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Molecular detection of minimal residual cancer in surgical margins of head and neck cancer patients

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Abstract. A great disappointment in head and neck cancer surgery is that 10–30% of head and neck squamous cell carcinoma (HNSCC) patients develop local recurrences despite histopathologically tumor-free surgical margins. These recurrences result from either minimal residual cancer (MRC) or preneoplastic lesions that remain behind after tumor resection. Distinguishing MRC from preneoplasic lesions is important to tailor postoperative radiotherapy more adequately. Here we investigated the suitability of quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using human Ly-6D (hLy-6D) transcripts as molecular marker to detect MRC in surgical margins.

Submucosal samples of deep surgical margins were collected from 18 non-cancer control patients and 67 HNSCC patients of whom eight had tumor-positive surgical margins. The samples were analyzed with hLy-6D qRT-PCR, and the data were analyzed in relation to the clinicohistological parameters.

A significant difference was shown between the group of patients with histopathological tumor-positive surgical margins and the non-cancer control group ($p < 0.001$), and the group of patients with histopathological tumor-free surgical margins ($p = 0.001$).

This study shows a novel approach for molecular analysis of deep surgical margins in head and neck cancer surgery. The preliminary data of this approach for detection of MRC in deep margins of HNSCC patients are promising.

Keywords: Head and neck cancer, hLy-6D, minimal residual cancer, molecular diagnosis, qRT-PCR, surgical margin

1. Introduction

Despite significant advances in treatment modalities over the last decades, the 5-year survival rates of head and neck squamous cell carcinoma (HNSCC) patients improved only moderately. An explanation for this observation is the high frequency of local relapse. Even when the surgical margins have been diagnosed as tumor-free by routine histopathology, the local recurrence rate is still 10–30% [1]. It has been shown that approximately 50% of locally recurrent tumors in patients with histopathologically tumor-free surgical margins arise from residual cancer cells in the surgical margins that are not detected by routine histological examination: minimal residual cancer (MRC) [2]. The remaining half of the local recurrences in these patients develop as new tumors in preneoplastic lesions that were not completely resected as these are invisible and large; and these were designated as second field tumors [2–4]. These findings hamper stratification of patients with histopathologically tumor-free surgical margins for postoperative management. Ideally, patients with MRC should receive postoperative radiotherapy, while those with remaining preneoplastic lesions should probably receive frequent and long-term surveillance, and radiotherapy might not be indicated. For patients with neither MRC nor preneoplastic le-

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sions, adjuvant treatment could be omitted if neck features allow, and regular surveillance will probably suffice.

Several prognostic factors for locoregional recurrence have been described, of which histologically tumor-free surgical margins, the dimensions of the primary tumor (T-stage) and the histopathological status of the neck nodes (N-stage) are the most significant [1]. Furthermore, some adverse histopathological parameters, such as severe grade epithelial dysplasia in the surgical margins, perineural growth, diffusely infiltrating growth-pattern and vaso-invasive growth, have been proposed to have prognostic value in HNSCC, although the results of the various studies are not concordant [5–8]. Postoperative radiotherapy is therefore usually applied when one or more surgical margins are histologically tumor-positive, in case of tumor-growth in bone, the presence of multiple tumor-positive lymph nodes in the neck or extranodal spread. Furthermore, postoperative radiotherapy is discussed in case of advanced T-stage ($\geqslant T3$), close surgical margins (distance between tumor and surgical margin <5 mm) or presence of severe dysplasia in the surgical margins, perineural growth, vaso-invasive growth, and diffusely infiltrating growth-pattern. Obviously, radiotherapy should be applied only when necessary because it results in acute morbidity [9], long-term sequels and, as if applied, a treatment option may be lost in case of a second primary or recurrent tumor.

Particularly the tumor-free status of the surgical margins appears as most important parameter to apply postoperative radiotherapy, but histopathological examination meets limitations with respect to sensitivity as still a considerable number of patients with tumor-free margins develop recurrences. Molecular analysis of surgical margins seems superior over histopathology to identify patients at high risk for local recurrence and tumor-related death [10]. Molecular analysis should therefore enable better selection of patients for postoperative radiotherapy. Several studies showed that *TP53*-mutated DNA in surgical margins can serve as molecular marker to identify patients at increased risk for local recurrence [2,10,11]. However, this approach is limited by the fact that only 60% of HNSCC has a mutation in *TP53* and measurement of *TP53*-mutated DNA does not allow differentiating between MRC and preneoplastic lesions. Moreover, this method can give false-positive results and the molecular assay is relatively laborious [2,10]. At present there is no reliable and simple method for detection of MRC in surgical margins.

For MRC detection in tissue samples or body fluids two main strategies are applied: immunostaining of cyto-histological preparations and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) [12,13]. For tissue analysis the sensitivity of immunostaining methods is determined by the number of sections analyzed, and usually only a few sections can be screened routinely. QRT-PCR has shown to be much more suitable for screening large tissue volumes without sampling error [13–15].

We hypothesized that MRC might be detected by human Ly-6D (hLy-6D) qRT-PCR. The hLy-6D antigen is a squamous cell-specific surface antigen recognized by monoclonal antibody E48, which is expressed in normal, transitional and malignant squamous epithelia [16], and is therefore a potential marker for HNSCC [17]. In HNSCC the hLy-6D antigen is expressed in approximately 80–90% of the primary tumors [13]. Previously, the suitability of qRT-PCR using hLy-6D as a molecular marker has been demonstrated for the detection of MRC in various tissue samples including lymph nodes, bone marrow and blood [18–20]. We hypothesized that this same assay might also be suitable for detection of MRC in subepithelial deep surgical margins of HNSCC patients.

The aim of this study is to evaluate hLy-6D qRT-PCR for the detection of MRC in deep surgical margins of HNSCC patients. The ultimate aim is to guide postoperative clinical management based on these findings.

2. Materials and methods

2.1. Patients and samples

Eighteen patients, surgically treated in the head and neck region for other indications than cancer, were included as negative control patients (Table 1). Sixty-seven patients with primary HNSCC in the oral cavity, oropharynx, hypopharynx or larynx, who were primarily surgically treated, were included in this study. Patient characteristics are listed in Table 2. The study was approved by the Institutional Review Board, and informed consent was obtained from all patients. Samples from the resection margins that were analyzed by qRT-PCR were taken at the operation room as follows. After tumor resection, the surgical field was extensively rinsed using Dakin's solution (0.5% sodium hypochlorite pH 11.5 and 0.2% sodium carbonate) to prevent tumor cell contamination. Subsequently, surgical instruments and gloves were changed and five to

Table 1
Characteristics of the non-cancer control patients and molecular analysis

Patient	Gender	Age	Location	Histology	hLy6D qRT-PCR pos	# OR-samples	# Pos. OR-samples	Highest hLy-6D (ng)
Control 1	m	43	pg	pa	x	2	0	0.14
Control 2	m	30	n	tc	x	4	0	0.36
Control 3	m	45	pg	wt	x	1	0	0.12
Control 4	m	61	pg	wt	x	5	0	0.16
Control 5	m	34	n	tc	x	3	0	0.00
Control 6	f	59	pg	pa	n	1	0	0.02
Control 7	f	72	pg	pa	n	2	0	0.03
Control 8	f	63	pg	pa	n	5	0	0.03
Control 9	f	70	pg	pa	n	6	0	0.02
Control 10	f	49	pg	pa	n	5	0	0.07
Control 11	f	51	pg	wt	n	3	0	0.05
Control 12	f	68	pg	pa	n	5	0	0.03
Control 13	f	55	pg	bca	y	3	2	0.56
Control 14	f	37	pg	pa	n	6	0	0.01
Control 15	m	66	pg	wt	n	3	0	0.15
Control 16	m	48	pg	pa	n	1	0	0.02
Control 17	f	66	pg	pa	n	5	0	0.03
Control 18	m	67	pg	wt	n	3	0	0.07

Abbreviations: pg, parotid gland; n, neck; pa, pleomorfic adenoma; tc, thyroglossal duct cyst; wt, Warthin's tumor; bca, basal cell adenoma; x, used to determine hLy-6D cut-off level; hLy-6D qRT-PCR pos, positive results hLy-6D qRT-PCR analysis; # OR-samples, total number of analyzed OR-samples; # pos. OR-samples, total number of hLy-6D positive OR-samples; highest hLy-6D (ng), highest hLy-6D qRT-PCR value of the analyzed OR-samples in nanogram.

fifteen samples, evenly distributed in the deep surgical margins, of approximately five millimeters in diameter were collected from the deep subepithelial surgical margins. Sampling was done at the operation room and not from the tumor resection specimen, first because this might influence the results of the routine histopathological examination necessary for clinical decision-making, and second to prevent contamination. The deep surgical margin samples were collected from submucosal tissues to prevent mucosal epithelial contamination, and consisted of muscle, glandular tissue, fat, or connective tissue. The samples were snap-frozen in liquid nitrogen and stored at -80°C. These subepithelial deep margin samples will be referred to as "OR-samples". The resection specimen was sent to pathology for full histopathological workup.

2.2. Histopathology

All tumor resection specimens were routinely examined by a pathologist and scored according to the standard criteria of the World Health Organization Classification international histological classification of tumors [21]. The surgical margins of the resection specimen were paraffin-embedded and examined separately as part of the routine histological workup. These margins will be referred to as "RS-margins". Analysis of these RS-margins was performed as suggested by Batsakis: clear when the minimal distance from tumor to deep surgical margin was more than 5 mm; close when the distance was less than 5 mm, but no evidence tumor at the deep margin, or involved when tumor was found in one or more surgical margins [22]. The OR-samples were not histopathologically examined, because the entire sample was required for qRT-PCR.

2.3. RNA isolation

The OR samples taken from the patient at the operation room were homogenized using an electric hand-held pestle in one ml RNA-Bee (Campro Scientific, Veenendaal, The Netherlands). RNA isolation was further performed as indicated by the manufacturer. In addition, RNA was re-precipitated by adding $0.1\times$ volume 3 M sodium acetate pH 5.2 (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and $2.5\times$ volume 100% ethanol. After centrifugation at $12,000 \times g$ for 30 min at 4°C, the pellet was washed in 400 µl 70% ethanol, and then centrifuged at $12,000 \times g$

Table 2
Characteristics of the HNSCC patients and molecular analysis

Patient number	Gender	Age	Location	RS-margins	Stage	Differentiation	Tumor morphology	RS-margins morphology	Treatment	hLy6D qRT-PCR pos	Strict RT-indication	# OR-samples	# Pos. OR-samples	Highest hLy-6D (ng)	IHC E48 expression
1	m	58	oc	inv	IVA	m	ig	scc	surg+rt	y	y	9	3	43.5	100
2	m	51	oc	inv	II	p	ig	scc	surg+rt	y	y	4	4	178.3	5
3	f	42	oc	inv	IVA	m	ig	scc	surg+rt	y	y	4	2	440.0	80
4	m	84	oc	inv	II	m		scc	surg+rt	y	y	4	1	1.00	30
5	m	70	oc	inv	II	m	ig	scc	surg+rt	y	y	7	4	165.7	30
6	m	52	hp	inv	IVA	m	pn+vg	scc	surg+rt	n	y	5	0	0.15	90
7	m	70	oc	inv	IIII	p	ig	scc	surg+rt	y	y	7	2	120.4	0
8	f	71	L	inv	IVA	p		scc	surg+rt	y	y	6	1	5.37	80
9	m	50	oc	close	IVA	p	ig		surg+rt	n	y	8	0	0.10	50
10	m	62	oc	clear	III	p			surg+rt	n	n	6	0	0.20	20
11	f	70	oc	close	II	m	ig		surg+rt	n	n	7	0	0.23	100
12	m	61	oc	close	IVA	m	ig+vg	dys	surg+rt	n	y	5	0	0.17	10
13	m	57	oc	close	III	m		dys	surg+rt	n	n	7	0	0.03	80
14	m	57	oc	close	III	m			surg+rt	y	n	6	1	18.75	30
15	f	83	oc	clear	I	m		dys	surg	n		5	0	0.09	80
16	m	80	oc	close	I	m		dys	surg	y		5	1	2.59	10
17	m	49	oc	clear	I	m		dys	surg	n		4	0	0.13	0
18	m	68	oc	clear	II	m			surg	n		5	0	0.27	30
19	m	49	oc	clear	I	w	ig		surg	n		3	0	0.23	100
20	f	52	op+oc	close	IVA	m	ig+pn	dys	surg	y		6	1	0.58	50
21	m	67	oc	close	I	m	ig+pn+vg	dys	surg+rt	y	n	6	1	12.0	50
22	f	52	op	close	III	m		dys	surg+rt	n	n	6	0	0.00	80
23	f	80	L	clear	II	m	ig+vg		surg	n		6	0	0.08	05
24	m	63	oc	close	II	p	pn+vg	dys	surg+rt	n	n	7	0	0.03	0
25	m	67	L	close	IVA	m	ig		surg+rt	n	n	4	0	0.06	30
26	m	64	oc	close	III	m	vg		surg+rt	n	n	6	0	0.05	100

Table 2
(Continued)

Patient number	Gender	Age	Location	RS-margins	Stage	Differentiation	Tumor morphology	RS-margins morphology	Treatment	hLy6D qRT-PCR pos	Strict RT-indication	# OR-samples	# Pos. OR-samples	Highest hLy-6D (ng)	IHC E48 expression
27	m	77	oc	clear	IVA	m	ig	dys	surg+rt	y	n	6	3	248.2	80
28	m	72	oc	close	IVA	p			surg+rt	n	y	5	0	0.04	5
29	m	57	op	close	III	p	ig	dys	surg+rt	n	n	7	0	0.22	80
30	m	70	L	close	IVA	w			surg+rt	n	n	5	0	0.23	100
31	m	68	oc	close	IVA	p	vg		surg+rt	n	y	5	0	0.03	30
32	f	54	oc	clear	III	m	ig	dys	surg	n		4	0	0.02	50
33	m	62	oc	clear	III	m	ig		surg	n		3	0	0.07	70
34	m	71	oc	close	III	m	ig+pn		surg+rt	n	n	6	0	0.03	70
35	m	69	L	clear	II	m	vg		surg	n		6	0	0.04	80
36	m	54	L	close	IVA	m	ig		surg+rt	y	y	6	1	2.94	50
37	f	58	op	close	IVA	p			surg+rt	y	y	6	3	259.3	0
38	m	73	op	clear	III	p	ig+pn		surg+rt	n	n	5	0	0.04	100
39	f	58	oc	clear	II	m		dys	surg	n		5	0	0.12	20
40	m	64	oc	close	IVA	p			surg+rt	y	y	6	4	377.4	5
41	m	60	oc	close	II	m	ig		surg+rt	n	n	5	0	0.12	40
42	m	56	oc	close	III	m	pn		surg+rt	n	n	3	0	0.18	5
43	m	64	oc	close	IVA	m			surg+rt	n	y	3	0	0.00	70
44	m	52	oc	close	I	m	ig		surg+rt	y	n	7	1	2.19	80
45	m	43	hp	close	IVA	m	ig+vg	dys	surg+rt	n	y	4	0	0.05	0
46	m	78	oc	close	IVA	m	ig+vg	dys	surg+rt	n	n	4	0	0.03	40
47	f	64	oc	close	IVA	m	ig+vg	dys	surg+rt	n	y	5	0	0.04	100
48	f	88	oc	close	IVA	w	ig+pn+vg		surg+rt	n	y	5	0	0.03	50
49	m	48	op	close	IVA	p		dys	surg+rt	y	y	6	1	49.7	100
50	m	55	L	close	IVA	m		dys	surg+rt	n	n	5	0	0.03	10
51	m	66	oc	clear	II	m			surg	n		6	0	0.08	0
52	m	59	oc	clear	III	p			surg	y		4	1	1.13	10

Table 2
(Continued)

Patient number	Gender	Age	Location	RS-margins	Stage	Differentiation	Tumor morphology	RS-margins morphology	Treatment	hLy6D qRT-PCR pos	Strict RT-indication	# OR-samples	# Pos. OR-samples	Highest hLy-6D (ng)	IHC E48 expression
53	m	61	oc	close	IVA	m	ig+pn	dys	surg+rt	n	y	5	0	0.03	10
54	m	77	op	close	IVA	m	ig+pn	dys	surg+rt	y	y	6	1	170.0	60
55	m	74	op	close	IVA	p		dys	surg+rt	n	y	3	0	0.02	20
56	m	62	L	close	IVA	m	ig+pn		surg+rt	n	n	10	0	0.03	10
57	m	82	oc	clear	III	m	ig+pn		surg	n		6	0	0.11	60
58	m	70	L	close	IVA	m	pn+vg	dys	surg+rt	n	n	7	0	0.12	50
59	f	80	oc	clear	III	m		dys	surg	n		3	0	0.11	90
60	f	33	oc	close	III	m	ig		surg	n		5	0	0.11	60
61	f	56	oc	close	I	m			surg	n		6	0	0.05	100
62	m	52	L	clear	I	m			surg	n		5	0	0.19	100
63	f	59	oc	clear	II	m	ig		surg	n		6	0	0.12	10
64	m	66	oc	close	III	m	ig+pn+vg	dys	surg+rt	n	n	3	0	0.06	50
65	f	61	oc	close	III	m	pn	dys	surg+rt	n	n	8	0	0.26	25
66	m	73	hp	clear	IVA	m	ig	dys	surg	n		6	0	0.11	50
67	m	70	L	close	IVA	m			surg+rt	n	n	3	0	0.01	70

Abbreviations: oc, oral cavity; op, oropharynx; L, larynx; clear, distance between tumor and surgical margin >5 mm; close, distance between tumor and surgical margin <5 mm, but no tumor present in surgical margin; inv, tumor involved surgical margin; p, poorly differentiated; m, moderately differentiated; w, well differentiated; ig, infiltrative growth pattern of the tumor; pn, perineural growth; vg, vaso-invasive growth; scc, squamous cell carcinoma; dys, dysplasia in surgical margin; surg, surgery; surg+rt, surgery and postoperative radiotherapy; hLy-6D qRT-PCR pos, positive results hLy-6D qRT-PCR analysis; # OR-samples, total number of analyzed OR-samples; # pos. OR-samples, total number of hLy-6D positive OR-samples; highest hLy-6D (ng), highest hLy-6D qRT-PCR value of the analyzed OR-samples in nanogram; IHC hLy-6D expression, percentage of hLy-6D expression in tumor determined by immunohistochemical staining.

for 10 min at 4°C. The pellet was dissolved in 22 µl RNAse-free water and the RNA quantity was measured in duplicate by the absorbance at 260 nm by a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). Finally, a concentration of 100 ng/µl total RNA was prepared. The additional ethanol precipitation improved the quality of the preparation as indicated by the OD260/280 ratio.

2.4. Primers and probes

Primers and probes for the hLy-6D transcript have been previously described [19]. To control the RNA quality we used mRNA transcribed from the gene encoding for β-glucuronidase gene (BGUS), which is considered to be a constantly expressed housekeeping gene [23]. The sequences of the primers and probe for BGUS are listed in Table 3.

2.5. cDNA synthesis

In total 5 µl RNA (250 ng) of the OR-samples was heated to 65°C for 5 minutes and immediately cooled on ice. Next, 15 µl of RT mixture was added. The RT mixture consisted of 2 µl 10× RT buffer (600 mM KCL, 30 mM $MgCl_2$, 500 mM pH 8.0 TRIS.HCL), 10 µl 2 mM dNTP (0.5 mM each deoxynucleotide triphosphate, Boehringer, Ingelheim, Germany), 2 µl 10 mM DTT, 1 µl 25 pmol/µl reverse primer hLy-6D or BGUS, 0.05 µl 40 U/µl RNAsin ribonuclease inhibitor (Promega, Leiden, the Netherlands), 0.1 µl 10 U/µl AMV-RT (Promega, Leiden, the Netherlands). The reaction mixture was incubated for 2 hours at 42°C. All reactions were performed in triplicate.

2.6. Quantitative Real-Time RT-PCR amplification

PCR amplification was done in 40 cycles using 5 µl of cDNA. The cDNA was added to 45 µl reaction mixture, containing 5 µl 10× TaqMan buffer A (Applied Biosystems, Foster City, USA), 10 µl 25 mM MgCl2, 0.5 µl 100 mM dNTP, 0.6 µl sense and antisense primers of hLy-6D or BGUS (25 pmol/µl each), 1.5 µl 5 pmol/µl fluorescent probe hLy-6D or BGUS, 26.55 µl sterile H_2O (Baxter, Utrecht, The Netherlands), and 0.25 µl 5 U/µl AmpliTaq ™Gold DNA polymerase (Applied Biosystems, Foster City, USA).

Real-time RT-PCR was performed in an ABI Prism 7500 (Applied Biosystems) with the following conditions: 1 cycle of 10 minutes at 95°C, followed by 40 cycles of 15 seconds 95°C and 60 seconds 60°C. In each experiment a serial dilution of tumor cell line UM-SCC-22A cDNA ranging from 500 to 0.05 ng was run in parallel as calibration curve for hLy-6D as well as BGUS. Preparations without RNA template were used as negative control.

2.7. E48 immune peroxidase staining and qRT-PCR of tumors

Immunohistochemical staining was performed on formalin-fixed paraffin embedded (FFPE) biopsies of the tumor and used to assess the hLy-6D expression of the tumor. After deparaffinization, the slides were washed in phosphate buffered saline and incubated with 0.1% pepsin (Sigma Aldrich, Zwijndrecht, The Netherlands) in 0.02 N HCL for 30 minutes at 37°C. Subsequently the slides were washed in demineralized water and then in phosphate buffered saline. The slides were incubated with 2% normal rabbit serum (Dako Cytomation, Glostrup, Denmark) for 20 minutes and afterwards the normal rabbit serum was poured off. All tumor sections were stained with monoclonal antibody E48 that specifically detects hLy-6D, as well as normal mouse IgG (Dako Cytomation 100 mg/l, Glostrop, Denmark) as control. StreptABComplex/horse radish peroxidase (Dako Cytomation, Glostrop, Denmark) was used as indicated by manufacturer. The slides were scored for the percentage of hLy-6D positive cells in the tumor by two independent reviewers, and a consensus score was taken as final readout.

To determine the correlation between E48 immune peroxidase staining and hLy-6D qRT-PCR we performed a hLy-6D qRT-PCR on eleven frozen tumor specimens. From the frozen tumor specimens two 5-micron sections were cut for hematoxilin and eosin staining, then twelve 10-micron sections for RNA-isolation and again two 5-micron sections for hema-

Table 3
Primers and probes of BGUS used for qRT-PCR

Primers/Probe	Sequence 5′–3′
BGUS forward	GAAAATATGTGGTTGGAGAGCTCATT
BGUS reverse	CCGAGTGAAGATCCCCTTTTTA
BGUS probe	FAM-CCAGCACTCTGGTCGGTGACTGTTCA-TAMRA

toxilin and eosin staining. From the specimens with at least 50% tumor in the hematoxilin and eosin slides RNA was isolated and a hLy-6D qRT-PCR was performed as described above with 50 ng RNA input.

2.8. Data analysis

Threshold values (CT) were determined and the original amount of hLy-6D and BGUS mRNA in each OR-sample was calculated from the calibration curves of cell line UM-SCC-22A. An OR-sample was defined as representative when it had a minimum amount of 25 ng BGUS mRNA relative to UM-SCC-22A. The cut-off level for designating an OR-sample as MRC-positive was determined using 15 samples of five non-cancer control patients, and was set at 0.41 nanogram hLy-6D RNA, being the t-value $(n-1) \times \mathrm{SD} + \mathrm{mean}$, which corresponds to the 99% confidence interval. The samples used for determining the cut-off level were not used for further analysis. Patients were determined MRC-positive when the hLy-6D value of one or more OR-samples exceeded the cut-off value, irrespective of the hLy-6D expression in the tumor, and MRC-negative when the hLy-6D value of all OR-samples was below the cut-off value and the tumor showed at least 5% hLy-6D expression. Patients were defined as non-diagnostic when the tumor showed less than 5% hLy-6D expression and all OR-samples appeared to be hLy-6D qRT-PCR negative. The groups of patients with clear (>5 mm), close (<5 mm) and involved surgical margins determined on the resection specimen were compared with each other in SPSS14 using chi-square tests.

3. Results

3.1. E48 immune peroxidase staining and hLy-6D qRT-PCR of tumors

First we determined whether hLy-6D RNA-expression could be monitored reliably on basis of immunostaining. Figure 1 shows the relation between the hLy-6D expression of FFPE tumors and the hLy-6D expression determined by qRT-PCR of frozen tumors. One frozen tumor specimen with low hLy-6D expression appeared to have less than 50% tumor in the hematoxilin–eosin stained section, and was excluded. Statistical analysis showed significant association between E48 immune peroxidase staining on FFPE tissue and hLy-6D qRT-PCR on frozen samples ($p = 0.023$).

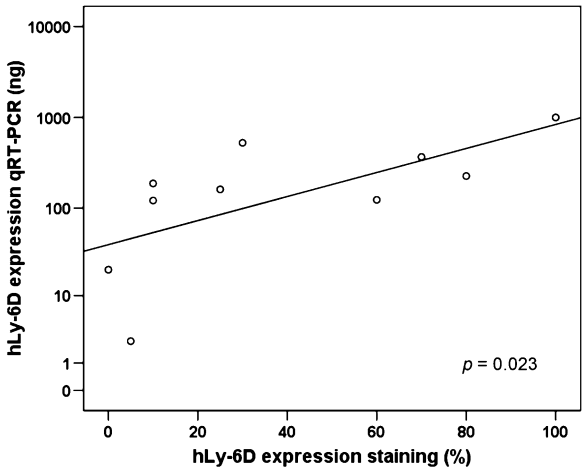

Fig. 1. E48 immunohistochemical staining and hLy-6D qRT-PCR of tumors. To determine the correlation between E48 immune peroxidase staining and hLy-6D qRT-PCR we performed hLy-6D qRT-PCR on ten selected frozen tumor specimens: four with high hLy-6D expression (>50%) in paraffin embedded biopsies of the tumor, four with intermediate expression (6–50%) and two with low expression (⩽5%). On the x-axis the percentage of hLy-6D expression determined by immunostaining is shown and on the y-axis the hLy-6D qRT-PCR value in nanogram determined by hLy-6D qRT-PCR.

Immunostaining showed high hLy-6D expression (⩾50% expression) in 37/67 (55%) of the FFPE tumor specimens (Fig. 2A). It appeared that in total 10 tumors (15%) had low expression (⩽5%) and 20 tumors (30%) had intermediate expression (6–49%) of hLy-6D (Fig. 2B) that often presented as heterogeneous immunostaining.

We also investigated whether there were indications that particularly the invasive front of the tumors showed low hLy-6D expression**.** In heterogeneously staining tumors that do not show expression of hLy-6D in the tumor borders, the more invasive cells, might cause false negative qRT-PCR results in OR-samples containing tumor cells. Therefore we reviewed the infiltrative growing tumors to investigate this point in more detail. In 33 cases the tumor showed an infiltrative growth pattern. The invasive cell nests were highly positive for E48 antigen in 23/33 cases, while in six of the 33 cases intermediate hLy-6D expression was found. In only four of the 33 cases hLy-6D expression was absent in the invasive cell nests, but it appeared that all these four cases did not show expression of hLy-6D in the tumor. Hence there seems not much evidence that low expression is specifically present in the tumor borders.

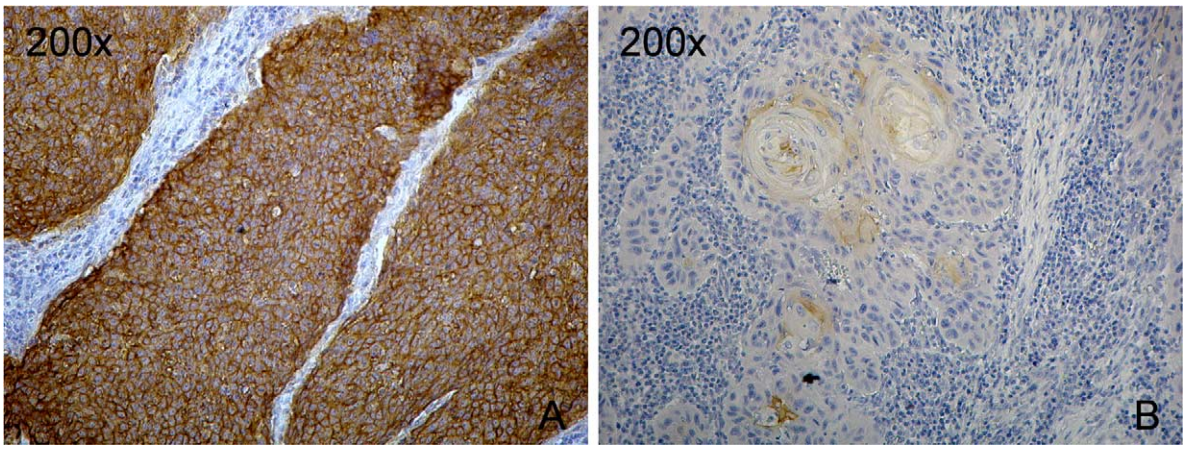

Fig. 2. E48 immunostaining. Representative example of E48 immune peroxidase staining of a tumor with high (100%) hLy-6D expression (A) or low (5%) hLy-6D expression (B).

3.2. hLy-6D qRT-PCR of deep surgical margins

HLy-6D analysis was performed on 48 submucosal samples of 13 non-cancer control patients. It appeared that twelve out of thirteen control patients had a hLy-6D negative test. The one patient with a positive test, control 13, had two borderline positive samples (Table 1).

Of four of the 67 HNSCC patients all OR-samples appeared to be hLy-6D qRT-PCR negative and the tumor showed no hLy-6D expression as well: patient 17, 24, 45 and 51 (Table 2). These cases were considered as non-diagnostic and therefore excluded.

Eight of 63 patients had histologically tumor-positive surgical margins in the resection specimen. Seven of them showed positive OR-samples analyzed by the hLy-6D qRT-PCR assay (range 1–4 per patient, median 3) (Table 2). Approximately 41% (17/41) of the analyzed OR-samples of these seven patients appeared to be positive in the test. The hLy-6D qRT-PCR value per positive OR-sample varied over a 3-log range from 0.44 to 440 ng, median 55 ng. Five OR-samples were analyzed from the patient with histologically tumor-positive margins whose margin samples scored hLy-6D qRT-PCR negative (patient 6). Histopathological review showed one tumor-positive surgical margin, and all other RS-margins were tumor-free. Staining of the tumor showed high expression of hLy-6D, therefore this case has to be considered as false-negative. In total, 295 OR-samples of 55 patients (range 3–10 per patient, median 5), who were diagnosed as histopathologically tumor-free based on the RS-margins of the resection specimen, were analyzed (Table 2). Twelve of 55 (22%) patients had one or more positive OR-samples (range 1–4, median 1), indicating the presence of MRC. As can be seen in Table 2, three of the twelve MRC positive patients did not receive postoperative radiotherapy: patient 16, 20 and 52. Of the 43 patients with histologically tumor-free resection RS-margins who tested MRC-negative, 28 patients received radiotherapy. Eleven of these 28 patients were irradiated based on strict criteria such as tumor growth in bone.

3.3. Frequency analysis

The qRT-PCR results of the OR-samples taken at the operation room were compared to the histopathological results of the resection specimen. Statistical analysis showed a significant difference in the frequency of patients who scored hLy-6D qRT-PCR positive between the group of patients with histopathological tumor-positive RS-margins and the group of non-cancer control patients ($p < 0.001$). There was also a significant difference in the frequency of patients who scored hLy-6D qRT-PCR positive between the group of patients with histopathological tumor-positive RS-margins and the group of patients with histopathological tumor-free RS-margins ($p = 0.001$) (Fig. 3A). When the histopathological tumor-free surgical margin group was divided in one group with clear surgical margins (>5 mm) and a second group with close surgical margins (<5 mm), it appeared that both groups were significantly different from the group of patients with histopathological tumor-positive surgical margins (resp. $p < 0.001$ and $p = 0.001$, Fig. 3B). There was no significantly difference between the groups of patients with clear surgical margins and close surgical margins ($p = 0.227$). No sig-

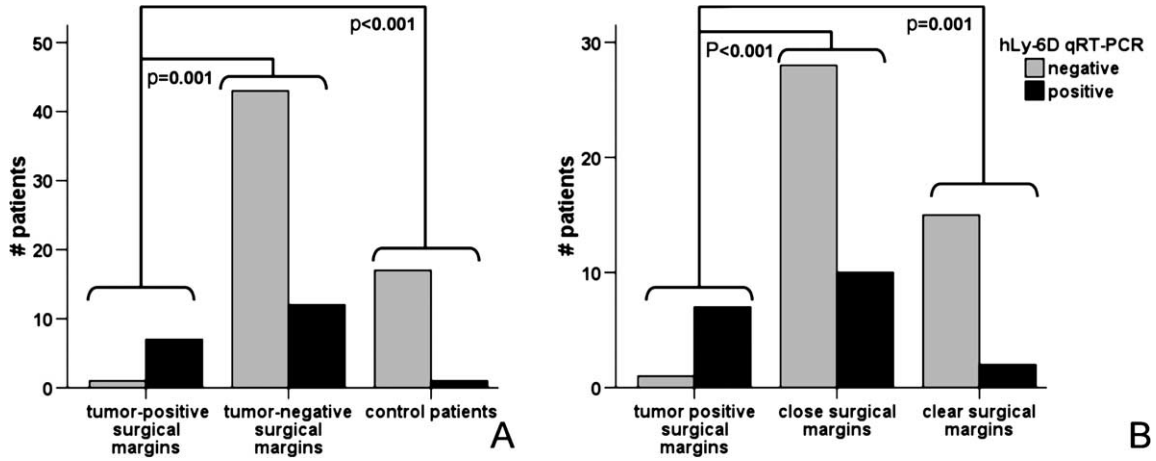

Fig. 3. Relationship between histopathological status of surgical margins and presence of hLy-6D qRT-PCR results. (A) On the x-axis the groups of HNSCC patients are shown with either tumor-positive surgical margins, tumor-negative surgical margins. The number of patients with either positive (black bars) or negative (grey bars) OR-samples as determined by hLy-6D qRT-PCR is indicated on the y-axis. The non-diagnostic cases were excluded. The groups were compared with each other using chi-square tests. p-values below 0.05 were considered significant. (B) No significant difference was shown between the groups of patients with clear surgical margins ($\geqslant$5 mm) and close surgical margins ($<$5 mm) ($p = 0.227$).

nificant association was shown between hLy-6D qRT-PCR results in the OR-margin samples and dysplasia, vaso-invasive growth, peri-neural growth or diffusely infiltrating growth-pattern as determined on the resection specimen.

4. Discussion

The present study is the first report describing MRC-detection in submucosal surgical margins of HNSCC patients by hLy-6D qRT-PCR. One of 13 non-cancer control patients had two borderline positive samples. A possible explanation for this finding is that we chose cut-off levels based on the 99% Confidence Interval indicating that approximately 1% of the control samples will be false-positive. Based on these initial data it might be worthwhile to shift the cut-off levels to the borders determined by the 99.9 or 99.99% Confidence Interval. However, also contamination with epithelial cells cannot be excluded.

Seven of the eight patients with histopathological tumor-positive RS-margins had one or more positive OR-samples using this assay. Approximately 41% of the OR-samples of these patients tested positive, demonstrating that analysis of multiple OR-samples is necessary to prevent sampling-error. The histopathological report of the one false-negative patient indicated that tumor cells were present in only one RS-margin of the resection specimen. Hence, the false-negative results from the OR-samples are most likely explained by sampling-error, and this again demonstrates the importance of analyzing multiple OR-samples.

In total, there were 17 HNSCC patients with clear surgical margins, and 38 patients with close surgical margins as determined by histopathological examination of the resection specimens. Two of 17 (12%) patients with clear margins, and ten of 38 (26%) patients with close margins tested positive in one or more of the OR-samples, indicating the presence of MRC. The frequency of patients with MRC in the group of patients with clear surgical margins was lower than that of the group of patients with close surgical margins, but the difference was not significant. This may be due to the limited sample size, as there seemed a tendency to a higher frequency in the close margin group. This would be in line with previous studies, in which it was demonstrated that close surgical margins do increase the risk for local recurrences [24–26].

It appeared that $17/43$ patients with histologically tumor-free RS-margins and who tested MRC-negative, received postoperative radiotherapy based on less strict criteria. Particularly in this group tailoring of radiotherapy using molecular analysis might be of interest. Obviously a large prospective study and association of the molecular analysis of OR-samples with outcome needs to be awaited for stronger conclusions.

In four of 67 patients with histologically tumor-free resection margins MRC could not be assessed because

the immunostaining of the tumor did not show hLy-6D expression and all OR-samples were hLy-6D qRT-PCR negative. A solution to overcome this problem is the use of a panel of markers, instead of a single marker. Promising markers for HNSSC-detection are cytokeratin 19 [27,28], SCCA [28,29], PTHrP [28,29], EGFR [29] and squamous cell-specific splice variants of the CD44v6 antigen [30].

Some patients with tumors with low hLy-6D expression did show hLy-6D positive OR-samples. An explanation for this finding is that the tumor load in these OR-samples was large enough for a positive hLy-6D qRT-PCR assay despite the low expression. This is not unexpected as transcript analysis is much more sensitive than immunohistochemical staining. A difference between positive and negative hLy-6D immunostaining might be only 2-log, and even when such a decreased protein-expression level is reflected by a 2-log decreased number of transcripts, it will still allow a sensitive detection level. This is shown in the experiment in which E48 immune peroxidase staining was compared with hLy-6D qRT-PCR. There were two patients with tumors with low hLy-6D expression determined by immunostaining, but hLy-6D qRT-PCR of the frozen tumor samples showed a hLy-6D expression far above the cut-off level (Fig. 2).

The findings of this study suggest a high sensitivity and specificity of this approach, but it should be stressed that only eight patients with tumor-positive RS-margins and thirteen non-cancer control patients were tested so far. A second remark is that the sampling in the non-cancer controls is not exactly identical to that in the head and neck cancer patients. These patients are mostly scheduled for surgery in the salivary glands, and not in the oral cavity, oropharynx or larynx. This might have a reflection on the chosen cut-off point for a positive test and it might be worthwhile to perform statistical associations with clinical outcome in prospective studies not only with the final test results based on the presented cut-off point, but in addition with the tertiles or quartiles of the test results. This seems at present the only possibility to solve this problem. Obviously ROC curves might be calculated with different cut-off values using the outcome data, but the consequence of such a training approach is that an independent study needs to be carried out to validate the findings.

The true clinical value of this approach can only be proven when the results of our assay correlate with the outcome of the patients. However, both the number of patients as well as the follow-up time of the patients described is at present not adequate to correlate our findings to survival and the development of relapse. Nevertheless, the association of our data with the routine histopathological parameters is interesting to the field and might stimulate the research in this area.

In conclusion, the preliminary data of this study on the application of hLy-6D qRT-PCR to detect MRC in surgical margins of HNSCC patients are promising, and support the design of subsequent larger prognostic trials using treatment outcome as endpoint.

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