

PH-20

A Novel Tumor Marker for Laryngeal Cancer

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Objective: To determine whether levels of PH-20, a hyaluronidase similar to that found in human sperm, are elevated in laryngeal cancer tissue.

Design: In this case-control study, reverse transcription polymerase chain reaction was used to measure levels of PH-20 messenger RNA in tissue taken from laryngectomy specimens.

Setting: A university medical center.

Patients: We compared tissue samples taken from 11 patients with laryngeal cancer, and from 2 metastatic lymph nodes, with samples of normal, healthy laryngeal tissue and prostate cancer tissue (positive control).

Main Outcome Measure: PH-20 complementary DNA expression as quantified by densitometric analysis.

Results: Expression of PH-20 was significantly higher in nonirradiated laryngeal cancer specimens than in normal laryngeal tissue ($P < .01$). Metastatic lymph nodes also had higher levels of PH-20 expression than did primary laryngeal cancer tissue ($P = .11$) and normal laryngeal tissue ($P < .01$). Irradiated laryngeal cancer specimens had PH-20 levels comparable to normal.

Conclusions: We report the first data on PH-20 expression in laryngeal cancer tissue. PH-20 expression is significantly elevated in primary laryngeal cancer tissue and seems to be even higher in metastatic lesions compared with normal laryngeal tissue. PH-20 may be a useful tumor marker and prognostic tool for laryngeal cancer.

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IN THE United States, laryngeal cancer accounts for approximately 1% of all new cancer diagnoses. Overall 5-year survival for all patients with laryngeal squamous cell carcinoma is approximately 67%. Advanced disease with regional or distant metastasis, however, portends a much worse prognosis.¹ One property that is a prerequisite for metastasis is the ability of the tumor to degrade connective tissue extracellular matrix and basement membrane components. These structures are important barriers for invading tumor cells.² Hyaluronic acid, a proteoglycan in the extracellular matrix, constitutes part of this invasion barrier and is instrumental in maintaining normal tissue architecture. Hyaluronidase is the enzyme responsible for degrading hyaluronic acid into its small angiogenic fragments.³

Although hyaluronidase activities have been reported in animal cells for decades, the molecular identity of hyaluronidase was revealed only recently. A protein (PH-20) required for the binding of guinea pig sperm to the zona pellucida was identified in 1985.⁴ The complementary DNA (cDNA) for

PH-20 was cloned and found to have significant features homologous with the bee venom hyaluronidase gene.⁵ The human PH-20 gene was later cloned and sequenced.⁶ Using this sequence, primers were developed and used in reverse transcription polymerase chain reaction (RT-PCR) to show that a hyaluronidase similar to that found in human sperm is expressed by metastatic human melanoma, colon carcinoma, glioblastoma cell lines, and tumor biopsy specimens from patients with colorectal carcinomas but not by normal colon tissue.³ Recently, it has been shown⁷ that high-grade bladder cancer tissue expresses elevated levels of hyaluronidase compared with normal bladder tissue and that levels of hyaluronidase are also elevated in the urine of patients with high-grade bladder cancer. Hyaluronidase levels in prostate cancer tissue were found to be significantly elevated and to correlate well with the tumor grade.⁸ Based on the results of these investigations, we hypothesized that PH-20 would be expressed at elevated levels in laryngeal carcinoma tissue. We report the first data on PH-20 expression in laryngeal cancer tissue.

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PATIENTS, MATERIALS, AND METHODS

PATIENTS

This project was determined to be exempt research in accordance with federal regulations 45 CFR 46.101(b)(4) (study of existing and unidentified specimens) by the Institutional Committee on Use of Human Subjects, Tulane University School of Medicine, New Orleans, La. Eleven patients underwent total laryngectomy for laryngeal cancer, with 2 patients having had previous irradiation. Tissue specimens were taken from the gross cancer sites and from 2 metastatic lymph nodes from 1 patient. Another patient underwent laryngopharyngectomy for a large tonsillar carcinoma extending into the pyriform sinus. Tissue was taken from the true cords of this specimen and used as a control. Histologically, this control tissue was without tumor; however, it exhibited mild chronic inflammation and mild atypia. A specimen of prostate cancer tissue was used as a positive control.

TISSUE COLLECTION AND PCR PROTOCOL

Tissue samples were collected from the operating room as soon as the laryngectomy specimen was removed from the operative field. They were then snap frozen in liquid nitrogen to halt any degradation of the RNA and stored at -80°C . The RNA was extracted from the tissue by adding 1 mL of Trizol reagent (Gibco BRL, Gaithersburg, Md) and homogenizing the tissue in a "tissue mizer" (Tekmar). The RNA from the homogenate was precipitated by 0.5 mL of isopropyl alcohol after extraction with 0.2 mL of chloroform. The RNA pellet was then washed with 0.5 mL of 70% ethanol, dried, and redissolved in 30 μL of double-distilled water. The concentration of RNA in the solution was determined by spectroscopy. Complementary DNA was then synthesized using Superscript II reverse transcriptase (Gibco BRL). Total RNA, 5 μg , was used in the reverse transcriptase reaction with 5 μL of oligo(dT) at a concentration of 100 $\mu\text{g}/\text{mL}$.

For the PCR, Taq DNA polymerase (Gibco BRL) was used. The basic PCR protocol consisted of $10\times$ PCR buffer

(5 μL), 10-mmol/L deoxynucleotriphosphate mixture (3 μL), 50-mmol/L magnesium chloride (3 μL), primers (P1, P2, P3, and hypoxanthine guanine phosphoribosyl transferase [HPRT]: 2 μL each of 1- $\mu\text{g}/\mu\text{L}$ solution [see the following section]), template cDNA (5 μL), Taq polymerase (0.5 μL of 5-U/ μL solution), and autoclaved distilled water (to 50 μL total volume). Mineral oil was added to prevent evaporation. Using a Robocycler (Stratagene, La Jolla, Calif), samples were initially denatured at 95°C for 5 minutes; 35 cycles of PCR amplification were then performed as follows: denature at 94°C for 1 minute, anneal at 58°C for 1 minute, and extension at 72°C for 2 minutes.

PH-20 BAND VISUALIZATION AND QUANTIFICATION

The PCR products were then separated on 1.5% agarose gel and visualized after ethidium bromide staining (0.4 mg/mL). Two pairs of hyaluronic acid-specific primers were used on each sample (nested primers). The expected product from P1 (5'-CCA TGT TGC TTG ACT CTG AAT TTC A-3') and P3 (5'-CCG AAC TCG ATT GCG CAC ATA GAG T-3') was 759 base pairs (bp). The expected product from P2 (5'-CCA GAA GAT TTC CTT ACA AGA CC-3') and P3 was 504 bp. Both of these products were expressed at equal intensities for each sample (**Figure 1**). An internal control primer set for HPRT (Clontech, Palo Alto, Calif) was used to evaluate the quality and quantity of cDNA for each specimen. The expected product from the HPRT RT-PCR was 467 bp (**Figure 1**). The expression of hyaluronidase was quantified with an imaging densitometer (Bio-Rad, Hercules, Calif) by comparing the densities of each 759 bp product with the HPRT product and the normal, healthy laryngeal tissue.

STATISTICAL ANALYSIS

All data were recorded and entered into a statistical software package (SPSS 6.1.1 for the Macintosh; SPSS Inc, Chicago, Ill). The 2-tailed *t* test was used to assess statistical significance between sample means. Because of the small sample sizes in various groups, the nonparametric Wilcoxon rank sum test was used to confirm all levels of significance.

RESULTS

The expression of PH-20 was significantly higher in non-irradiated laryngeal cancer specimens than in normal laryngeal specimens (**Figure 2**). All specimens were examined by a minimum of 3 runs of RT-PCR to determine the mean densitometric values. Each densitometric value was also normalized to each specimen's HPRT internal control. The mean \pm SD value of PH-20 levels for 9 non-irradiated laryngeal cancer specimens averaged together (147.42 ± 77.98) was compared with the mean PH-20 level of normal laryngeal tissue (58.34 ± 27.13) and was found to be significantly greater ($P < .01$) (**Figure 3**). Mean PH-20 levels for each of the nonirradiated laryngeal cancer specimens are depicted in **Figure 4**.

Mean PH-20 levels from the 2 previously irradiated specimens were not significantly greater than the nor-

mal. Average PH-20 expression in 2 metastatic lymph nodes (mean values, 157.91 ± 27.38 and 102.89 ± 28.83) was higher than in the primary tumor ($P = .11$) and significantly higher than in normal laryngeal tissue ($P < .01$). The **Table** displays the mean PH-20 values and tumor grade for each individual specimen. Specimen 9 seems to have a low PH-20 value even though the tumor was poorly differentiated; however, this lesion metastasized, and the PH-20 level of the metastasizing lesion was higher than that of the primary tumor (**Figure 2**).

COMMENT

In the present study, we measured the levels of PH-20, a hyaluronidase similar to that found in human sperm, in laryngeal cancer specimens by RT-PCR. We hypothesized that PH-20 expression would be elevated in laryngeal cancer tissue based on results of reported stud-

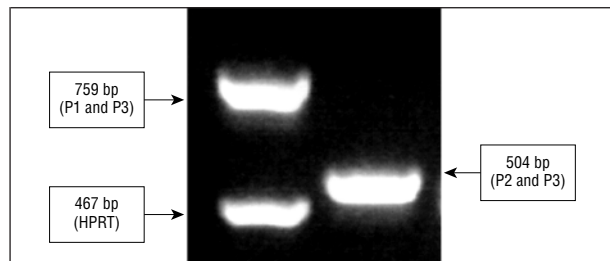


Figure 1. PH-20 complementary DNA expression in laryngeal cancer tissue. HPRT indicates hypoxanthine guanine phosphoribosyl transferase; bp, base pair.

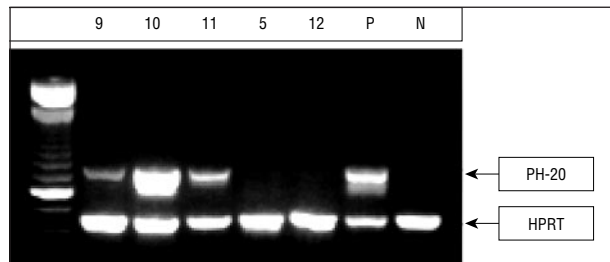


Figure 2. PH-20 complementary DNA expression in cancerous and normal laryngeal tissue. Lane 1 is a DNA ladder. Specimen 9 is from a primary cancer, specimens 10 and 11 are metastatic lymph nodes, specimens 5 and 12 are cancer specimens that had been previously irradiated. HPRT indicates hypoxanthine guanine phosphoribosyl transferase; P, prostate cancer (positive control); and N, normal control.

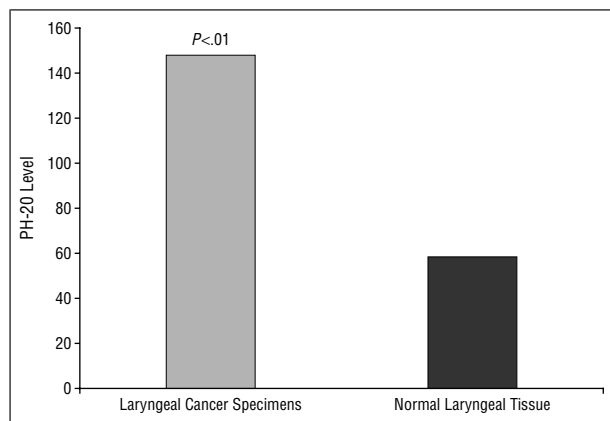


Figure 3. Mean PH-20 levels for 9 nonirradiated laryngeal cancer specimens averaged together compared with the mean PH-20 level of normal, healthy laryngeal tissue.

ies^{3,7,8} with melanoma, colon, bladder, and prostate cancer tissues. Levels of PH-20 were significantly elevated in laryngeal cancer specimens compared with normal laryngeal tissue. Metastatic lesions had even higher levels of PH-20 than did the primary tumor, indicating that elevated PH-20 levels might assist in tumor spread and metastasis. This correlates with observed PH-20 expression in melanoma cell lines. PH-20 RT-PCR products were detected in metastatic melanoma cells but not in primary melanoma cells or in normal melanocyte messenger RNA.³ External beam irradiation reduced the expression of PH-20 in 2 specimens collected from salvage laryngectomies. It would have been interesting to know what the PH-20 levels were in these 2 specimens before radiation exposure to determine whether high levels of PH-20 correlated with radiation therapy failure.

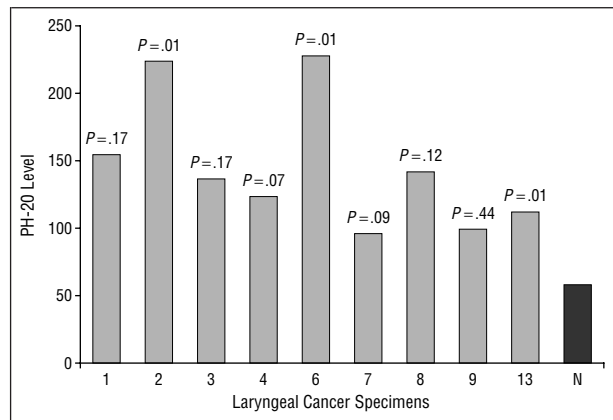


Figure 4. Mean PH-20 levels for each of the nonirradiated laryngeal cancer specimens. N indicates normal.

Characteristics of Patients With Laryngeal Cancer

Patient No.	TNM	Cancer Stage	Tumor Grade	Mean PH-20*
8	T1 N0 M0	I	Moderate	141.76
7	T2 N0 M0	II	Moderate	96.49
3	T2 N0 M0	II	Moderate	136.83
13	T3 N0 M0	III	Moderate	112.48
2	T3 N0 M0	III	Moderate	223.64
4	T2 N0 M0	II	Poor	123.48
1	T3 N0 M0	III	Poor	154.65
9	T2 N2 M0	IV	Poor	99.53
6	T4 N2 M0	IV	Poor	227.93

*Mean PH-20 in normal, healthy tissue is 58.34.

The data presented herein indicate that PH-20 might be a useful tumor marker for laryngeal cancer. Only a small amount of tissue is required for RT-PCR (15-20 mg), which also enhances the usefulness of this test for screening purposes.

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