Long-term Survival Is Linked to Serum LDH and Partly to Tumour LDH-5 in NSCLC

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Abstract. *Background: Lactate formation is up-regulated in tumorous cells by lactate dehydrogenase (LDH). High serum LDH level is linked to many malignancies with poorer survival, but tumour LDH-5 has not been well investigated in non-small cell lung cancer (NSCLC). Patients and Methods: In 89 patients operated on for NSCLC stage I-III, the serum LDH level was assayed and immunohistochemistry for tumour LDH-5 was performed. Impact on long-term survival and correlation was analysed. Results: High serum LDH was associated with poorer survival (p<0.001). No correlation was revealed between serum LDH and the tumour LDH-5. Only in tumours greater than 3 cm were high tumour LDH-5 values associated with higher serum LDH values (p=0.04) and in this subgroup, high tumor LDH-5 was associated with poorer long-term survival (p=0.024). Conclusion: High serum LDH has a negative impact on long-term survival in NSCLC, whereas for tumour LDH-5, this was seen only in a subgroup of patients with larger tumours.*

Lung cancer is a devastating tumour entity, with a five-year mortality of 11-16% (1). The prognosis is more favourable in localized or limited advanced stages (1, 2). For prognosis estimation, different clinical and molecular markers are available, but only a few have any clinical impact on survival (3). The strongest predictor of mortality is the TNM classification (2).

A substantial need for energy of tumour cells is by their increased consumption of glucose. The glycolysis product pyruvate is transformed either by oxidative decarboxylation to acetyl-CoA or by lactate dehydrogenase (LDH) to lactate. These processes are competitive (4). Even under optimal oxygen delivery in malignancies, pyruvate transformation to lactate is up-regulated (5). LDH is composed of four different polypeptide chains (subunits M or H) in which the H subunit catalyses the oxidation of lactate to pyruvate and the M subunit catalyses the reduction of pyruvate to lactate. The higher the proportion of subunit M, the higher the capacity for transformation from pyruvate to lactate. The isozyme LDH-5 is composed of four M subunits and is preferentially expressed in cancer tissue (6, 7). Expression of LDH-5 in tumour cells of non-small cell lung cancer (NSCLC), especially in adenocarcinoma (ADC) or squamous cell carcinoma (SCC), can be a surrogate parameter for independency of oxygen delivery, resistance to therapeutic targets and a molecular marker of prognosis. We analysed the correlation and prognostic impact of serum LDH (sLDH) and tumour LDH-5 (tLDH) levels of NSCLC patients after curative surgical resection.

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Patients and Methods

Patients. For curative major lung resection (lob-, bilob- or pneumectomy), 280 patients were operated on between January 2000 and July 2006 in our institution. Indication for curative resectional surgery was given by an interdisciplinary tumour board. In 89 patients with either ADC or SCC sLDH levels were available immediately before surgical resection. Postoperative staging was carried out according to the Revised Classification of the International Association for the Study of Lung Cancer (2, 8). Approval of the local Ethical Committee was given.

sLDH was measured with standard biochemical assays according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) from blood samples in all patients preoperatively and in 75 patients (84%) postoperatively. A high sLDH value was defined as that being above the sum of the mean plus one standard deviation.

For follow-up, a structured questionnaire was send to oncologists, pneumologists and general practitioners. Long term follow-up is 98.8% complete. For testing the influence of tLDH

expression, long-term survival was analysed with respect to lung cancer-related death. Perioperative death was defined as death within 90 days.

Tumour LDH-5 immunohistochemistry. Formalin-fixed and paraffin-embedded primary lung tumour tissue was selected by the pathologist defining the vital tumour tissue area. For further immunohistochemical staining, a two-step indirect streptavidin-biotin technique was used. In brief, after deparaffinization and rehydration, peroxidase was quenched with 3% H_2O_2 (S2023; Dako, Hamburg, Germany). Washing was carried out with Tris-buffered saline. The primary polyclonal antibody for cytoplasmatic LDH-5 (ab9002; Abcam, Cambridge, UK) derived from sheep immunized against human placenta, was used. The antibody ab6747 against sheep IgG (Abcam, UK) was used as a secondary antibody. After multiple experiments for antibody dilution optimization, the optimal concentration of the ab9002 was found to be 16 μg ml^{-1} (1:500); application was carried out overnight at 4˚C. After labelling the secondary antibody, diaminobenzidine chromogen was applied and sections counterstained with hemalaun.

Evaluation of LDH-5 expression. Detection of vital tumour tissue and cancer cells was using the haematoxylin-eosin-stained sections and only these regions were the target areas. Expression analysis was performed by semi-quantitative scaling with five levels (<10%, 10-25%, 25-50%, 50-75%, >75%, respectively) relative to the signal intensity and frequency. Up to 10 fields per section (mean of 9.4 fields) were analysed at a magnification of ×100 by two investigators; the median was calculated and the section was categorised to a low (under 25th percentile), high (above 75th percentile), medium expression level (25-75 percentile). These levels (low, medium, high) were used for further analysis.

Statistical analysis. Data are expressed for categorical variables as proportions (%) or number (n), and for continuous variables as mean±standard deviation (SD). Differences in categorical variable were analysed by Fisher's exact test. Differences in continuous variables were analysed by independent Student's *t*-test, controlled by the Levene test for equal variances. Results were considered significant if *p*-values were less than 0.05. Logistic regression analysis was performed if parameters reached the significance level in univariate calculation. Long-term follow-up was determined by the Kaplan-Meier method and differences between groups were tested with the log-rank test. Association analysis was performed for correlation of sLDH and tLDH and is given as Kendall tau-rank correlation coefficient T. Statistical modelling and testing were performed with SPSS® statistical software (SPSS 15.0 for Windows Evaluation Version; SPSS Inc., Chicago, Illinois, USA).

Table I. *Characteristics of the patient cohort*.

Characteristic	All patients (n=89)	Long-term survival analysis (n=71)
Age (years)	64.25±9.0	64.29±9.1
Gender		
Male	80 (89.9%)	64 (90.1%)
Female	9 (10.1%)	7 (9.9%)
Cigarettes (packs/year)	39.5±19.6	41.3±20.7
Serum LDH pre (U l^{-1})	192±44	190±43
Serum LDH post (U l^{-1})	218±70	205±61
CRP pre ($mg^{*}l^{-1}$)	29±57	34±63
Haemoglobin pre (g l^{-1})	13.2±1.6	13.4±1.6
Haematocrit pre (%)	40.1±4.6	40.3±4.4
Leucocytes pre (10^3 l^{-1})	8.8±3.6	8.9±3.7
Calcium pre (mmol l^{-1})	2.40±0.18	2.40±0.20
Chemotherapy, pre	13 (14.6%)	11 (15.5%)
Operation type		
Pneumectomy	23 (25.8%)	16 (22.5%)
Lobectomy	57 (64.1%)	48 (67.6%)
Bilobectomy	9 (10.1%)	7 (9.9%)
Lymph nodes resected (n)	19.8±8.8	19.8±8.3
Lymph nodes positive (n)	1.2±2.3	1.2±2.3
Pathological stage (UICC2002)		
IA	22 (24.7%)	17 (23.9%)
IB	24 (27.0%)	22 (31.0%)
IIA	4 (4.5%)	2 (2.8%)
IIB	15 (16.9%)	12 (16.9%)
IIIA	19 (21.3%)	14 (19.7%)
IIIB	5 (5.6%)	4 (5.6%)
Histological type		
ADC	28 (31.5%)	23 (32.4%)
SCC	61 (68.5%)	48 (67.6%)
Grading		
1˚	3 (3.3%)	2 (2.8%)
2˚	61 (68.5%)	48 (67.6%)
3˚	25 (28.1%)	21 (29.6%)
N-Status		
N0	55 (61.8%)	42 (62.0%)
N1	16 (18.0%)	14 (19.7%)
N2	16 (18.0%)	11 (15.5%)
N3	2 (2.2%)	2 (2.8%)
Chemotherapy, adjuvant	15 (16.9%)	14 (19.7%)
Radiotherapy, adjuvant	14 (15.7%)	12 (16.9%)

Continuous values are mean±SD. ADC, Adenocarcinoma; CRP, C-reactive protein; LDH, lactate dehydrogenase; post, postoperative; pre, preoperative; SCC, squamous cell carcinoma.

Results

Clinical characteristics. The mean age was 64.2±9.0 (range 42.2-79.3) years; gender was male in 80 (89.9%) patients. Localized lung cancer (stage I and II) was present in 65 (73%), SCC in 61 (68.5%) patients, ADC was predominate in women (77.8%). Pneumonectomy was performed in 25.8%. The mean serum LDH was 192.9±43.9 U l^{-1} preoperatively and 218.6±70.6 U l^{-1} postoperatively. At the time of follow-up (mean time 2.6±1.9 years), 37 (41.6%) patients were alive. Lung cancer-related death occurred in 34 patients (38.2%). Other causes of death were non-cancer related in 7 patients, a distinct secondary malignancy in 1 patient and unknown in 1 patient; 9 patients died within 90 days postoperatively. These patients were excluded from the analysis of long-term survival for tLDH. Detailed clinical characteristics of patients are presented in Table I.

Assessment of sLDH and tLDH expression. The expression of tLDH was low in 17 patients (19.1%), medium in 54 (60.7%)

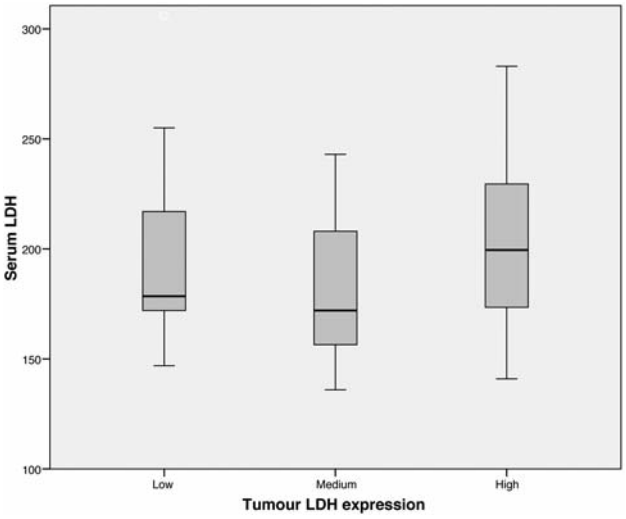

Figure 1. *Serum LDH values (U l^{-1}) according to tumour LDH level, all patients.*

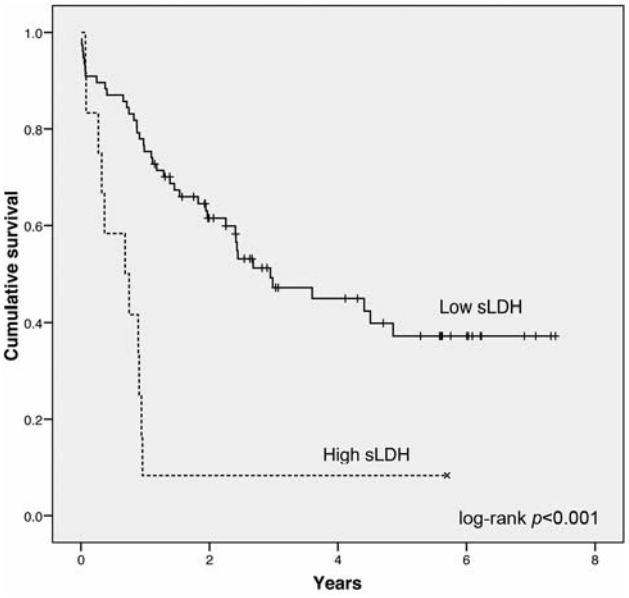

Figure 2. *Overall survival curve discriminated for serum LDH levels.*

and high in 18 (20.2%) patients. There was no significant difference between the tLDH expression and the sLDH level (Figure 1). There was no correlation of sLDH and tLDH expression for any of the stages. Only in UICC stage I (n=39) was a slight positive correlation between the tLDH expression and the sLDH value observed, but this did not reach statistical significance between the groups (T=0.23, p=0.79). In patients with a high tLDH *versus* medium tLDH level, mean sLDH value was increased (229±64 U l^{-1} *versus* 182±34 U l^{-1}, p=0.04), but only for tumours with a diameter more than 3 cm. Neither for UICC stages nor for histological type were any differences between sLDH or tLDH levels noted.

Assessment of sLDH and long-term follow-up. A high serum LDH level was determined as being above 237 U l^{-1} and was seen in 12 patients (13.5%). The mean sLDH value in the high sLDH group was 271.7±35.9 U l^{-1} and for the normal sLDH group was 180.6±30.3 U l^{-1}, p<0.001. The mean overall survival was significantly lower for patients with high sLDH levels *versus* those with normal sLDH level (median 0.68 years, confidence interval, CI: 0.34-1.34 *versus* 2.95 years, CI: 1.72-4.18; p<0.001; Figure 2). Concerning the characteristics listed in Table I, only the preoperative haemoglobin level was significantly lower in the high sLDH group. In multivariate analysis, these factors did not reach statistical significance for long-term survival.

Assessment of tLDH expression and long-term follow-up. The clinical characteristics (see Table I) concerning the groups of tLDH expression levels (low, medium, high) revealed no differences except that the pneumonectomy rate was significantly higher in the low tLDH group (47.1% *versus* 20.8%; p=0.035). No survival difference between the three tLDH group was observed for patients overall. In patients with lobectomy (n=66), there was a decreased survival time for those with high tLDH *versus* medium or low tLDH levels, but without significance (median 1.53 *versus* 2.98 years, log-rank p=0.220). Among the patients with a tumor diameter greater than 3 cm (n=36), the long-term survival was poorer in those with high tLDH level *versus* medium or low tLDH level (median 1.45 years, CI

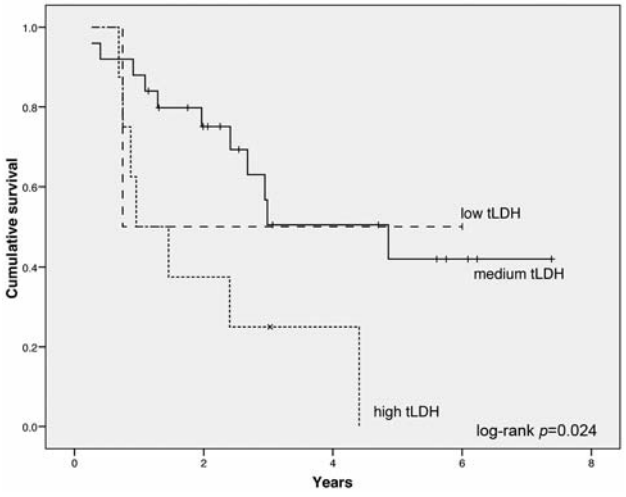

Figure 3. *Survival curve for subgroups of patients with a tumour diameter greater than 3 cm and lobectomy discriminated for tumour LDH expression level.*

0.01-2.91 *versus* 4.86 years, CI 1.49-8.23, log-rank p=0.024; Figure 3). In univariate analysis of this subgroup, the calcium level and sLDH value were higher in the high tLDH group, but this did not reach significance in multivariate analysis (Table II).

Table II. *Multivariate analysis for subgroup of patients with a tumour diameter greater than 3 cm and lobectomy (n=36).*

Characteristic	Odds ratio	95% Confidence interval	p-Value
Calcium level	0.381	0.06-2.58	0.383
sLDH value	1.00	0.98-1.02	0.850
High tLDH level	9.26	0.99-86.14	0.050

LDH, Lactate dehydrogenase; sLDH, serum LDH; tLDH, tumour LDH-5.

Discussion

Due to the increased glycolysis of tumour tissue not only is pyruvate reduction to lactate accelerated but also the oxidative phosphorylation of pyruvate is diminished (9, 10). The increased production of lactate and H^+ ions subsequently reduces the extracellular pH. The acidic environment may increase the ability for invasion (11) and macrophage-mediated angiogenesis (12). This could explain the worse survival of patients with increased sLDH. In our study, high sLDH had a significant negative impact on survival, also in the subgroup analysis. Mostly this has been previously shown only for advanced stages in NSCLC or for small cell lung cancer (13, 14), but it also has an impact on short-term survival after curative resection in NSCLC (15).

Under physiological conditions, the isozyme proportion of LDH-5 in serum is about 10% (16); in cancer, the proportion increases to 20 and 50%, depending on histology (6, 17). In our cohort, we did not find any correlation between the sLDH and tLDH level in patients overall, not even for ADC. In tumours with a diameter >3 cm, higher sLDH values were found in patients with a high tLDH expression level. This could be explained by a higher need for aerobic glycolysis in large tumours (18). Nevertheless, the proportion of LDH-4 and LDH-5 is increased in the sera of cancer patients compared to healthy humans (17). In a mouse model with a SCC line, a correlation of tumour volume and sLDH was not found, on the other hand, tLDH did increase with tumour volume (19). In a recent study, a correlation between sLDH and tLDH was noted (20), but a rather low number of patients was investigated.

In colorectal cancer, there was a negative impact of high tLDH expression levels (18, 21). The impact of tLDH level on survival in NSCLC was questioned in one study, but in multivariate analysis it reached no significance (20). Overall survival in our cohort was not significantly different between the groups with different tLDH expression; only in the subgroup analysis for large tumours in patients with performed lobectomies did a high tLDH level have a negative impact on survival. The findings from this subgroup confirm those of Koukourakis *et al.* (20). Because pneumectomy results in a higher mortality rate in the short and long term (22, 23), these patients have a negative influence on survival analysis.

The incongruent results compared to previous reports may have following causes. (i) The subunit M of LDH was found as the main subunit in cancer cells but there is a proportion of up to 25% of H subunits, subsequently total LDH activity and lactate production is equivalently high (6). In serum electrophoresis of patients with malignancy, an additional LDH-1ex was found which was correlated with a higher range of LDH-4 and LDH-5 (17). Tumour LDH-5 was also found to be as a cofactor with hypoxia-inducible factor I and carbonic anhydrase 9 (20). These isozymes are not dedected by LDH-5 immunohistochemistry. (ii) Hypoxia is postulated as a trigger for aerobic glycolysis with an increase of lactate production (24), but failed to increase tLDH in SCC (19); latent factors may exist which trigger energy delivery in tumor cells. (iii) Suppression of LDH-A, which encodes the M subunit, in a mouse model resulted in a higher rate of oxidative phosphorylation with reduction of proliferation activity and tumour cell growth (25), but metastatic behavior was not evaluated. (iv) Expression of tLDH was very inhomogeneous in the vital tumour compartments. We found a high variability of expression levels in the same tumour, which may indicate different levels of activity levels within the same tumour.

In conclusion, we found a high sLDH level had a statistically significant negative impact on survival in patients with adenocarcinoma or squamous cell lung cancer. No correlation of sLDH and tLDH levels was revealed; and high tLDH expression levels only have a negative impact on survival of the subgroup of patients with a tumour diameter larger than 3 cm.

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