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CATHEPSIN D CONCENTRATION IN PRIMARY LARYNGEAL CANCER: CORRELATION WITH CLINICO-PATHOLOGICAL PARAMETERS, EGFR STATUS AND PROGNOSIS

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Sixty-three patients with primary laryngeal squamous-cell carcinoma were followed up for a median of 33 months after surgery. Cathepsin D (Cath D) concentration was assayed using a solid phase 2-site immunoradiometric assay in which the first monoclonal antibody (MAb) was coated on the ELISA solid phase and the second one, M1G8 radiolabeled with I125-EGF, was used as the tracer. The median value of Cath D (13.8 pM/mg protein) was chosen as cut-off. Cath D ≥ median value was closely related to neck lymph node involvement at presentation and to a short metastasis-free survival (MFS) and actual overall survival (OS). The 5-year MFS was 71% for patients with Cath D < median value tumors as compared with 0% for patients with Cath D ≥ median value tumors. Lymph node status at presentation was not related to a short MFS and OS. Cox's univariate regression analysis using Cath D as a continuous variable showed that Cath D levels are correlated with neck lymph node metastasis. On multivariate analysis, Cath D status proved to be an independent factor for predicting a short MFS. Cath D assay may prove to be particularly useful in identifying laryngeal cancer patients who, with or without neck lymph node involvement at presentation, are at high risk of metastatic disease and poor outcome.

The prognostic clinical characterization of laryngeal squamous-cell carcinoma (SCC) remains inadequate because the outcome may differ considerably in comparably staged and treated patients. Attempts should thus be made to identify new factors which could be useful in predicting clinical behavior.

Considerable efforts have been made to identify the role of oncogenes, such as p53, c-*ras*, c-*myc* and EGFR expression or amplification (Anderson *et al.,* 1992; Scambia *et al.,* 1991, 1994; Irish and Bernstein, 1993; Maurizi *et al.,* 1992). For laryngeal squamous-cell carcinoma, we need better methods for distinguishing patients whose disease is localized and who will be cured by removal of the primary tumor from those with lymph node micrometastases who need additional elective neck dissection.

It is likely that proteases, as well as oncogene products and peptidic growth factors (EGF, EGFR), affect tumor invasion and metastasis. Cathepsin D (Cath D) is an aspartyl endopeptidase that, apart from its involvement in tumor invasion as a protelytic enzyme, has also been demonstrated to have a mitogenic effect *in vitro* (Vignon *et al.,* 1986). Higher Cath D concentrations were found in breast, endometrial and ovarian cancers than in their normal counterparts (Garcia *et al.,* 1990), which suggests that elevated Cath D levels may be associated with abnormal cell growth. Spyratos *et al.* (1989) showed that the total concentration of Cath D was a significant independent factor for identifying breast cancer patients with poor prognosis and at high risk of lymph node metastatic disease.

In a previous report we described significantly higher Cath D concentrations in laryngeal cancer tissues than in their normal mucosa counterparts, which suggests that Cath D could be involved also in laryngeal tumor cell growth processes (Ferrandina *et al.,* 1992).

In this study we have compared Cath D with EGFR expression levels and other established prognostic factors in the prediction of metastasis-free survival (MFS), relapse-free survival (RFS) and actual overall survival (OS).

MATERIAL AND METHODS

Sixty-three untreated consecutive primary laryngeal SCC patients were studied. The clinico-pathological features of the patients are shown in Table I. Tumor site was classified as supraglottic (18/63) and transglottic (supraglottic and glottic sites; 45/63). Tumors were staged according to the TNM classification and graded as well (G1), moderately (G2) or poorly differentiated (G3).

At our institution, all primary laryngeal cancer patients receive standard therapeutic management: therapeutic surgical treatment (curative surgery) of the primary tumor (T) related to the lesion extension; therapeutic neck node dissection when there is lymph node involvement at clinical presentation (N^+) according to the "wait and see" policy under strict follow-up conditions; post-surgical radiotherapy for local advanced tumors (T4) and neck lymph node metastasis with extranodal spread. All patients of this study have been treated according to this standard procedure. Twenty-six patients underwent total laryngectomy, 18 underwent supraglottic laryngectomy and 19 hemilaryngectomy. Eight patients with advanced tumors (5T4N0, 3T4N+, stage IV) had post-surgical radiotherapy. At surgery, 16 patients with clinically positive neck nodes underwent a therapeutic neck dissection. The median follow-up period was 33 months (range = 7–77 months).

Preparation of cytosolic and membrane fractions

Tissue specimens collected during surgery were frozen on dry ice shortly after surgical removal and stored at −80°C until processed. Cytosolic and membrane fractions were prepared as described elsewhere (Battaglia *et al.,* 1988). Briefly, tumor specimens were finely minced and homogenized in 5 vol of ice-cold buffer consisting of 25 mM Tris, 1.5 mM EDTA, 5 mM NaN3, 0.1% monothioglycerol and 20% glycerol (TENMG), by applying several intermittent bursts of an Ultra-turrax homogenizer. The crude homogenate was centrifuged at 105,000 *g* for 75 min at 0°C to obtain a cytosolic fraction for Cath D and a membrane fraction for EGFR assay.

Cath D measurements

Cath D concentration was assayed using a solid phase 2-site immunoradiometric assay (CIS Bioindustries, Gif-sur-Yvette, France) in which the first MAb is coated on the ELISA solid phase and the second, M1G8 radiolabeled with ^{125}I-EGF, is used as the tracer (Brouillet *et al.,* 1990). Protein concentration was measured using the Bradford (1976) method and was reset to about 1 mg/ml before the assay. Cytosols were then diluted 1/40 and 1/80 with the diluent contained in the kit. Results were expressed as pM Cath D/mg cytosol protein. Intra- and

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inter-assay variations were 7.5% and 10%, respectively. Cath D status was defined using the cut-off value of 13.8 pM/mg protein, which corresponded to the median concentration value (Cath D^- as Cath D < median value; Cath D^+ as Cath D ≥ median value).

EGFR measurements

The membrane pellet was resuspended in 25 mM Tris, 1.5 mM EDTA, 5 mM NaN3, 20% glycerol and 10 mM $MgCl_2$ and the EGFR assayed on the membrane fraction as previously described using 125I-EGF (800,000 Ci/mmol; NEN, Dupont, Wilmington, DE) as the radiolabeled ligand (Scambia *et al.*, 1991). Results were expressed as fmol/mg protein. EGFR status was defined using the best discriminating cut-off value of 8 fM/mg protein.

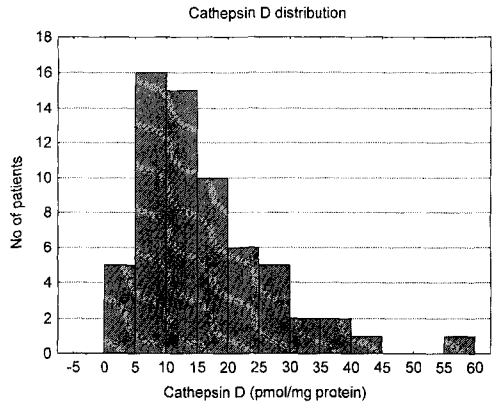

FIGURE 1 – Distribution of patients (n = 63) as a function of Cath D concentrations.

Statistical analysis

Statistical analysis was performed by the Statistica package (rel. 4.5) and by the BMDP package (vers. 7) for multivariate analysis only. Distribution of Cath D and EGFR levels according to clinico-pathological parameters was analyzed by the 2 × 2 table of contingency. Fisher's exact 2-tailed *p* was obtained from 2 × 2 contingency tables. Cox's proportional hazards method was used to evaluate the prognostic role of Cath D values as a continuous variable (Cox, 1972). All medians and life tables were computed using the product-limit estimate by Kaplan and Meyer (1958). All survival curves were examined by means of the log-rank test (Mantel, 1966), grouping by clinico-pathological parameters, EGFR and Cath D status. Multivariate analysis was performed by Cox's proportional hazards model. Five-year MFS and RFS were calculated from the date of primary surgery to the date of clinical or pathological regional lymph node involvement or local recurrence of disease. Actual 5-year OS was calculated from the date of first surgery to the date of death (median follow-up 33 months, range 7–77 months). Patients who died of intercurrent disease were considered censored.

RESULTS

The distribution of Cath D concentrations is represented in Figure 1. Cath D levels ranged from 3.26 to 57.60 pM/mg protein, with a median value of 13.80 pmoles/mg protein. Using arbitrary cut-off values of 13.8 pM/mg protein, 51% of tumors were considered Cath D^+ (Cath D ≥ median value). Table I shows no significant relationship between Cath D concentration and age, sex, T classification, stage of disease and histopathological grading, while a significant relationship

TABLE I – DISTRIBUTION OF EGFR AND CATH D LEVELS ACCORDING TO CLINICAL-PATHOLOGICAL PARAMETERS IN 63 PRIMARY LARYNGEAL CANCER PATIENTS

	Number	EGFR			Cath D			p^1
		Median (fmoles/mg protein)	Range	% of EGFR >8.0	Median (pmoles/mg protein)	Range	% of Cath D ≥13.80	
Total	63	7.70	0.–64.80	48	13.80	3.26–57.60	51	
Sex								
Males	59	7.60	0.–64.80	46	13.60	3.26–57.60	46	
Females	4	16.15	0.–49.90	50	15.90	10.60–25.30	5	
Age (yr)								
≤60	25	7.00	0.–61.70	16	10.40	3.26–57.60	17	
>60	38	8.54	1.2–64.80	32	14.65	4.80–42.30	33	0.4
Tumor site								
Supraglottic	18	8.85	0.–61.70	17	16.10	4.86–57.60	17	
Transglottic	45	7.00	0.–64.80	30	13.00	3.26–42.30	33	0.4
T classification								
1	10	6.80	0.–64.80	8	10.76	4.86–25.90	6	
2	27	5.40	0.–61.70	17	14.80	4.60–57.60	22	
3	18	10.05	0.–49.90	14	13.80	3.26–42.30	16	
4	8	17.00	1.2–35.40	8	14.05	4.80–32.00	6	0.8
Lymph-node involvement								
No	47	7.60	0.–64.80	33	12.84	4.20–57.60	32	
Yes	16	8.85	1.2–49.90	14	16.75	3.26–34.80	19	0.04
Histopathological grading								
G1	15	5.40	0.–64.80	10	13.00	5.10–42.30	11	
G2	32	8.25	0.–61.70	25	14.15	3.26–57.60	29	
G3	16	8.25	0.–35.40	13	12.06	4.86–39.15	11	0.6
Stage								
I	9	4.80	0.–64.80	6	10.40	4.86–25.90	6	
II	20	5.20	0.–61.70	11	12.87	4.60–57.60	13	
III	22	10.65	0.–49.90	21	14.30	4.20–42.30	21	
IV	12	11.15	1.2–35.40	10	15.60	3.26–34.80	11	0.2
EGFR								
>8 fmoles/mg protein	33				12.84	3.26–35.40	19	
≤8 fmoles/mg protein	30				16.50	4.20–57.60	32	0.02

[1]*p* value: Fisher exact 2-tailed *p* from 2 × 2 contingency table.

was found between Cath D$^+$ and neck lymph node positivity (N$^+$) at presentation ($p = 0.04$) and EGFR expression ($p = 0.02$). During the follow-up period, local recurrence and metastatic cervical lymph node involvement were observed in 23 and 23 cases, respectively. At the end of the study, 18 patients had died of cancer.

Cox's univariate regression analysis, using Cath D as a continuous variable, showed that Cath D levels correlated with neck lymph node metastasis ($p = 0.01$).

Figures 2 and 3 show the survival curves according to Cath D status. On univariate analysis, a significant relationship was found between Cath D$^+$ and shorter 5-year MFS ($p = 0.005$) and OS ($p = 0.04$). In fact, 5-year MFS was 71% for patients with Cath D$^-$ (Cath D < median value) tumors as compared with 0% for patients with Cath D$^+$ tumors ($p = 0.005$). Similarly, 5-year OS was 74% for patients with Cath D$^-$ tumors as compared with 61% for patients with Cath D$^+$ tumors ($p = 0.04$). Age significantly correlated with shorter OS ($p = 0.008$). EGFR status, T classification and stage of disease also significantly correlated with shorter OS, RFS and MFS (Table II) in univariate analysis.

Table III shows the multivariate analysis of prognostic variables for survival in laryngeal cancer patients. T classification and stage proved to be independent prognostic factors in MFS, RFS and OS. EGFR status was a prognostic factor in MFS and OS. Cath D status was an important independent prognostic factor in MFS only ($p = 0.01$).

DISCUSSION

We show that Cath D concentration is a significant independent prognostic factor for identifying laryngeal SCC patients at high risk of neck node metastasis. High Cath D concentration is associated with neck lymph node involvement at clinical presentation and shorter MFS. Moreover, analysis of logarithmically transformed Cath D values shows that the risk of lymph

TABLE II – UNIVARIATE ANALYSIS OF PROGNOSTIC VARIABLES FOR SURVIVAL IN 63 PRIMARY LARYNGEAL CANCER PATIENTS

Prognostic variable	Number	Overall survival			Relapse-free survival			Metastasis-free survival		
		% 5-year survival	RR[1]	p	% 5-year survival	RR	p	% 5-year survival	RR	p
Age (yr)										
≤ 60	25	85	1		64	1		64	1	
> 60	38	55	3.3	0.008	44	1.5	0.07	40	1.2	0.2
Tumor site										
Supraglottic	18	70	1		59	1		56	1.1	
Transglottic	45	67	1.04	0.4	53	1.1	0.4	54	1	0.2
T classification										
1–2	37	81	1		68	1		68	1	
3–4	26	48	2.2	0.01	30	2.2	0.002	31	2.2	0.002
Lymph-node involvement										
No	47	71	1		55	1		56	1	
Yes	16	58	1.5	0.08	56	1.04	0.2	52	1.3	0.1
Histopathological grading										
G1–G2	47	69	1		52	1		54	1.2	
G3	16	64	1.1	0.3	60	1.04	0.3	61	1	0.5
Stage										
I–II	29	87	1		68	1		76	1	
III–IV	34	49	3	0.003	38	1.9	0.006	33	3.1	0.0003
EGFR status										
≤ 8.0 fmoles/mg protein	33	79	1		69	1		74	1	
> 8.0 fmoles/mg protein	30	53	2.2	0.02	34	1.7	0.03	27	2.1	0.01
Cath D status										
< 13.80 pmoles/mg protein	31	74	1		61	1		71	1	
≥ 13.80 pmoles/mg protein	32	61	1.6	0.04	29	1.1	0.1	0	1.8	0.005

[1]RR, relative risk corresponds to the number of adverse events in 1 category expressed as a proportion of reference-category events.

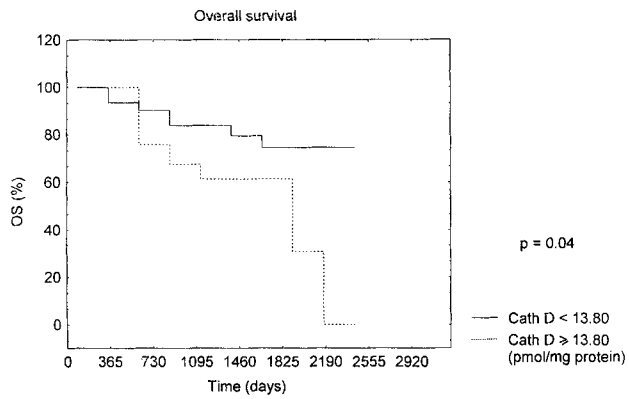

FIGURE 2 – Actual 5-year OS according as Cath D status in 63 primary laryngeal cancer patients. OS was 74% for patients with Cath D < median value tumors as compared with 61% for patients with Cath D ≥ median value tumors.

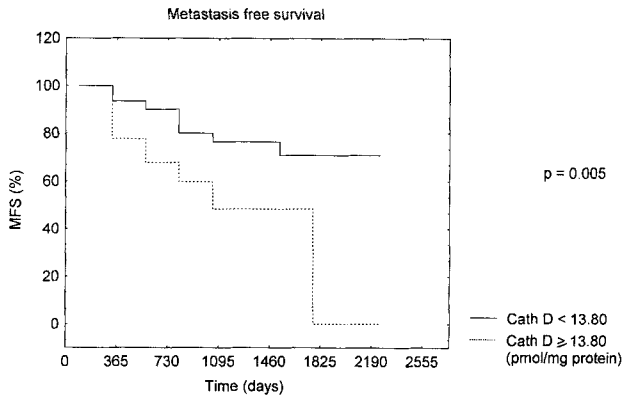

FIGURE 3 – Five-year MFS according to Cath D status in 63 laryngeal cancer patients. MFS was 71% for patients with Cath D < median value tumors as compared with 0% for patients with Cath D ≥ median value tumors.

TABLE III – MULTIVARIATE ANALYSIS OF PROGNOSTIC VARIABLES FOR SURVIVAL IN 63 PRIMARY LARYNGEAL CANCER PATIENTS

Prognostic variable	Number	Overall survival			Relapse-free survival			Metastasis-free survival		
		RR[1]	χ^2	p	RR	χ^2	p	RR	χ^2	p
Age (yr)										
≤ 60	25									
> 60	38	3.9	6.37	0.01	—	2.3	0.1	—	0.5	0.5
Tumor site										
Supraglottic	18									
Transglottic	45	—	0.14	0.7	—	0.06	0.8	—	0.6	0.4
T classification										
1–2	37	2.9	4.9	0.03	3.3	8.0	0.005	3.4	8.1	0.005
3–4	26									
Lymph-node involvement										
No	47									
Yes	16	—	1.7	0.2	—	0.4	0.5	—	1.0	0.3
Histopathologic grading										
G1–G2	47									
G3	16	—	0.37	0.5	—	0.3	0.6	—	0.0	1.0
Stage										
I–II	29									
III–IV	34	4.1	7.56	0.06	3.0	6.3	0.01	5.0	12.0	0.0005
EGFR status										
≤ 80 fmoles/mg protein	33									
> 80 fmoles/mg protein	30	2.7	4.18	0.04	2.2	3.3	0.07	2.7	5.2	0.02
Cath D status										
< 13.80 pmoles/mg protein	31									
≥ 13.80 pmoles/mg protein	32	2.4	3.17	0.08	—	1.1	0.3	3.1	6.6	0.01

[1]RR, relative risk taking into account all variables of the table.

node metastasis increases with Cath D values in a significant way. These data agree with the findings of Tandon *et al.* (1990) and Maudelonde *et al.* (1990), thereby confirming a clear association between high cytosolic Cath D and an increased metastatic potency of tumor cells (Garcia *et al.*, 1990).

The prognostic significance of EGFR status in laryngeal SCC patients has already been described (Maurizi *et al.*, 1992). The correlation between EGFR and Cath D levels could confirm the experimental evidence that EGF increases Cath D levels (Cavailles *et al.*, 1988) and suggests that the EGF/EGFR system is involved in the regulation of Cath D synthesis and/or secretion. Both EGFR and Cath D status appear to represent biological factors which provide useful information for predicting outcome and individualizing treatment in laryngeal cancer patients.

In particular, Cath D could identify a subset of laryngeal cancer patients with a high risk of metastatic neck node involvement, similar to what happens in breast cancer (Spyratos *et al.*, 1989). The explanation of the association of Cath D with neck node metastasis may lie in its property to degrade proteoglycans and extracellular matrix material (Rochefort *et al.*, 1988).

In our study, the prognostic value of the clinical neck status did not confirm published data probably because of fewer N^+ than N0. Clinical examination of neck lymph nodes remains critical because the presence of tumor cells in apparently negative lymph nodes correlates with metastatic spread and survival (Snow *et al.*, 1982). At present, the proper treatment of patients without apparent neck disease remains uncertain. More information is needed to facilitate a decision on elective neck dissection justified by the probability of occult disease and, thus, to avoid under- and over-treatment. Elective neck dissection rather than a "wait and see" policy under strict follow-up conditions is an unsettled issue. The introduction into current clinical practice of biological markers to predict the capacity of a tumor to invade extracellular matrices would represent an important step ahead.

In conclusion, our results suggest that assessment of Cath D status at the time of initial surgery may identify a subset of laryngeal cancer patients with increased metastatic potency and permit therapy to be adapted accordingly. Patients bearing tumors with high Cath D levels should undergo elective neck dissection, elective neck irradiation or post-surgical irradiation for neck lymph node metastasis even without extranodal spread.

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