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Lactate Dehydrogenase 5 Expression in Squamous Cell Head and Neck Cancer Relates to Prognosis following Radical or Postoperative Radiotherapy

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Key Words
Squamous cell head and neck cancer · Lactate dehydrogenase · Hypoxia-inducible factor-1α

Abstract
Objectives: We assessed the expression and the prognostic role of lactate dehydrogenase 5 (LDH5, the major LDH isoenzyme involved in anaerobic glycolysis) in patients with squamous cell head and neck cancer (SCHNC). ***Methods:*** LDH5 was assessed immunohistochemically in whole tissue sections from 141 patients with SCHNC. Of these, 102 were subjected to surgery with (90 patients) or without (12 patients) postoperative radiotherapy (group A), while 39 patients were treated with radical radiotherapy (group B). ***Results:*** Mixed nuclear/cytoplasmic LDH5 expression was detected in 72.5% of group A and 61.5% of group B patients. This was significantly related to T4-stage ($p = 0.04$) and hypoxia-inducible factor-1α (HIF-1α) expression ($p = 0.002$). In group A, high LDH5 was linked with poorer distant metastasis-free survival ($p = 0.01$) and disease-specific overall survival (OS; $p = 0.009$). In multivariate analysis, LDH5 ($p = 0.002$) and HIF-1α ($p = 0.01$) were independently linked with distant metastasis. LDH5 was also linked with death events ($p = 0.005$). In group B, high LDH5 expression was significantly associated with poorer local relapse-free survival ($p = 0.009$) and OS ($p = 0.01$). In multivariate analysis, only T stage was a significant predictor of death events ($p = 0.04$). ***Conclusions:*** LDH5 is highly expressed in SCHNC and is linked with local relapse, survival and distant metastasis, suggesting that LDH5 is a marker of radioresistance and a target for therapeutic interventions.

Introduction

Hypoxia has been considered as a major factor in defining the radioresistance of tumors since the early days of radiobiological research [1]. Indeed, a large number of clinical studies confirmed the ominous prognostic and predictive role of intratumoral oxygen tension in the postradiotherapy outcome of cancer patients, including patients with squamous cell head and neck carcinomas (SCHNC) [2].

More recently, several hypoxia-regulated proteins have been identified, most of them being under the direct control of a group of key transcriptional factors, i.e.

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hypoxia-inducible factors (HIF) [3]. Under hypoxia, the activity of enzymes involved in the degradation of HIF (i.e. prolyl and asparaginyl hydroxylase) is blocked and the concentration of HIF is increased in the cytoplasm and nuclei of cells triggering the transcription of a large number of proteins involved in angiogenesis, anaerobic glycolysis and apoptosis. Apart from the strong biological rationale linking HIF with radioresistance [4], several clinicopathological studies revealed the important predictive role of HIF in patients with SCHNC treated with radiotherapy [5–7].

The induction of anaerobic glycolysis is an important step for normal and cancer cells to survive and obtain energy (ATP) when the Krebs cycle is blocked in the absence of oxygen. Under such conditions pyruvate, the end product of glycolysis, does not enter the Krebs cycle but is converted to lactate, a reaction catalyzed by lactate dehydrogenase (LDH) [8]. LDH is hypoxia-regulated [9] and the LDH-A gene is under the direct transcriptional regulation of HIF-1α [10]. LDH is composed of 4 muscle (M) and/or heart (H) subunits, encoded by 2 distinct genes, LDH-A and LDH-B [8]. There are 5 different isoenzymes of LDH as a result of different subunit combinations. LDH5, which contains the highest number of M subunits, is the enzyme with the highest efficiency in catalyzing the transformation of pyruvate to lactate [8]. A high expression of LDH5 therefore secures a strong anaerobic metabolic activity which, apart from being an index of tissue hypoxia, may also be important in the survival of cells under hypoxic and stressful conditions.

In this study, we examined the expression of LDH5 in a series of SCHNC patients receiving postoperative or radical radiotherapy, aiming to investigate the predictive and prognostic role of a high expression of this hypoxia-regulated enzyme. Moreover, we assessed the association of LDH5 expression with HIF expression predicted by the transcriptional regulation of LDH-A by HIF.

Materials and Methods

The assessment of LDH5 expression was performed in 2 different series of patients. Group A consisted of 102 consecutive cases of SCHNC treated with primary surgery at the Radcliffe Infirmary, Oxford, UK. Of these, 90 cases had also received postoperative radiotherapy (total biological dose of 50 Gy, 2 Gy/fraction, in 5 weeks, directed to the whole neck and supraclavicular areas with 2-D planning) and were used for survival analysis. Group B was comprised of 39 consecutive cases of SCHNC treated with radical accelerated hypofractionated radiotherapy at the University Hospital of Alexandroupolis, Greece. This group received 2.7 Gy/fraction to the neck/tumor and supraclavicular area with a concomitant boost of 0.7 Gy to the tumor for 14 or 15 fractions within 4 weeks, up to a total biological dose for $\alpha/\beta = 4$ Gy of 70–74 Gy, using 3-D-conformal techniques. Patient and disease characteristics are shown in table 1. Tissue specimens containing apparently normal mucosa of tongue and larynx were also included.

Table 1. Patient and disease characteristics

		Group A (n = 102)	Group B (n = 39)
Age	≤60 years	51 (50.0)	15 (38.40)
	>60 years	51 (50.0)	24 (61.60)
Gender	Male	71 (69.6)	32 (82.05)
	Female	31 (30.4)	7 (17.95)
Region	Larynx	21 (20.59)	14 (35.90)
	Oral cavity	31 (30.39)	5 (12.82)
	Oropharynx	35 (34.31)	4 (10.26)
	Hypopharynx	15 (14.71)	3 (7.69)
	Nasopharynx	0 (0)	13 (33.33)
T stage	T1	19 (18.63)	6 (15.38)
	T2	19 (18.63)	4 (10.26)
	T3	19 (18.63)	18 (46.15)
	T4	45 (44.11)	9 (23.08)
	Recurrent	0 (0)	2 (5.13)
N stage	N0	34 (33.34)	22 (56.41)
	N1	20 (19.61)	1 (2.56)
	N2	43 (42.15)	4 (10.26)
	N3	5 (4.90)	10 (25.64)
	Recurrent	0 (0)	2 (5.13)
Grade	1	10 (9.80)	11 (28.21)
	2	42 (41.17)	10 (25.64)
	3	50 (49.03)	18 (46.15)

Values in parentheses denote percentage.

Immunohistochemistry

LDH5 expression was assessed on representative tumor areas mounted on multitissue array slides (group A) or on 3-μm tissue sections of biopsy specimens (group B). The samples were taken from the surgical specimen for group A and from biopsy performed before radiotherapy for group B. The material used was formalin fixed and paraffin embedded and was processed at the time of study. The ab9002 polyclonal antibody raised against human LDH5 (Abcam, Cambridge, UK) was used for immunohistochemistry [11].

A modified streptavidin technique was used as previously reported [12]. Sections were deparaffinized and peroxidase was quenched with methanol and H_2O_2 3% for 15 min. Microwaving for antigen retrieval was used (3 × 5 min). The primary antibody (25 μg/ml) was applied for 75 min. After washing in TBS, the sections were incubated with a secondary antibody (Kwik Kit, Cat. No. 404050; Thermo Shandon, Pittsburgh, Pa., USA) for 15 min and washed again in TBS. Kwik Streptavidin peroxidase reagent was applied for 15 min and the sections were again washed in TBS.

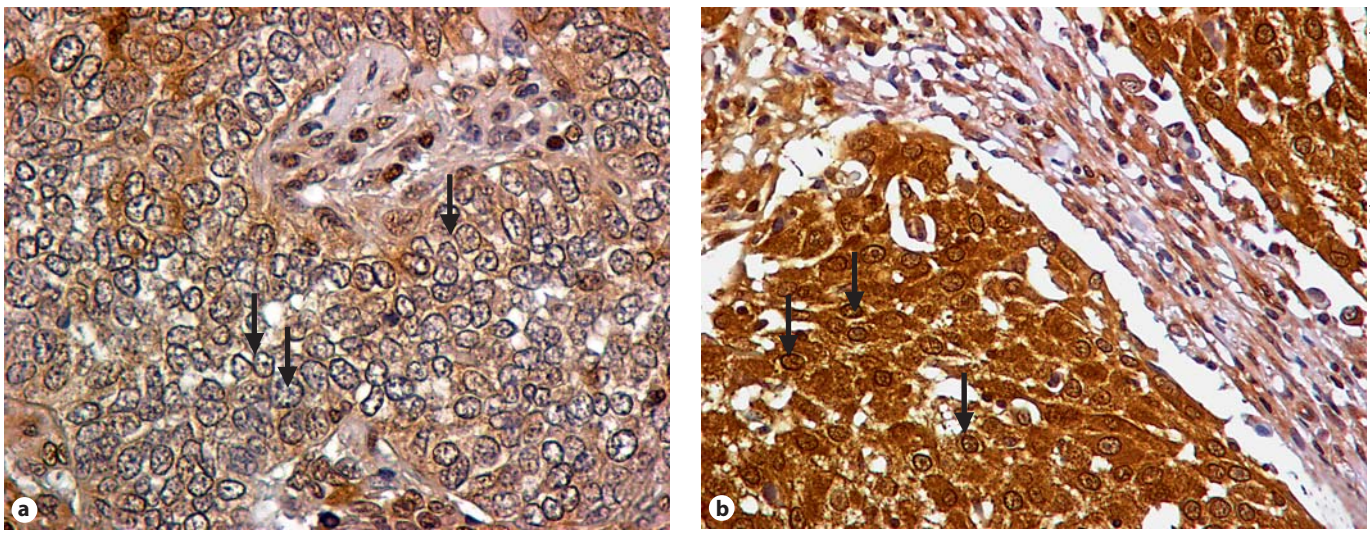

Fig. 1. Immunohistochemical expression patterns of LDH5 in SCHNC. **a** Lack of or sporadic cytoplasmic expression in cancer cells (arrows). **b** Intense cytoplasmic and nuclear expression in cancer cells (arrows).

The color was developed by 15-min incubation with DAB solution and the sections were weakly counterstained with hematoxylin. Appropriate positive and negative controls were used.

The percentage of cancer cells expressing LDH5 was assessed in all optical fields at magnification ×200 (1 on multitissue array slides and 2–4 on biopsy specimens). The percentage of positive cells/optical field was recorded and the mean value of all fields used to obtain the final score for each case. The extent and intensity of cytoplasmic and the extent of nuclear expression of LDH5 was first assessed separately and subsequently combined according to a grading system as previously proposed [7]. Thus, cases with nuclear LDH5 expression in >10% of cancer cells and/or strong cytoplasmic expression in >50% of cancer cells were considered as being of high LDH5 reactivity.

The above-mentioned immunohistochemical technique and scoring system had also been used in the past to assess the expression of HIF-1α and HIF-2α in samples from both groups. Details have previously been published [13, 14]. Data from HIF-1α and HIF-2α expression in tissue samples were used in the present study in comparison with LDH5 data.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 5.0 and the Instat 3.1 package (GraphPad Software, Inc., La Jolla, Calif., USA). Fisher's exact test was used for testing relationships between categorical variables. The unpaired two-tailed t test was used to assess the statistical association between groups of continuous variables. The Kaplan-Meier survival curves were used to assess the impact of various variables on patients: (1) local relapse-free survival (LRFS); (2) distant metastasis-free survival (DMFS); (3) overall relapse-free survival (RFS), and (4) overall disease-specific survival (OS). A Cox proportional hazard model was used to estimate the effect of assessed parameters on death events. $p<0.05$ was considered significant.

Results

LDH5 was expressed in all cancer cases examined. The reactivity ranged from limited and weak to extensive and strong cytoplasmic expression with or without a varying percentage of nuclear localization. Figure 1 shows typical immunostaining patterns of LDH5 expression. LDH5 was not expressed in normal head and neck mucosa.

Using the scoring system reported in the Materials and Methods section, 74/102 cases (72.5%) treated with surgery and postoperative radiotherapy (group A) and 24/39 cases (61.5%) treated with radical radiotherapy (group B) had high LDH5 expression. In group A, a significant association of LDH5 expression with tumor infiltration into the adjacent anatomical structures (T4; $p = 0.04$) was noted. High LDH5 expression was also significantly more frequent in tumors in oropharyngeal location ($p = 0.04$). In group B, there was no association of LDH5 with any of the histological and patient variables (table 2).

Association of LDH5 with HIF Expression

Using the above-mentioned scoring system, 52/90 (57.7%) cases receiving postoperative radiotherapy had high HIF-1α and 35/90 (38.8%) high HIF-2α expression. A significant association of HIF-1α with LDH5 expression was noted as 44/65 (67.6%) cases with high LDH5 expression had high HIF-1α reactivity in contrast to

Table 2. Association of LDH5 expression with patient and disease characteristics

		Group A LDH5			Group B LDH5		
		low (n = 28)	high (n = 74)	p	low (n = 15)	high (n = 24)	p
Age	≤60 years	13	38	0.82	7	8	0.50
	>60 years	15	36		8	16	
Gender	Male	20	51	0.99	12	20	0.99
	Female	8	23		3	4	
Region	Larynx	4	17		5	9	
	Oral cavity	7	24	0.04[a]	2	3	0.47
	Oropharynx	14	21		1	3	
	Hypopharynx	3	12		0	3	
	Nasopharynx	0	0		7	6	
T stage	T1	7	12		3	3	
	T2	5	14	0.05[b]	2	2	0.35
	T3	8	11		6	12	
	T4	8	37		3	6	
	Recurrent	0	0		1	1	
N stage	N0	9	25		8	14	
	N1	8	12	0.54	0	1	0.82
	N2	10	33		2	2	
	N3	1	4		4	6	
	Recurrent	0	0		1	1	
Grade	1	3	7		4	7	
	2	12	30	0.94	3	7	0.74
	3	13	37		8	10	

[a] Comparing oropharynx with all other locations.
[b] Comparing T4 with all other T stages.

8/25 (32%) cases with low LDH5 expression (p = 0.003). There was no significant association of HIF-2α with LDH5.

Using the scoring system applied, 27/39 (69.2%) and 20/39 (51.2%) cases in group B had high HIF-1α and HIF-2α reactivity, respectively. The mean percentage of cancer cells with LDH5 expression was significantly higher in tumors with high HIF-1α reactivity (52 ± 26 vs. 24 ± 24%; p = 0.002). No association between HIF-2α and LDH5 was noted.

Survival Analysis in Group A

Survival analysis was performed in 90/102 cases who received postoperative radiotherapy, excluding patients who received surgery alone. In univariate analysis, advanced N stage (N2, 3 vs. N0, 1) defined a poorer DMFS (p = 0.05), and a trend for poorer LRFS was noted for high-grade (G3 vs. G1, 2) cases (p = 0.11). HIF-1α was significantly related to poorer LRFS (p = 0.04) while no significant prognostic relevance of HIF-2α was noted. No other significant association was noted between histological variables, age and prognostic variables (LRFS, DMFS, RFS and OS).

High LDH5 expression was significantly associated with poorer DMFS (p = 0.01) and with disease-specific OS (p = 0.009). A marginal association with LRFS (p = 0.08) and a significant association with RFS were also noted (p = 0.001) (fig. 2). The analysis of the whole series of 102 cases provided similar results with p = 0.04, 0.04 and 0.005 for LRFS, DMFS and OS, respectively.

In multivariate analysis, LDH5 was an independent variable linked with distant metastasis (p = 0.002; t ratio = 3.13) and death events (p = 0.005; t ratio = 2.87). HIF-1α was also an independent predictor of distant metastasis (p = 0.01; t ratio = 2.47). A marginal (not significant) association of histological grade with local recurrence was also noted (table 3). In a multivariate model excluding HIF-1α and HIF-2α, LDH5 was an indepen-

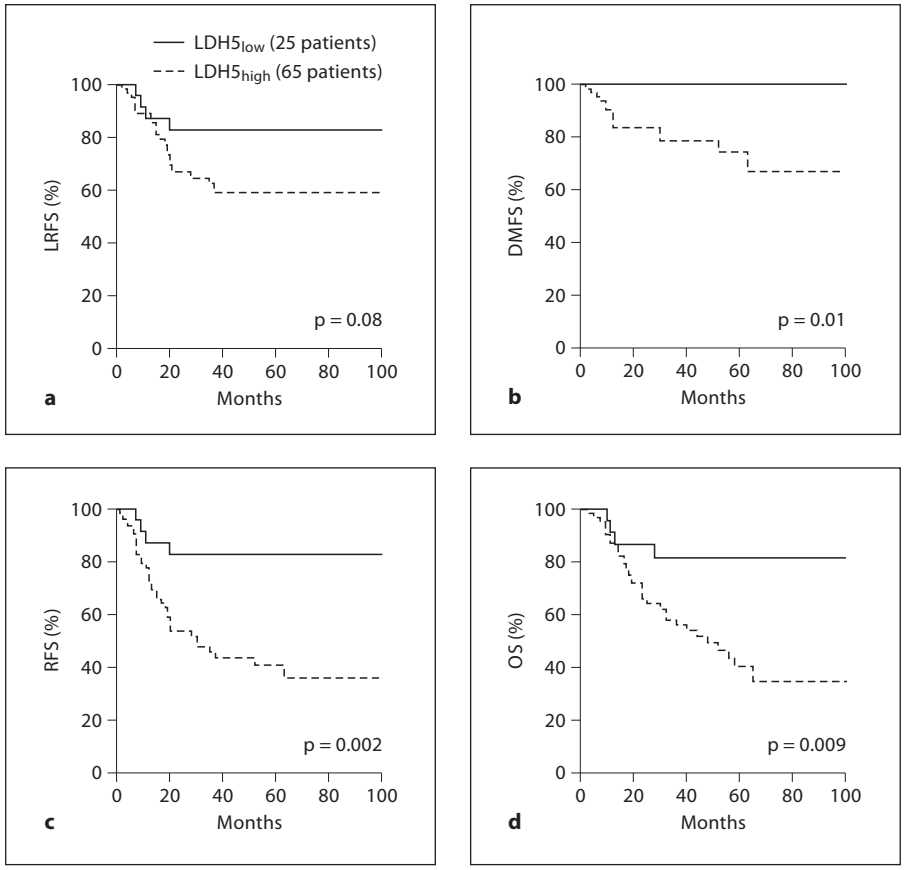

Fig. 2. Kaplan-Meier survival curves of patients treated with surgery and postoperative radiotherapy.

dent variable linked with relapse (p = 0.001; t ratio = 3.35) and death events (p = 0.004; t ratio = 2.93).

Survival Analysis in Group B

In univariate analysis, there was no association of histology and patient characteristics with any of the prognostic variables examined (LRFS, DMFS, RFS and OS). High LDH5 expression was significantly associated with poorer LRFS (p = 0.009) and with OS (p = 0.01) (fig. 3).

In multivariate analysis, LDH5 approached significance in defining death events (p = 0.09; t ratio = 1.71). T stage was an independent prognostic parameter for death events (t ratio = 2.12; p = 0.04) (table 3). In a multivariate model excluding HIF-1α and HIF-2α, T stage, N stage and LDH5 expression were independent prognostic parameters for death events (t ratio = 2.55, p = 0.01; t ratio = 0.04, p = 0.04; t ratio = 2.19, p = 0.03, respectively).

Discussion

LDH is a major enzyme involved in anaerobic glycolysis, a metabolic pathway providing energy to cells under conditions that prevent the usage of pyruvate by the Krebs cycle. Pyruvate is transformed to lactate that is extruded outside the cells through the activity of monocarboxylate transporters [15]. In this way, acidic cellular death is avoided at the expense of an increased extracellular accumulation of lactate which, together with carbonic acid, is responsible for low intratumoral pH [16–18]. Acidic conditions facilitate local invasion and metastasis by stimulating the secretion of specific molecules such as cathepsin and gelatinase and counteract the activity of weakly basic chemotherapeutic agents such as anthracyclins [19, 20]. LDH, being directly regulated by hypoxia and HIFs [10], is also a direct indicator of intratumoral hypoxia, which renders this protein a putative marker of radioresistance.

Indeed, high serum LDH levels have been recognized as an ominous prognostic marker in various human ma-

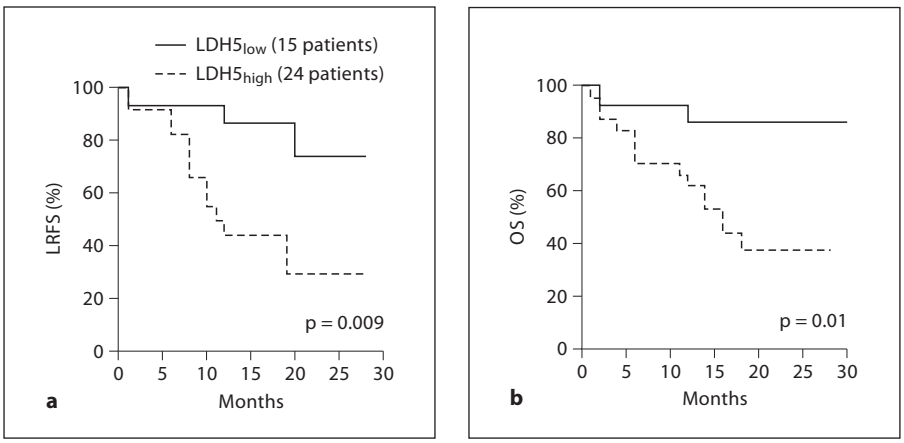

Fig. 3. Kaplan-Meier survival curves of patients treated with radical radiotherapy.

lignancies. Increased LDH serum levels were noted in patients with head and neck cancer (HNC) as early as 1968 [21]. Singh et al. [22] reported a study on HNC patients, in which high LDH serum levels were linked with poor histological differentiation and metastasis. The increased incidence of metastatic disease in nasopharyngeal cancer patients with high serum LDH levels has also been confirmed in subsequent studies [23–25]. High LDH serum levels are also an important factor predicting locoregional control of nasopharyngeal cancer following conformal radiotherapy [26]. Although serum LDH measurement refers to all LDH isoforms, the increased LDH levels in cancer patients should rather be attributed to M-subunit-containing isoenzymes, including LDH5, as LDH-A is the main gene upregulated by hypoxia and LDH1 was found to be equally expressed in normal and cancer cells [12]. Other studies have also focused on the end product of LDH5's catalytic activity, namely lactate. Increased lactate tumor content in nude mice xenografted with different SCHNC lines showed a marked radioresistance [27], a finding clinically confirmed by Brizel et al. [28] in a series of HNC patients treated with postoperative or radical radiotherapy.

Immunohistochemical techniques and antibodies produced by recognizing specific isoenzymes of LDH made it possible to study LDH in cancer cells in paraffin-embedded surgical or bioptic material [11, 12]. LDH5, composed of 5 M subunits entirely encoded by the LDH-A gene, is the most potent LDH isoenzyme for catalyzing the transformation of pyruvate to lactate. Using a specific polyclonal antibody recognizing human LDH5 and a previously established scoring system [7], we investigated the expression of LDH5 in 2 series of SCHNC patients treated with surgery and postoperative radiotherapy or with radical radiotherapy. In both series, strong extensive expression of the enzyme in the cytoplasm and nuclei of cancer cells was a common finding (72.5 and 61.5% of cases). High LDH5 expression was linked with tumor infiltration into adjacent structures (T4), suggesting that LDH5 promotes local invasive abilities compat-

Table 3. Multivariate analysis in groups A and B, using as end points local recurrence, development of distant metastasis and death events

	Group A		Group B	
	t ratio	p	t ratio	p
Local recurrence				
T stage	0.71	0.47	1.26	0.21
N stage	0.26	0.79	1.33	0.19
Grade	1.84	0.07	1.24	0.22
LDH5	1.09	0.27	1.26	0.21
HIF-1α	1.32	0.18	1.50	0.14
HIF-2α	0.30	0.75	1.09	0.28
Distant metastasis				
T stage	1.14	0.25	0.78	0.43
N stage	1.66	0.09	1.14	0.26
Grade	0.72	0.47	1.92	0.06
LDH5	3.13	0.002	1.05	0.29
HIF-1α	2.47	0.01	1.52	0.13
HIF-2α	0.43	0.66	0.95	0.34
Death events				
T stage	0.13	0.89	2.12	0.04
N stage	0.28	0.77	1.42	0.16
Grade	0.96	0.35	0.41	0.68
LDH5	2.87	0.005	1.71	0.09
HIF-1α	0.48	0.63	1.50	0.14
HIF-2α	0.71	0.47	0.19	0.85

ible with the known induction of stromalytic proteins under acidic conditions [19, 20]. A significant association of high LDH5 with death from distant metastasis also stresses the importance of the enzyme in the metastatic process, confirming the close link of high serum LDH with metastasis shown in previous studies [23, 25].

In patients treated with postoperative radiotherapy, high tissue LDH5 levels showed a marginal association with local recurrence, but this association was highly significant in patients treated with radical radiotherapy. This may be a result of the different tumor burden that radiotherapy has to eradicate in the 2 groups, which results in a stronger impact of LDH5 on the locoregional control of tumors treated with radical radiotherapy. This finding stresses the importance of the LDH pathway in radioresistance in accordance with the study by Brizel et al. [28]. High LDH5 tissue levels significantly compromised the OS both in patients treated with postoperative and radical radiotherapy. Although the anaerobic metabolism may per se affect cancer cell sensitivity to radiation, presumably by interfering with apoptotic pathways [29], the increased LDH5 levels may also reflect the upregulated HIF pathway and the overexpression of a multitude of proteins involved in angiogenesis and apoptosis regulation. Thus, LDH5 may be linked with radioresistance by being a marker of hypoxia and hypoxia-regulated pathways. Indeed, high LDH5 expression was significantly linked with high HIF-1α expression, a protein previously shown to be associated with the resistance of SCHNC to radiotherapy [5–7, 13].

Although LDH5 is a key enzyme involved in glycolysis and tumor acidity, implying a direct involvement in the tumor biology of invasion and metastasis, an alternative explanation for the link between LDH5 and the ominous prognostic features would be that LDH5 is simply a marker of hypoxia and, therefore, rather predicts tumor behavior than that it participates in the biology of aggressiveness. This point could be further investigated in experimental radiobiology studies using blockers of LDH activity such as metal bipyridyls and queuine [30–32]. In the current clinical series of patients, the expression of HIF-1α and LDH5, although it significantly coexists, did not overlap, suggesting an LDH5 regulation also via HIF-independent pathways so that HIF and LDH5 are not overlapping markers of tumor biology. In multivariate analysis, both LDH5 and HIF-1α were independent predictors of death events.

Nuclear LDH5 featured in several of the cases analyzed in this study although LDH does not possess a conventional nuclear localization signal. The nuclear pattern has been reported in several studies in colon, lung and other carcinomas [33, 34]. Phosphotyrosine-containing LDH was shown to be localized exclusively in the nuclei of neoplastic cells [35]. In cell lines, LDH-A and GAPDH are components of the OCA-S complex, an S-phase-dependent transactivator of the histone *H2B* gene [36]. The biological significance of nuclear LDH is unknown and it will be of interest to investigate its role in tumor biology.

In conclusion, LDH5 is overexpressed in a large fraction of SCHNC and linked with radioresistance, survival and distant metastasis. Whether increased acidity inferred from lactate production or the close association of LDH5 with an active HIF pathway is the reason for our findings demands further investigation. A prospective study focusing on the serum LDH and tissue LDH5 levels of patients with SCHNC undergoing radiotherapy would be important to establish the clinical value of this marker.

Acknowledgments

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