Molecular Profiling Using Tissue Microarrays as a Tool to Identify Predictive Biomarkers in Laryngeal Cancer Treated with Radiotherapy

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Abstract. *Aim: To explore the usefulness of the expression of five potential cancer biomarkers in predicting outcome in patients with laryngeal cancer. Materials and Methods: In the present study, the Swedish National Cancer Registry databases were used to identify patients with laryngeal cancer diagnosed during the years 1978-2004 in the Uppsala-Örebro region and treated with radiotherapy. The expression of Ki-67, MutS homolog 2, (MSH2), p53, B-cell CLL/lymphoma 2 (Bcl-2) and cyclin D1 in the cancer cells was assessed immunohistochemically using tissue microarrays (TMAs) and its predicitve value on survival and relapse was analyzed using Cox regression models. Results: A total of 39 patients were included in the present study. Nuclear MSH2 staining was statistically significantly correlated to Ki-67 expression (p=0.022). However, univariate and multivariate Cox analyses showed no statistically significant association between the expression of the investigated biomarkers and overall survival or relapse. Conclusion: The present exploratory study does not show any significant predictive value of the biomarkers examined with respect to survival or relapse. However, with larger patient cohorts, we believe that protein profiling using TMAs and immunohistochemistry is a feasible strategy for prognostic and predictive biomarker screening in laryngeal cancer.*

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Head and neck cancer is the sixth most frequent cancer in the world and the fourth most common cancer in men in the European Union after cancer of the lung, prostate and colorectum (1, 2). Laryngeal squamous cell carcinoma is in turn the most common neoplasm of the head and neck region, with 159,000 new cases and 90,000 deaths annually reported world-wide (1, 2). Two important contributors to the development and progression of laryngeal cancer are tobacco smoking and alcohol use (3). Their role as independent risk factors is well established and in addition they may act synergistically, thereby multiplying the risk (4). Early-stage laryngeal cancer is successfully treated either by external beam radiation therapy, or a combination of surgery and irradiation, with an overall 5-year survival of >65% independent of treatment modality (5, 6). The treatment of advanced laryngeal cancer is complex as the possibility of cure must be weighed against the functional consequences of any given treatment approach with curative intent, such as total laryngectomy. In recent years, the management of advanced laryngeal cancer has shifted towards avoidance of initial total laryngectomy whenever possible, saving it as a mean of salvage therapy after failed conservative management. As a consequence, identifying the patients with

a poor prognosis who would benefit from a radical approach presents a great challenge. Today however, partly due to the lack of consistent prognostic factors, the treatment of choice remains controversial and is to a large extent dependent on geography and local treatment traditions (7). In Sweden, the standard curative treatment of laryngeal neoplasms is external beam radiotherapy, with surgery generally reserved for salvage or resection of small primary tumors (*e.g.* carcinoma *in situ*) or for very advanced tumors, where surgery is always combined with radiotherapy. Unfortunately, a considerable fraction of tumors treated with radiotherapy prove to be radioresistant, recurring at the original site (8, 9). To predict which patients are most likely to fail radiotherapy, clinicians have traditionally used clinicopathological factors such as clinical stage, histological grade and the location of the tumor. However, the reliability of these traditional prognostic factors have been questioned and deemed as inadequate for the treatment protocols currently in use, which is why clinicians have shifted their attention towards cellular and biomolecular markers in order to develop more accurate predictors of clinical outcome. Although much effort has been put into research in molecular characterization of tumors, the knowledge of the molecular mechanisms underlying radioresistance is still sparse and the studies made to date have been unable to reach a consensus. Molecular markers have been subjects of investigation in this field, including proteins related to apoptosis such as p53 and B-cell CLL/lymphoma 2 (Bcl-2), proliferation markers such as Ki-67, regulators of the cell cycle such as cyclin D1 and proteins involved in DNA mismatch-repair such as MutS homolog 2 (MSH-2). The aim of this study was to investigate the expressions of the aforementioned proteins and elucidate any association they may have with the clinical outcome of patients treated with primary radiotherapy.

Materials and Methods

Patients. Sweden is divided into six health care regions and the Uppsala-Örebro region, which was used as the base population for this study, was the largest of these during the time period studied, with approximately 2 million inhabitants. In Sweden it is mandatory by law to report all cases of cancer to the National Cancer Registry (NCR). We identified all patients with invasive laryngeal cancer that had been treated with radiotherapy in the Uppsala-Örebro region through the NCR. The medical records and radiotherapy charts for patients diagnosed during the years 1978-1998 were retrospectively reviewed. Since 1998 there has been an ongoing prospective registration in a clinical database at the Regional Oncologic Centre in the Uppsala-Örebro region, and these patients were also included in the study with the clinical data taken from the clinical database. The Swedish Cancer Registry registered 1004 patients with laryngeal cancer during 1978-2004; clinical data were found for 961 patients and in 945 of these, it was possible to obtain follow-up data. These patients constitute the population analyzed, thus encompassing 94% of all cases. We were able to obtain tumor specimens (biopsies and surgical specimens) from the hospitals in Uppsala and Gävle and of these, 45 cases contained enough tumor material to be eligible for tissue microarray (TMA). Of these cases, four had not been treated with radiotherapy, which excluded them from the study. In two cases, the tissue sections were washed away during the immunohistochemical staining procedure (see later) resulting in the final total number of 39 patients.

Table I. *Grade distribution of protein expression in laryngeal squamous cell carcinoma.*

Grade	Nuclear p53	Nuclear Ki-67	Cytoplasmic Bcl-2	Cyclin D1		Nuclear MSH2
				Nuclear	Cytoplasmic	
0	9 (23%)	3 (8%)	35 (90%)	4 (10%)	32 (82%)	0 (0%)
1	3 (8%)	0 (0%)	0 (0%)	4 (10%)	5 (13%)	1 (3%)
2	4 (10%)	7 (19%)	4 (10%)	6 (15%)	2 (5%)	8 (21%)
3	23 (59%)	29 (74%)	0 (0%)	25 (64%)	0 (0%)	30 (77%)

All patients were classified according to the TNM system; age at the time of diagnosis, gender, histological diagnosis, tumor site, and treatment data (surgery, radiotherapy and chemotherapy) were registered. Patients were followed up initially within one month after radiation treatment and then generally every third month up to five years or relapse. The recorded events were relapse or death.

Generation of tissue microarrays. Tissue microarrays (TMAs) were created essentially as previously described (10). For all tumors, hematoxylin-eosin or van Gieson stained slides were available for comparison with the paraffin tissue blocks to enable identification of representative tumor material for transfer to the TMA. From each paraffin donor block, cylinders of tumor tissue (0.6 mm in diameter) were collected and transferred into recipient blocks using an automated tissue microarrayer (Beecher Instruments, Silver Springs, MD, USA). To increase representativity, two cylinders were taken from each tumor.

Immunohistochemistry. All primary antibodies used in the study had previously been tested and validated for protein profiling using immunohistochemistry on TMAs. Protein expression data is available at the Human Protein Atlas (www.proteinatlas.org) (11). Primary antibodies used were anti-p53, M7001, and anti-Ki-67, M7240, both from Dako, Glostrup, Denmark; anti-cyclin D1, NCL-L-CYCLIN D1-GM, from Novocastra, Newcastle upon Tyne, UK; and anti-MSH2, sc-22771, from Santa Cruz Biotechnology, Santa Cruz, CA, USA. From each TMA block, 4 μm-thick tissue sections were cut and applied to glass slides. The slides were deparaffinized in xylene and rehydrated through graded alcohols. Prior to immunostaining, heat-induced epitope retrieval (HIER) was performed using a Decloaking chamber® (Biocare Medical, Walnut Creek, CA, USA). All the primary antibodies were applied diluted at 1:100. All immunostainings were performed using an automated immunostaining instrument (Labvision, Fremont, CA, USA) and Ultravision LP detection kit including diaminobenzidine (DAB) as chromogen (Labvision). The slides were counterstained with Mayers hematoxylin, dipped in lithium chloride and dehydrated through graded alcohols and xylene. Finally, the slides were mounted with Pertex (Histolab AB, Gothenburg, Sweden).

Table II. *Correlation analysis of protein expression for the investigated biomarkers.*

	Nuclear p53	Nuclear Ki-67	Cytoplasmic Bcl-2	CyclinD1		Nuclear MSH2
				Nuclear	Cytoplasmic	
Nuclear p53	1.0					
Nuclear Ki-67	–0.029	1.0				
Cytoplasmic Bcl-2	0.027	0.13	1.0			
CyclinD1						
Nuclear	0.17	–0.076	0.24	1.0		
Cytoplasmic	0.19	–0.036	–0.11	0.012	1.0	
Nuclear MSH2	0.19	0.37 (p=0.022)	0.22	0.13	0.18	1.0

Scoring of immunostainings. The evaluation of the immunostaining was blinded with respect to the clinical parameters of the patients and performed by two independent observers. For each TMA, immunostained tissue spots were scored as previously described (12). In brief, positive immunohistochemical staining in tumor cells was evaluated with respect to the fraction of positive tumor cells and the intensity of positive staining. From these scoring data, three categories were defined: Grade 3: Strong immunoreactivity in >25% of tumor cells; Grade 2: moderate immunoreactivity in >25% of tumor cells, or strong immunoreactivity in <25% of tumor cells; Grade 1: weak immunoreactivity in >25% of tumor cells, or moderate immunoreactivity in <25% of tumor cells; Grade 0: Lack of immunostaining (negative), or weak immunostaining in <25% of tumor cells (Table I).

Statistics. Patients' characteristics at diagnosis were analyzed with standard descriptive statistics. Association among different markers with respect to TMA staining (grades) was evaluated using Spearman's correlation coefficients. The association between relapse and TMA staining for different markers was analyzed using Fisher's exact test.

Univariate and multivariate Cox regression models estimated the impact of different covariates on prognosis. In the survival analyses, time to event was defined as the time from the date of diagnosis to death or last follow-up until 06-09-2007. Results are presented as relative hazards (RH) with 95% confidence intervals (95% CI). In addition, *p*-values were given, but should be interpreted in a descriptive manner.

Results

Clinical data. In the present study, a total of 39 patients, 34 men and 5 women, were included. The median age of the patients was 65 years at diagnosis (range 44-82 years). The UICC stage distribution was as follows: stage I, 7 patients; stage II, 15 patients; stage III, 7 patients; and stage IV, 9 patients. For one patient, the stage was not known. Of the tumors, 32 were glottic, 6 were supraglottic and 1 was of subglottic origin. All patients received primary radiation treatment, 37 of these with curative intent. Radiation treatment consisted of a daily fraction of 2 Gy, however one patient received fractions of 1.2 Gy *b.i.d*. and another patient received fractions of 1.7 Gy *b.i.d*. For one patient, information about fractionation was missing. The median total dose of irradiation in the present study was 66 Gy (minimum 31.2 Gy, maximum 72.8 Gy). Of the included patients, 32 had surgery after radiotherapy, either as part of the initial treatment, because of recurrence, or because they were never tumor free, the latter occurring in 6 patients after primary radiotherapy. Two of the patients were operated on twice after radiotherapy. Despite the treatment regimens, 24 patients relapsed during follow-up. Clinical data of the patients and their association with protein expression are shown in Table V.

Protein expression in laryngeal carcinomas. The staining pattern of p53 was distinctly nuclear. Non-malignant cells showed no p53 expression, except for some scattered stained nuclei in the basal layer of proliferating laryngeal epithelia. A similar staining pattern was observed for Ki-67. Bcl-2 was only expressed in four of the tumors and only in the cytoplasm of the tumor cells. The expression was weak in all but one tumor. Strong Bcl-2 expression was noted in the lymphocytes surrounding the malignant cells. Cyclin D1 expression was mainly confined to the nucleus, its intensity ranging from weak to strong. The staining of MSH2 was homogeneously weakly to moderately positive in almost all the tumor cell nuclei observed as well as in the nuclei of non-malignant cells such as lymphocytes. The grade distribution of the protein expression is shown in Table I.

Associations among biomarkers and their relation to clinical outcome. Nuclear MSH2 staining was statistically significantly correlated to Ki-67 expression (p=0.022). No other statistically significant correlation between the investigated biomarkers was found (all other *p*-values were >0.14). Correlation analysis of the investigated parameters is shown in Table II.

Table III. *Univariate Cox analyses of overall survival for the clinical variables gender, location, relapse, and tumor-free status and the investigated biomarkers.*

Variable	Hazard ratio (95% CI)	*p*-Value
Nuclear p53	1.13 (0.81-1.58)	0.470
Nuclear Ki-67	1.12 (0.69-1.81)	0.648
Bcl-2 Cytoplasmic	1.24 (0.60-2.63)	0.551
Cyclin D1 Nuclear	0.99 (0.63-1.55)	0.960
Cytoplasmic	1.18 (0.51-2.76)	0.699
Nuclear MSH2	1.32 (0.58-3.05)	0.510
Gender		
Female (ref.)		
Male	0.37 (0.12-1.14)	0.085
Location		
Glottic/subglottic (ref.)		
Supraglottic	2.3 (0.80-6.64)	0.123
Relapse		
No (ref.)		
Yes	0.63 (0.27-1.47)	0.286
Tumor-free		
Yes (ref.)		
No	1.86 (0.66-5.28)	0.243

Table IV. *Multivariate Cox analyses of overall survival for each of the investigated biomarkers controlling for the clinical variables gender, location, relapse and tumor-free status.*

Variable	Hazard ratio (95% CI)	*p*-Value
Nuclear p53	1.24 (0.86-1.78)	0.25
Nuclear Ki-67	1.08 (0.66-1.77)	0.75
Cytoplasmic Bcl-2	1.53 (0.69-3.37)	0.30
Cyclin D1 Nuclear	0.92 (0.57-1.47)	0.71
Cytoplasmic	1.25 (0.50-3.13)	0.63
Nuclear MSH2	1.02 (0.41-2.56)	0.97

Univariate survival analysis of our clinical data showed that tumor localization and gender had a borderline statistically significant association with survival (p=0.12 and p=0.085, respectively). There was a tendency for patients with localization of tumors in the supraglottis to have poorer survival on average than those with other localizations. In addition, female patients tended to have a poorer survival than males. Neither never being tumor-free nor relapse were significantly associated with survival (p=0.24 and p=0.29, respectively).

When the investigated TMA stainings were studied in conjunction with relapse data, none of the investigated biomarkers showed any statistically significant association with relapse The smallest p-value was observed for nuclear expression of MSH2 (p=0.15).

Univariate overall survival analysis of the data of the investigated biomarkers showed no statistically significant association with the overall survival of the patients (p-values between 0.47-0.96). All HRs were, however, >1 except for nuclear expression of cyclin D1, for which the HR was 0.99 (95% CI 0.63-1.55). Univariate analysis of overall survival for the clinical variables and the investigated biomarkers is shown in Table III.

To further evaluate the significances of these biomarkers with respect to survival, we performed a series of multivariate analyses (Table IV). Each of these included one of the investigated biomarkers together with the clinical variables gender, relapse, tumor localization and never being tumor-free. These analyses showed small fluctuations in HRs as compared with the corresponding univariate analyses. As in the univariate analyses, all HRs, except for nuclear expression of cyclin D1, were >1, but none of the investigated biomarkers showed any statistically significant association with the overall survival. The lowest p-value was noted for nuclear expression of p53 (p=0.25).

Discussion

The conventional factors which are commonly used today as prognostic factors in head and neck cancer include tumor site, TNM stage and histopathological grade. However, due to the erratic nature of many neoplasms, the reliability of these established factors of prediction have been questioned and much research has been carried out with the purpose of finding novel biomarkers that better aid the clinicians to find a feasible treatment protocol for the individual patient. In recent years, cellular and biochemical markers have emerged as novel predictors of clinical outcome in different types of cancers. However, concerning head and neck cancer in general, and laryngeal squamous cell cancer in particular, the results from studies of these new markers have been controversial and there are still no established predictive molecular markers in clinical practice. Contributing to this is the fact that the number of patients included in most of the studies conducted to date has been rather small. In this retrospective study, by means of immunohistochemistry, we examined the expression of two proteins involved in apoptosis (p53 and Bcl-2), one proliferative marker (Ki-67), one cell cycle regulator (cyclin D1) and one mismatch-repair protein (MSH2), in order to clarify their relation to the clinical outcome of patients with laryngeal cancer treated with primary radiotherapy.

The *p53* gene is a tumor suppressor gene and plays a major role in preventing development of tumors. The derived protein is a transcription factor which halts cell growth and proliferation by several regulatory pathways. In the present study, we found that p53 was overexpressed (grade 2-3) in the nuclei in 27 (69%) out of the 39 patients. The overexpression of p53 in our study is comparable with reported percentages from other similar studies (13-15). The present study showed a tendency towards an association

Table V. *Distribution of protein expression according to clinical data of the patients.*

	Number of patients (%)	Nuclear p53				Cyclin D1							
						Nuclear				Cytoplasmic			
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 0	Grade 1	Grade 2	Grade 3	Grade 0	Grade 1	Grade 2	Grade 3
Gender													
Male	34 (87%)	8 (24%)	3 (9%)	27 (79%)	27 (79%)	27 (79%)	27 (79%)	6 (18%)	21 (62%)	0 (3%)	1 (3%)	8 (24%)	25 (74%)
Female	5 (13%)	1 (20%)	0 (0%)	5 (100%)	5 (100%)	5 (100%)	5 (100%)	0 (0%)	4 (80%)	0 (0%)	0 (0%)	0 (0%)	5 (100%)
Localization													
Glottic/subglottic	33 (85%)	7 (21%)	3 (9%)	28 (85%)	28 (85%)	28 (85%)	28 (85%)	6 (18%)	20 (61%)	0 (3%)	1 (3%)	6 (18%)	26 (79%)
Supraglottic	6 (15%)	2 (33%)	0 (0%)	4 (67%)	4 (67%)	4 (67%)	4 (67%)	0 (0%)	5 (83%)	0 (0%)	0 (0%)	2 (33%)	4 (67%)
Relapse													
Yes	24 (62%)	5 (21%)	2 (8%)	3 (13%)	3 (13%)	3 (13%)	3 (13%)	4 (17%)	15 (63%)	1 (4%)	0 (0%)	7 (29%)	16 (67%)
No	15 (38%)	4 (27%)	1 (7%)	2 (13%)	2 (13%)	2 (13%)	2 (13%)	2 (13%)	10 (67%)	0 (0%)	0 (0%)	1 (6%)	14 (93%)
Never tumor-free													
Yes	6 (15%)	2 (33%)	0 (0%)	4 (67%)	4 (67%)	4 (67%)	4 (67%)	2 (33%)	4 (67%)	0 (0%)	0 (0%)	0 (0%)	6
No	33 (85%)	7 (21%)	3 (9%)	28 (85%)	28 (85%)	28 (85%)	28 (85%)	4 (12%)	21 (64%)	0 (0%)	1 (3%)	8 (24%)	24 (73%)

	Number of patients (%)	Nuclear Ki-67				Nuclear MSH2				Cytoplasmic Bcl-2			
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 0	Grade 1	Grade 2	Grade 3	Grade 0	Grade 1	Grade 2	Grade 3
Gender													
Male	34 (87%)	3 (9%)	0 (0%)	7 (21%)	24 (71%)	0 (3%)	1 (3%)	8 (24%)	25 (74%)	31 (91%)	0 (0%)	3 (9%)	0 (0%)
Female	5 (13%)	0 (0%)	0 (0%)	0 (0%)	5 (100%)	0 (0%)	0 (0%)	0 (0%)	5 (100%)	4 (80%)	0 (0%)	1 (20%)	0 (0%)
Localization													
Glottic/ subglottic	33 (85%)	2 (6%)	0 (0%)	7 (21%)	24 (73%)	0 (3%)	1 (3%)	6 (18%)	26 (79%)	29 (88%)	0 (6%)	4 (12%)	0 (0%)
Supraglottic	6 (15%)	1 (17%)	0 (0%)	0 (0%)	5 (83%)	0 (0%)	0 (0%)	2 (33%)	4 (67%)	6 (100%)	0 (0%)	0 (0%)	0 (0%)
Relapse													
Yes	24 (62%)	2 (8%)	0 (0%)	4 (17%)	18 (75%)	1 (4%)	0 (0%)	7 (29%)	16 (67%)	22 (92%)	0 (0%)	2 (8%)	0 (0%)
No	15 (38%)	1 (7%)	0 (0%)	3 (20%)	11 (73%)	0 (0%)	0 (0%)	1 (6%)	14 (93%)	13 (87%)	0 (0%)	2 (13%)	0 (0%)
Never tumor-free													
Yes	6 (15%)	1 (17%)	2 (33%)	1 (17%)	4 (67%)	0 (0%)	0 (0%)	0 (0%)	6 (100%)	6 (100%)	0 (0%)	0 (0%)	0 (0%)
No	33 (85%)	2 (6%)	0 (24%)	6 (18%)	25 (76%)	0 (0%)	1 (3%)	8 (24%)	24 (73%)	29 (88%)	0 (0%)	4 (12%)	0 (0%)

between p53 overexpression and lower survival (HR>1) which was, however, not statistically significant with our limted number of patients. This is in agreement with data reported by other authors (14, 15), although there are also several reports on laryngeal cancer in which a statistically significant association between p53 overexpression and reduced survival has been shown (16, 17).

Ki-67 is strictly associated with cell proliferation. It is one of the most widely used markers for assessing the proliferative rate in tumors, thus estimating their grade of malignancy. In this study, Ki-67 was solely expressed in the nucleus and expression was noted in 36 (92%) out of the 39 patients, which is a rather high value compared to other studies (18, 19). We found no statistically significant association between Ki-67 expression and survival. Ki-67 has been deemed to predict shortened survival of laryngeal cancer patients according to some studies (20, 21), while others found no such associations of statistical significance (18, 22).

The Bcl-2 family of proteins are key regulators of cell death and survival, and individual family members can serve to inhibit or promote apoptosis, mainly at the level of the mitochondria. In our series, Bcl-2 was positively stained in the cytoplasm in only 4 out of 39 patients (10%). This is in line with a similar study by Fracchiolla *et al*. (23), although most of the literature report higher percentages of expression, generally of between 20-60% (24-26). Our results showed a tendency towards association between Bcl-2 expression and adverse survival (HR>1) which was, however, not statistically significant. However, considering the small number of Bcl-2 positive patients in our series, the results have to be interpreted with discretion. The results from previous studies are controversial, with some reports arguing in favour of a statistically significant correlation between Bcl-2 expression and improved survival (24, 27), while others were unable to find any such relationship (25, 28).

Cyclin D1 is one of the proteins regulating the G_1-S transition in the cell cycle and is considered to play an important role in advancing beyond the START checkpoint in the late G_1-phase, making the cell enter the S-phase, which subsequently leads to mitotic division. In the present study, cyclin D1 was overexpressed (grade 2-3) in the nuclei of 31/39 (79%) of the patients. In comparable studies, the nuclear expression generally ranged between 30-60% (29-31). In this study, we found no statistically significant association between cyclin D1 expression and survival or relapse. In other studies, cyclin D1 has been found to be associated with reduced survival (30, 32) and relapse (33), but there are also reports that failed to prove any such connection (34, 35).

The human MSH2 protein belongs to a family of highly conserved proteins involved in post-replication mismatch repair (MMR) (36). Loss of normal DNA MMR promotes tumorigenesis by accelerating the accumulation of mutations in oncogenes and tumor suppressor genes (37). Autosomal dominant disorder hereditary non-polyposis colon cancer can be caused by germline mutations in one of several MMR genes, most commonly in the MutL homolog 1 (*MLH1*) and *MSH2* genes (38). In the present study, a statistically significant correlation was found between nuclear MSH2 staining and the nuclear expression of Ki-67 ($r=0.37$; $p=0.022$). The correlation to the proliferative marker Ki-67 suggests that increased expression of MSH2 may be associated with more aggressive tumors and that the protein may be involved in the development of laryngeal carcinomas. However, we found no statistically significant association between MSH2 expression and survival.

The technique used in this study, namely TMA, was first described by Kononen *et al*. in 1998 (39). It represents a high-throughput method for the rapid assessment of tissue analysis in thousands of tumor samples by means of *in situ* molecular methods such as immunohistochemistry. This allows a comprehensive investigation of the tumor biology in a given malignancy in order to find novel molecular biomarkers. A common criticism of TMA technology relates to the small size of each tissue core, which means that due to tumor heterogeneity, biomarker scores obtained from small TMA cores will not necessarily be representative of the whole tumor mass (40). In addition, even though the tumor material needed to punch out the tissue cylinders is relatively small (diameter 0.6 mm), the smallest of biopsies have to be excluded, which makes studies, such as the present one, prone to selection bias. Moreover, as mentioned earlier, there are numerous studies with conflicting results as to the prognostic value of individual biomarkers such as p53 (14, 16) or Bcl-2 (27, 28), in a given malignancy.

In conclusion, this exploratory study does not show any correlation between the expression of the investigated biomarkers and survival in patients treated with radiotherapy for laryngeal squamous cell carcinoma. However, considering the small number of cases in the present study and the fact that the estimated HRs for all biomarkers except nuclear expression of cyclin D1 were >1 in both the univariate and the multivariate analyses, it is not possible to exclude the prospect that some of these biomarkers may have future use as supplements to conventional clinicopathological parameters when identifying those patients who may benefit from a more aggressive regimen. In addition, we believe that protein profiling using tissue microarrays and immunohistochemistry has a potential use for discovering novel biomarkers whose predictive value in laryngeal cancer is currently unknown. Thus, it is imperative that further trials with larger cohorts of patients are conducted in order to fully establish the value of molecular profiling using TMA for patients with laryngeal carcinoma.

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