Pergamon

*Oral Oncol, Eur J Cancer*, Vol. 30B, No. 1, pp. 51–55, 1994
Elsevier Science Ltd
Printed in Great Britain
0964–1955/94 $6.00 + 0.00

# Immunohistochemical and Immunoradiometric Evaluations of Total Cathepsin D in Human Larynx

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**By using a commercially available immunoradiometric technique (Cath-D-IRMA, Cis BioInt.) the distribution of total cathepsin D (cath-D) in 30 malignant and in the corresponding histologically-proven non-malignant fragments obtained from lymph node negative patients suffering from larynx cancer was investigated. In both tissues the oestrogen and progesterone receptors were also assayed. In 17 out of the 30 samples, the cath-D was also assayed by immunohistochemistry using the M1G8, a mouse monoclonal antibody raised against cath-D (Cis BioInt.). Our data indicate that cath-D is present in prismatic cells of the normal laryngeal epithelium and in the cancerous cells. In cancerous larynx, the outer cell layer of large tumour nests showed the highest degree of immunoreactivity, while fibroblasts and inflammatory cells always showed a very faint staining. Cathepsin D levels were significantly higher ($P < 0.0001$) in the cancerous fragments (with a mean of $33 \pm 3.4$ pmol/mg protein) than in the corresponding non-cancerous specimen (with a mean of $20.8 \pm 2$ pmol/mg protein). A significant positive association ($P < 0.001$) between cath-D and progesterone receptor (PR) concentration values in the cancerous larynx was observed; accordingly, tumours expressing PR had significantly ($P = 0.0005$) higher cath-D levels than the tumours which did not contain the receptor. In contrast, such a relationship was absent in the non-malignant specimens. As regard the oestrogen receptor, no significant relationship between this and cath-D was observed. We conclude that cath-D measured by IRMA in tissue cytosols is mainly derived from cancerous cells, the contribution from fibroblasts and inflammatory cells being negligible. Cathepsin D overexpression and association with the PR in the malignant part of the larynx could indicate a possible role of the receptor in the biology of this disease.**

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## INTRODUCTION

THE PROTEINASE cath-D is a lysosomal phosphoglycoprotein first secreted in a precursor form (pro-cath-D, 52 kD) and then processed in an intermediate form of 48 kD and mature forms of 34 kD and 14 kD. It has been found in a wide variety of human tissues including liver [1], placenta [2], gastric mucosa [3], and in neoplastic tissues such as breast, ovaries, endometrium, gastric mucosa, central nervous system. No evidence about the presence of cath-D in cancerous and non-cancerous larynx has been reported so far, apart from a work showing cath-D expression in head and neck carcinomas but without mentioning the anatomical location [4]. The assessment of such an expression is of potential interest in that cath-D is, at least in breast cancer, involved in the process of tumour invasion and metastatisation [5], and high level of this proteinase is a poor prognostic factor in that it can be used to identify patients who are at risk of metastatic disease. Moreover, cath-D is specifically induced, in human breast cancer cells, by oestrogens [5–7], and is associated to the functionality of ER [8]. In view of the fact that larynx cancer also possesses steroid receptors [9–12], the assessment of a relationship between them and cath-D would be of biological and prognostic interest, and provide new insights into the role of the steroid receptors in this disease.

This paper describes studies which examine the expression of total cath-D (52 kD, 48 kD, 34 kD and 14 kD forms) in cancerous and in non-cancerous larynx, and its correlation to the oestrogen (ER) and progesterone (PR) receptors.

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Received 22 Feb. 1993; provisionally accepted 10 Mar. 1993; revised manuscript received 30 Mar. 1993.

## MATERIALS AND METHODS

This study was performed on a group of 30 node-negative patients with histologically confirmed squamous cell carcinomas of the larynx who underwent larynx surgery in the past 12 months. All patients were adult men between 50 and 80 years of age (median age = 62). From all patients, a fragment of histologically proven non-malignant larynx adjacent to the tumour was also taken.

*Tissue handling*

Malignant and non-malignant larynx tissues were placed on ice immediately after excision and stored at −70°C until processing for measurements of ER, PR and cath-D. Tumour tissue was homogenised, using an Ultra-Turrax homogeniser, in glycerol phosphate buffer of low ionic strength (10% glycerol (v/v), 10 mM $K_2HPO_4/KH_2PO_4$, 1.5 mmol/l EDTA, 10 mmol/l $MgCl_2$, containing 1 μg/ml of each of the protease inhibitors soybean trypsin inhibitor, leupeptin and aprotinin, and 1 mmol/l of phenylmethylsulphonyl fluoride). The homogenate was centrifuged for 15 min at 900 *g* at 4°C. The supernatant centrifuged again for 60 min at 100 000 *g* and the resulting supernatant (termed "cytosol") was used for steroid receptors and cath-D determinations after having adjusted the protein content to 3 mg/ml and 0.05 mg/ml, respectively.

*Immunohistochemistry of cath-D*

Seventeen larynx cancer samples including contiguous normal and metaplastic laryngeal epithelium were formalin-fixed, paraffin-embedded and cut into 5 μm thick sections, collected on poly-L-lysine coated slides and stained with haematoxylin–eosin. The immunohistochemical studies were performed by using the alkaline-phosphatase/anti-alkaline-phosphatase (APAAP) technique [13] on haematoxylin–eosin consecutive sections. Sections were rehydrated in a xylene-graded alcohol scale and immersed in Tris-buffered saline (TBS) containing 0.1% BSA (fraction V), for 15 min. Slides were probed for 30 min at 25°C with primary mouse monoclonal antibody M1G8 (Cis BioInternational, Gif-sur-Yvette, France) raised against the C-terminal part of the total cath-D and diluted 1:5 in TBS. Next, rabbit anti-mouse antibody (Techno Genetics, Cassina de Pecchi, Milano, Italy) was applied at the sections at a 1:25 in TBS, and incubated for 30 min at 25°C. Subsequentely, APAAP complexes (Techno Genetics, Cassina de Pecchi, Milano, Italy) were added for 30 min at 25°C and detected with fuchsin chromogen containing 0.1% levamisole. Finally, the sections were counterstained for 1 min in Mayer's haematoxylin and coverslipped with an aqueous mounting medium.

In each run, control tissue sections were included where mouse monoclonal antibody of the same subclass of the M1G8 replaced the M1G8 itself. None of the negative controls showed positive staining.

*Immunoradiometric assay of cath-D*

Total cath-D assay was performed using a commercially available solid phase two site immunoradiometric assay according to the instruction provided with the CIS Biointernational Kit, Gif-Sur-Yvette, France. Such system has been validated by others [14] and used for total cath-D assay by many research groups [14–16]. This system uses two different monoclonal antibodies, the first, D7E3, attached to the ELSA solid phase, and the second one, M1G8, is iodinated and free in solution. The second antibody is the same one used in immunohistochemistry.

*Steroid receptor assays*

Because of the limited amount of tissue available, steroid receptors have been measured by single saturating dose assay, having shown previously [9] that receptor concentration values obtained with this system are in good agreement with the values obtained by multi-point assay followed by Scatchard analysis. We assayed the steroid receptors in the high-speed soluble fraction (termed "cytosol") because, as previously described [9], all of the cytosol steroid receptor positive biopsy specimens are also nuclear positive and the receptors in the cytosol are found in higher concentrations than in the nucleus.

*Protein estimation*

These were carried out using the method of Bradford [17] using BSA as the standard.

*Statistical analysis*

Statistical analysis of the correlations between concentration values of steroid receptors and cath-D was carried out using the Spearman's rank correlation and linear regression analysis. Mann–Whitney–Wilcoxon's rank sum test (MWW) was used to test differences in location of the above mentioned parameters in steroid receptors positive and negative tumours. Wilcoxon's signed rank sum test was used to test differences between paired samples. Analysis of the qualitative association between the receptor status and the cath-D distribution was performed using the Fisher's exact test.

A probability level of 0.05 or less was chosen to represent statistical significance.

## RESULTS

*Cathepsin D valuation by immunohistochemistry*

*Non-malignant larynx.* Figure 1(a) shows a section of normal laryngeal epithelium taken from a cancerous larynx. Prismatic cells show strong staining for cath-D. Immunoreactivity is also present in scattered fibroblasts and inflammatory cells in the chorion, but the level of staining was always much less intense than the one observed in the epithelium.

*Malignant larynx.* Figure 1(b) shows a section of the corresponding well differentiated squamous cell carcinoma. Cathepsin D immunoreactivity appears to be homogeneously distributed within tumour cells with a predominant intracytoplasmic staining pattern. A very faint staining is seen in stromal cells surronding tumour nests.

*Quantitation of cath-D by IRMA*

The cytosolic fraction of the tumours showed a relatively higher protein yield (mean value ± S.E. was 24.3 ± 4.3 mg cytosolic protein/g wet weight) than the non-malignant tissue (12.0 ± 3.5 mg cytosolic protein/g wet weight).

*Non-malignant larynx.* All non-malignant samples were cath-D positive (cath-D concentration ⩾5 pmol/mg protein was considered the lower limit of detection for the assay, i.e. a value significantly different from zero), with concentration values ranging from 5 to 46 pmols/mg protein, mean value (± S.E.) was 20.8 (± 2), median was 19.5.

*Malignant larynx.* All tumours expressed cath-D with concentration ranging from 5 to 82 pmol/mg protein, mean value was 33 (± 3.4), median was 28. Cathepsin D values in malignant samples were significantly higher than those

obtained in the corresponding non-malignant specimens (Wilcoxon's signed rank test: $P<0.0001$). When the tumours were divided in two subgroups having "high" and "low" cath-D levels, this overall trend appeared composed by two different patterns. More precisely, the "high" cath-D tumours expressed significantly higher levels of this protease than the corresponding non-malignant fragments ($P<0.00001$ by Wilcoxon's signed rank test), while no difference in cath-D expression was found between the "low" cath-D tumours and the corresponding non-malignant specimens (Fig. 2).

Tissue yield ratio (malignant/non-malignant) obtained in the 15 high cath-D tumours and paired non-malignant fragments was not significantly different (Student's t test: $P>0.5$) from the tissue yield ratio obtained in the 15 low cath-D tumours and paired non-malignant samples.

*Steroid receptors status*

*Non-malignant larynx.* Fourteen samples (47%) were ER positive (ER $\geq$ 3 fmol/mg cytosol protein), with concentration ranging from 3 to 41 fmol/mg protein, mean value ($\pm$ S.E.M.) was 5.2 ($\pm$ 1.5), median was 0.

PR positivity was observed in 20 out of the 30 (67%) cytosols (PR $\geq$ 3 fmol/mg protein), with concentration ranging from 3 to 44, mean value ($\pm$ S.E.M.) was 7.8 ($\pm$ 1.8), median was 5.

*Malignant larynx.* Sixteen tumours (53%) were ER positive (ER $\geq$ 3 fmol/mg cytosol protein), with concentration ranging from 3 to 33 fmol/mg protein, mean value ($\pm$ S.E.M.) was 4.3 ($\pm$ 1.5), median was 1.5.

PR positivity was observed in 17 out of the 30 (57%) tumour cytosols (PR $\geq$ 3 fmol/mg protein), with concentration ranging from 3 to 47, mean value ($\pm$ S.E.M.) was 7 ($\pm$ 1.9), median was 0.

No association between the concentration of steroid receptors in malignant and in non-malignant samples was observed (Spearman's rank correlation: $P>0.1$). Accordingly, no difference in ER and PR concentration between malignant and non-malignant was found (Wilcoxon's signed rank test: $P>0.1$).

*Association between steroid receptors and cath-D*

*Non-malignant larynx.* No significant association between cath-D and PR was observed by Spearman's rank correlation and by linear regression ($P>0.1$) (Fig. 3a). Associations between ER and cath-D were also not significant ($P>0.1$).

*Malignant larynx.* A highly significant association ($r_s=0.60$, $P<0.0001$) between cath-D and PR was observed by Spearman's rank correlation. Cathepsin D was also linearly correlated to PR ($P<0.01$) with linear regression coefficient of $r=0.45$ (Fig. 3b). Conversely, associations between ER and cath-D were not significant ($P>0.1$).

Choosing the cath-D median value as a cut-off point, two groups of patients having "high" ($\geq$28 pmol/mg protein) and "low" (<28 pmol/mg protein) cath-D levels were identified.

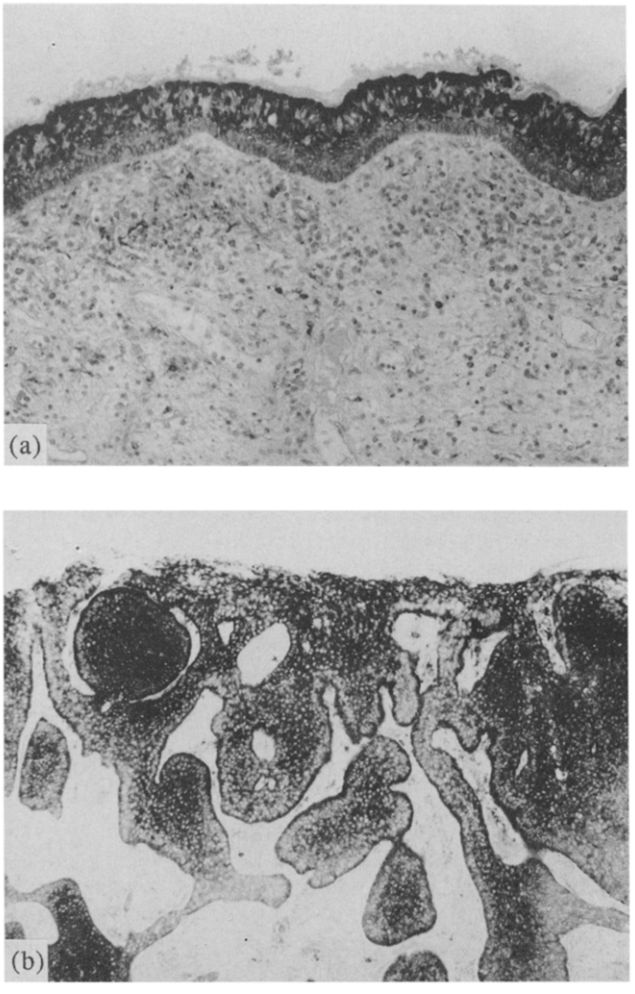


**Fig. 1. Cathepsin D immunostaining of larynx normal epithelium (a) (60×) and well differentiated squamous cell carcinoma of the larynx (b) (25×). Mouse monoclonal antibody M1G8 to human cathepsin D was used as described in Materials and Methods.**

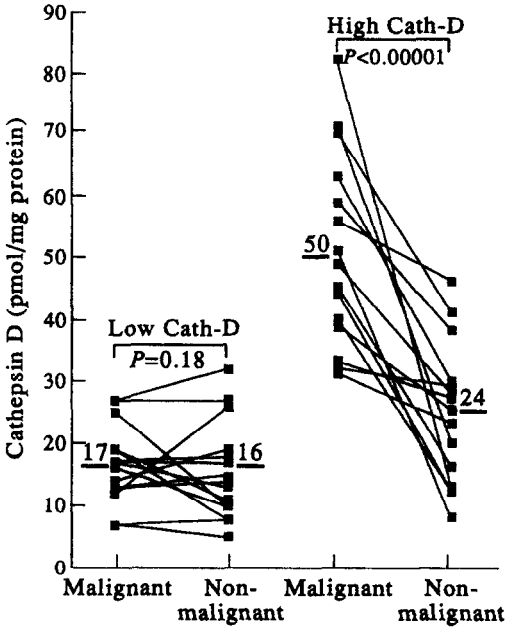


**Fig. 2. Cathepsin D concentration values in malignant and in the corresponding non-malignant larynx samples. Samples are divided on the basis of cathepsin D expression in the malignant specimens (low vs. high). Differences were tested using the Wilcoxon's signed rank sum test for paired samples.**

Fig. 3(b) shows that of the 15 tumours having high levels of cath-D, 13 (87%) expressed the PR, while only 3/15 (20%) low cath-D tumours were PR positive ($P=0.0003$ by Fisher's exact test). No significant relationships with ER was found (Fisher's exact test: $P>0.1$). Statistical analysis showed that PR positive tumours expressed higher cath-D level than the PR negative ones (MWW: $P=0.0005$). Similarly, PR in the "high" cath-D tumours was significantly higher (MWW: $P=0.002$) than PR obtained in "low" cath-D specimens.

## DISCUSSION

Since the cytosolic extracts used in IRMA quantification of total cath-D contain proteins derived from different cellular types, it is important to ascertain that cath-D is actually derived from cancerous cells and not from other cells that also express this proteinase. From this viewpoint, the major interest of immunohistochemistry as compared to IRMA is to define the antigen-producing cells. We here show that in all the tumours examined by immunohistochemistry, the participation of macrophages and fibroblasts in cath-D production is generally low (Fig. 1), so that the epithelial larynx cells are actually expressing the enzyme. The physiological roles of cath-D in laryngeal epithelium remains to be determined, but this proteinase could be involved in the self-destruct mechanisms of senescent or damaged epithelial cells. In cancerous larynx cath-D could have a role in tumour infiltration because immunoreactivity was often more evident in the outer cell layer of tumour nests. This agrees with the current opinion on cath-D role in breast cancer. Another finding of this study is the relative higher expression of cath-D in cancerous than in non-cancerous larynx (Fig. 2). Such higher relative cath-D concentration in malignant tissue is not peculiar to larynx cancer, since it has been observed in breast, ovarian and endometrial carcinomas [15, 18, 19]. Other cathepsins such as B and L, increase their synthesis in colorectal carcinoma with respect to the normal colorectal tissue [20]. Interestingly, a different pattern in endopeptidases' distribution between cancerous and non-cancerous tissues is observed in other organs; cathepsins D and E are present in normal gastric mucosa but absent from cancerous stomach [21], while no difference in cath-D concentration was reported between non-malignant and malignant central nervous system tissues [22], suggesting that the endopeptidases' deregulation in disease states can be very different. Cathepsin D over-expression in larynx cancer is neither related to the metastatisation processes, in that all the patients chosen for this study were lymph node negative, nor to differences in ER values. It is however related to PR concentration. In fact, tumours expressing PR had significantly higher levels of cath-D than those which did not show appreciable amounts of this receptor (Fig. 3b); also, cath-D was linearly correlated to PR (Fig. 3b). Such observations would be consistent with the hypothesis that exists a some degree of control of PR over cath-D. Because there is not significant difference in the expression of PR between malignant and non-malignant larynx, the possibility arises that in fast growing and rapidly dividing cancerous cells may exist PR-mediated mechanisms able to regulate cath-D expression and/or intracellular fate. That cath-D could be induced by progestin is not a novel issue in that it has been demonstrated in normal human endometrium [19]. Another point regarding the relative quantity of cath-D in cancerous larynx should be stressed. More precisely, cath-D relative concentration in cancerous samples was significantly higher than the corresponding non-cancerous specimens only in those tumours having cath-D concentrations higher than the median value (Fig. 2). On the contrary, cath-D levels expressed by the tumours having "low" concentration of this protease (lower than the median) were not different from those obtained in the corresponding non-malignant parts. Such observations could suggest that the median cath-D value observed in tumours (28 pmol/mg protein) may be considered as a break-down point that identifies cath-D over-expression. This is also supported by the fact that PR concentrations found in those tumours having "high" cath-D were higher than the concentrations measured in "low" cath-D specimens. In breast cancer, the prognostic outcome of the disease is taken to be reflected, at least partly, in cath-D content [23–25]. Accordingly, cath-D over-expression in larynx could also be clinically useful as an indicator of cancer aggressiveness and this hypotheses will be validated only after an adequate follow up of these patients.

In conclusion, we have shown that both normal and cancerous laryngeal epithelium express cath-D. All of the cancerous samples examined expressed cath-D the relative concentrations of which were significantly higher than in the corresponding non-malignant specimens. Moreover, in the cancerous larynx, cath-D was positively correlated with PR, while no such association was present in the non-malignant cells.

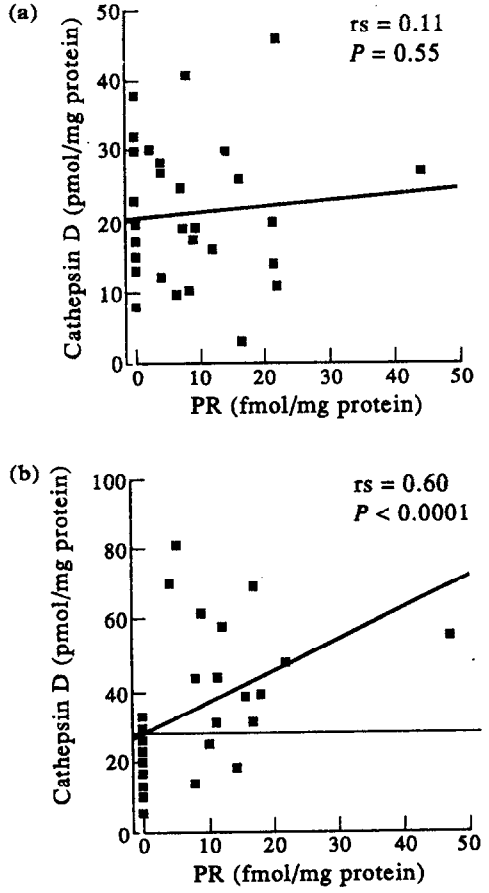


**Fig. 3. Scatterplots of PR and cathepsin D concentration values obtained in non-cancerous (a) and in cancerous (b) larynx samples. *P* values obtained by Spearman's rank correlation. Solid lines represent the linear regression between the two variables. Thin horizontal line (b) indicates the median cathepsin D value (28 pmol/mg protein).**

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