,cT1cN0)	Panendo+MLS	Glottis
#41	Malignant	Insufficient	Malignant	Malignant	Malignant	SCC (G2,pT3pN0)	LE+SND	Larynx
#42	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (G2, cT3 cN0)	Panendo	Supraglottis
#43	Malignant	Malignant	Malignant	Malignant	Malignant	SCC (G2, pT2 pN0)	PLR+SND	Supraglottis
#44	Malignant	**Benign**	Suspicious	Insufficient	**Benign**	SCC (S3, pT2 cN0)	MLS	Glottis
#45a	Malignant	Insufficient	Insufficient	Insufficient	Insufficient	SCC (G2, pT1 cN0)	Panendo	Glottis
#45b	Malignant	**Benign**	Suspicious	Insufficient	**Benign**	SCC (G2, pT1 cN0)	MLS	Glottis
#46	Benign	Insufficient	**Malignant**	**Malignant**	**Malignant**	Fibroid polyp	MLS	Glottis
#47	Benign	Benign	Benign	Benign	Benign	Inflammation + hyperplasia	MLS	Supraglottis/ Glottis
#48	Benign	Benign	Benign	Benign	Benign	Keratosis	MLS	Glottis
#49	Malignant	Malignant	Suspicious	Malignant	Malignant	SCC (G2, pT2 cN0)	MLS+SND	Glottis

Abbreviations: SCC, squamous cell carcinoma; panendo, panendoscopy; LE, laryngectomy; TN, classification to World Health Organization (WHO) TNM; PLR, partial laryngeal resection; LPE, laryngopharyngectomy; MLS, microlaryngoscopy with resection (laser); SND, selective neck dissection.

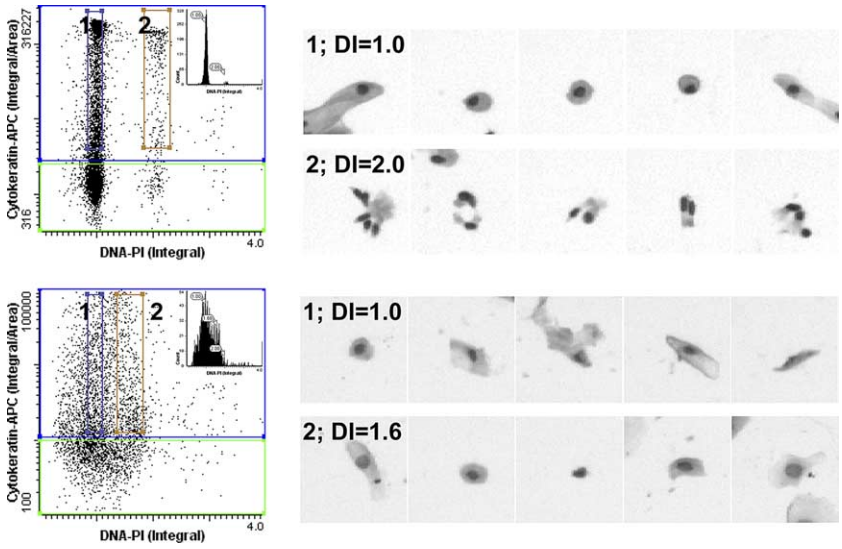

Fig. 1. Interpretation of LSC data. Swabs (top: #20, benign; bottom: #28, malignant) were analysed by LSC and stained with HE for re-localisation: gate 1 (top and bottom): DI = 1.0, diploid normal epithelial cells; gate 2 (top): DI = 2.0, doublettes; gate 2 (bottom): DI = 1.6, aneuploid malignant carcinoma cells. DNA-content (*x*-axis, linear) is plotted *vs.* APC-fluorescence per area (*y*-axis, logarithmic) (a 5% cut-off point is set in the control sample – data not shown). The inset shows a DNA histogram of cytokeratin-positive cells.

ventional haematoxylin–eosin (HE) staining was performed. Slides were then permanently covered using Eukitt (Kindler GmbH & Co., Freiburg, Germany).

2.2. Analysis by LSC

Slides were analysed in the LSC with the 20× objective as detailed earlier [10]. In general, 5000–10 000 cells were measured per area using the PI signal. Signals were acquired for the forward-scatter and the red channel after argon-laser excitation, and for the far-red channel after helium–neon-laser excitation. The photomultipliers were adjusted in such a way that signals did not exceed the range of sensitivity.

2.3. Data interpretation

Data were interpreted with the proprietary WinCyte-software in a blinded fashion. Specimens with less than 1500 cells or with massive staining artefacts were classified as ''LSC-insufficient''. Data interpretation was performed after HE-staining as described earlier in [12] and as exemplified in Fig. 1. A cut-off level of 5% was set for the negative control to detect cytokeratin-positive cells in a specifically immunostained sample. The 2c-channel for DNA diploid cells was set in the gated DNA-histogram for cytokeratin-negative cells, such as infiltrating/contaminating leucocytes. The DI of the different peaks for cytokeratin positive cells was then calculated. $0.95 < DI < 1.05$ were defined as DNA diploid, $1.9 < DI < 2.1$ was defined as DNA tetraploid; any other DI was defined as DNA aneuploid. Re-localisation of single cells confirmed the proper assignment of the different cell subsets. Ten consecutive cells were re-localised for each subset to estimate the cell loss by HE-staining.

A LSC sample was classified as ''LSC-malignant'' if DNA aneuploid peaks were detected. Otherwise, it was classified as ''LSC-benign''. Fisher's exact probability test was used to compare routine histopathology and the LSC classification. In addition to the sensitivity and the specificity, the positive (PPV) and negative predictive values (NPV) were calculated (Fig. 2).

2.4. Analysis by cytology

Following the LSC-analysis, slides were evaluated by an experienced cytopathologist for the presence of malignant cells based on the light-microscopic characteristics in a blinded manner. The specimens were scored as ''cytology-benign'', ''cytology-malignant'', or ''cytology suspicious/insufficient'', and were compared with routine histopathology based on a confusion matrix (Fig. 3).

2.5. Combined slide-based cytometric/cytological classification

In order to obtain a combined slide-based cytometric/cytological classification, we developed two alternative algorithms (see Fig. 4).

3. Results

Swabs typically yielded 5000–20 000 cells: a mean of 9339 and a median of 5897 cells per cell spot were ana-

	$Histo_{ben}$	$Histo_{mal}$	Sum		
LSC_{insuff}	3	4	7		
LSC_{ben}	13	5	18	PPV = 0.96	Sensitivity = 0.83
LSC_{mal}	1	25	26	NPV = 0.72	Specificity = 0.93
Sum	14	30	44	$P = 1.9 \times 10^{-6}$	
$Cytology_{susp\ or\ insuff}$	3	14	17		
$Cytology_{ben}$	12	6	18	PPV = 0.88	Sensitivity = 0.7
$Cytology_{mal}$	2	14	16	NPV = 0.67	Specificity = 0.86
Sum	14	20	34	$P = 1.6 \times 10^{-3}$	
Sum	17	34	51		

Fig. 2. Comparison of LSC and cytology *vs*. histopathology. The confusion matrix LSC *vs*. histopathology (above) and cytology *vs*. histopathology (below) of 51 swabs provides the PPV, the NPV, the sensitivity, and specificity per assay. Fisher's exact tests was used to determine the of *P* value.

	$Histo_{ben}$	$Histo_{mal}$	Sum		
$LSC\text{-}Cyto_{insuff}$	3	5	8		
$LSC\text{-}Cyto_{ben}$	11	1	12	PPV = 0.90	Sensitivity = 0.97
$LSC\text{-}Cyto_{mal}$	3	28	31	NPV = 0.92	Specificity = 0.79
Sum	14	29	43	$P = 4.8 \times 10^{-6}$	
$Cyto\text{-}LSC_{insuff}$	2	2	4		
$Cyto\text{-}LSC_{ben}$	12	4	16	PPV = 0.90	Sensitivity = 0.88
$Cyto\text{-}LSC_{mal}$	3	28	31	NPV = 0.75	Specificity = 0.8
Sum	15	32	47	$P = 10.1 \times 10^{-6}$	
Sum	17	34	51		

Fig. 3. Comparison of combined algorithms *vs*. histopathology. Swabs were classified according to LSC–Cyto-algorithm (above) and Cyto–LSC-algorithm (below) and classification was compared with routine histopathology. Fisher's exact test was used to determine the *P* value.

lysed. Erythrocytes were effectively destroyed by treatment with the lysis buffer. The morphology of the cells was well preserved.

Removal of the cover slip and HE re-staining did not lead to significant cell loss: none of 10 regularly re-localised cells within the different cell sub-sets (typically between 100 and 200 cells per sample) was missing. Even sporadically analysed sets of up to 50 consecutive cells were re-localised without missing cells, as has been shown for other applications [3,4,9,12].

Out of the 51 swabs, seven were classified as ''LSC-insufficient'' whereas only two swabs were insufficient for conventional cytology (Table 1). However, in an additional 15 swabs the cytological diagnosis was ''suspicious for malignancy'' which has the same clinical relevance as ''LSC-insufficient'': the swab has to be obtained a second time; making for cytology a total of 17 specimens.

In order to compare the predictive value of LSC versus cytology, the swabs that proved ''LSC-insufficient'' or ''cytology-insufficient or suspicious'' were excluded (Fig. 2). Out of a total of 34 malignant lesions, LSC detected 25 (74%), whereas cytology detected 14 (41%). LSC and cytology misclassified one and two benign lesions as malignant, respectively. Each method also misclassified five malignant lesions as benign.

Finally, we combined the two methods in a hierarchical way applying the LSC–Cyto algorithm and the Cyto–LSC algorithm (Figs. 3 and 4).

4. Discussion

Cytometry has had a major input in investigations of genetic changes [13]. Recent developments have concentrated on reducing the cell number needed for analysis. In order to minimise cell loss, we developed an assay using swabs that includes only one washing and spinning step prior to the immobilisation of the cells on a slide. This set-up has proven advantageous in a similar application for fine-needle aspiration biopsies [12]. Unlike flow cytometric assays, LSC gives stable specimens that can be stored as normal cytological slides which is of medico-legal importance.

Until now, DNA ploidy of solid carcinomas is determined by image analysis or flow cytometry. For both methods, numerous standardised protocols have been published [2,14–16]. However, they have weaknesses.

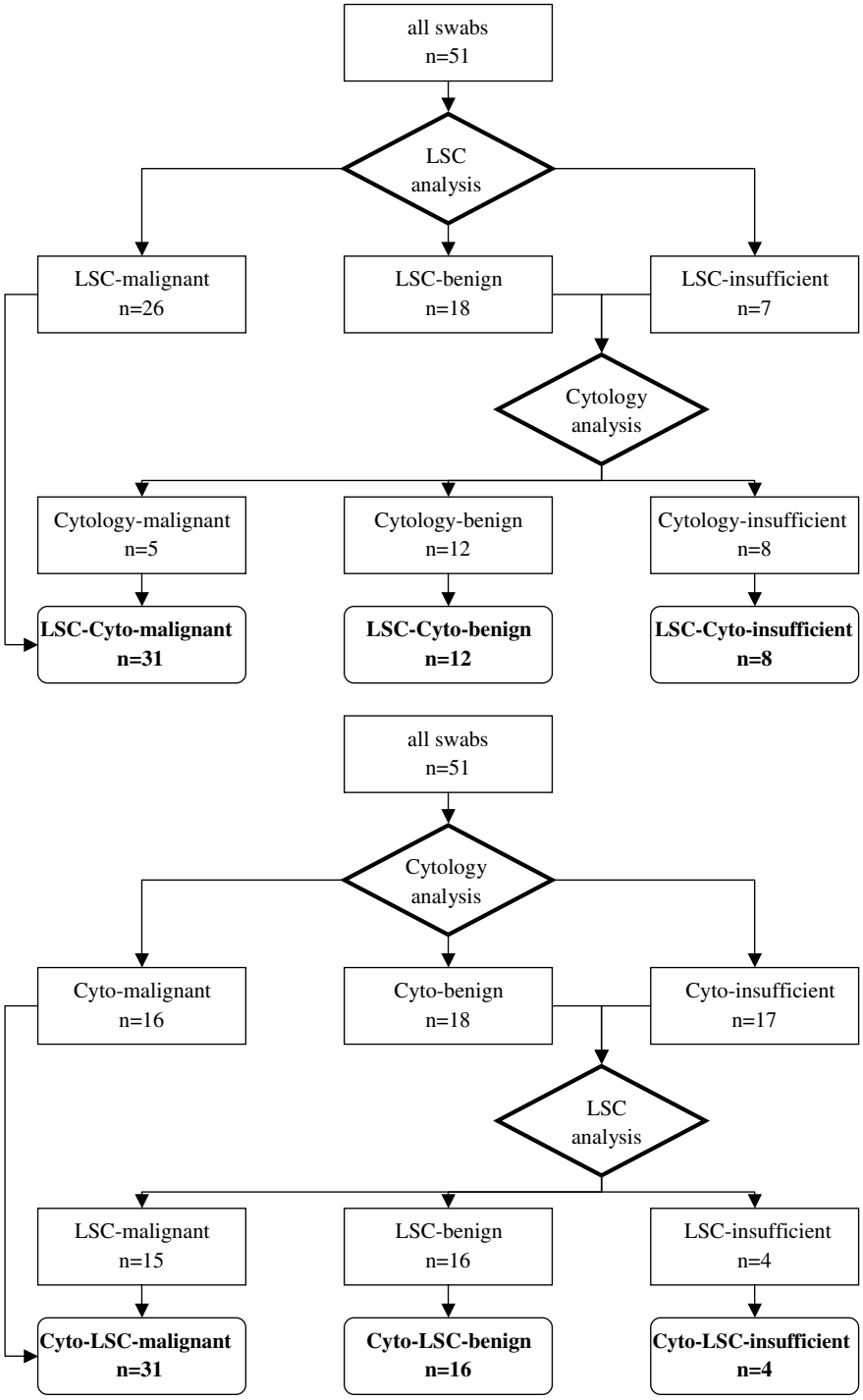

Fig. 4. Combined algorithms. The logical decision tree of the combined algorithms LSC–Cyto and Cyto–LSC is shown together with the respective number of swabs in each step.

By image analysis, only a few cells (150–300) are analysed, and only one parameter (i.e., DNA content) is determined in general, whereas LSC offers the capacity to sequentially stain the same cells for different characteristics, to re-analyse the cells, and to combine all the data on a single-cell basis [17]. In addition, image analysis is time-consuming and may give rise to inter-observer variations since the target cells are selected individually. By contrast, flow cytometry allows multiparameter analysis of bulk cell numbers, but physiological characteristics cannot be attributed to cellular morphology.

Although a relative small number of samples were analysed in this study ($n = 51$), they represent a typical

cross-section of patients presenting with changes of the laryngeal mucosa. Comparing LSC and routine cytology, we found that LSC has a higher specificity and higher PPV. The PPV of 96% could make LSC a useful tool for deciding upon surgical interventions. If LSC is coupled with routine cytology in those cases that are classified as ''LSC-benign'' or ''LSC-insufficient'' (i.e. applying the LSC-Cyto algorithm), the sensitivity is improved to 97% (whereas the Cyto–LSC algorithm did not yield any improvement); a high sensitivity is a prerequisite when screening for laryngeal cancer. In this context, specimens #31, #45a and #45b represent a peculiar limitation of all kinds of assays using swabs: they might have been taken from the non-malignant mucosa nearby yielding ''benign'' results both by LSC and cytology. This sample error might be even more pronounced if working with specimens obtained during local anaesthesia on an out-patient basis. The other four malignant specimens that were mis-classified as benign by LSC indicate another limitation of this assay: as long as DNA ploidy is used as the only criterion for malignancy, DNA diploid tumours cannot be classified as malignant by definition. In the case of cervical carcinoma, the detection of human papilloma virus [18,19] has proved useful; this could probably be transferred to laryngeal carcinoma. Nevertheless, at present, LSC yields valuable information for the patient and the clinician: LSC identified 14 malignancies that were not detected by cytology. Five of them were classified as benign by cytology, and nine as suspicious or insufficient. This highlights where quantitative analyses might be most highest beneficial: in carcinomas where only subtle morphological changes have taken place yielding indefinite cytology: most of these 14 swabs were from T1 and T2 carcinomas that were graded histologically as G2, the cell number sampled and the cytokeratin expression observed was in the same range as for the rest of the swabs, and swabs were obtained during the entire time-span of the study, i.e., the results did not reflect a learning curve.

Keeping in mind the limitations of this initial study, i.e., relatively small patient numbers, heterogeneity of the tumours and so on – the assay described here promises to generate objective preoperative data (i.e., DNA ploidy) on lesions of the laryngeal mucosa. If these data are confirmed by a large-scale multi-centre study, the results could be included in the decision-making process with regard to the subsequent therapeutic strategy. Since the entire assay can be completed in hours, it could be included in the daily routine of an out-patient clinic [20]: in cases of malignancy confirmed by LSC, the patient could decide immediately about any further therapy; in cases where malignancy was not confirmed, the further diagnostic tests/decisions could follow the present scheme.

5. Supports/grants

This work was not supported by any grants.

Conflict of interest statement

None declared.

Acknowledgements

The help of the team in the surgical ward headed by Mrs. C. Scheppukat and Mrs. S. Wiedenfeld and the assistance of Prof. Dr. rer. nat. O. Gerstner, Department of Mathematics, University of Erlangen, Germany, in performing the statistical analyses is gratefully acknowledged.

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