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ORIGINAL ARTICLE

Quantitative Assessment of Expression of Lactate Dehydrogenase and its Isoforms 3 and 4 may Serve as Useful Indicators of Progression of Gallbladder Cancer: A Pilot Study

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Abstract We have studied the expression of lactate dehydrogenase and its isoforms in gall bladder cancer, cholelithiasis and chronic cholecystitis. Quantitative and qualitative assays of lactate dehydrogenase and its various isoforms were carried out in the blood sera of patients and healthy controls along with parallel estimation of various liver function test enzymes. Statistical analysis was done using the software Graph Pad Prism. Significantly high expression of lactate dehydrogenase along with alkaline phosphatase and total bilirubin ($P \leq 0.05$) was observed in all the three clinical conditions as compared to controls. LDH showed an increasing trend from stage I to stage IV of GBC indicating a significant positive association with the disease progression. The levels of LDH 3 and 4 isoforms appeared significantly more elevated in GBC than cholelithiasis or chronic cholecystitis. We suggest that a careful estimation of total LDH and its isoforms 3 and 4 alone or along with alkaline phosphatase and total bilirubin during different clinical stages, like chronic cholecystitis, cholelithiasis and GBC, may prove to be a potentially useful biomarker in the prognostic management of gall bladder diseases, specifically GBC.

Keywords Gallbladder cancer · Lactate dehydrogenase · Cholelithiasis · Chronic cholecystitis

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Introduction

Gall bladder cancer (GBC) was first described in 1777 by DeStoll [1]. It is reported to be the most common malignancy of the billiary tract and the seventh most common gastrointestinal cancer. In India, the highest GBC incidence rate is reported in women (21.5/100,000) [2]. Our study in North Central India also showed women to be more prone to GBC than males (F:M = 2.314) [3]. Large number of studies have been carried out to identify different genetic and non-genetic risk factors for GBC, including epigenetic alterations, expression profiling, single nucleotide polymorphisms (SNPs), loss of heterozygosity (LOH), biochemical studies, dietary habits, environmental factors, family history, etc., but the problem of diagnosis, prognosis or therapy of GBC has still remained an enigma, yet to be clearly understood. By the time gall bladder cancer is diagnosed, resection remains the only way to rescue. About 32% 5 year survival rate is reported for lesions confined to the gallbladder mucosa, but only 10% 1 year survival rate for more advanced stages [4].

Lactate dehydrogenase (LDH) is an important enzyme catalyzing the reversible transformation of pyruvate to lactate [5]. Under aerobic conditions, pyruvate, the end product of glycolysis, enters the Krebs (citric acid) cycle via acetyl-coenzyme A, providing energy in the form of adenosine triphosphate (ATP) and dihydronicotinamide adenine dinucleotide (NADH) [6]. In mammalian cells, LDH is

expressed into five common isoforms (LDH 1, 2, 3, 4 and 5), generally expressed in a tissue specific manner [7–9]. The tetrameric isoforms are produced by the combination of two polypeptide chains encoded by two separate genes (i.e., M = muscle type and H = heart type) in different proportions (4 M or LDH5, 3M1H or LDH 4, 2M2H or LDH 3, 1M3H or LDH2 and 4H or LDH1). The LDH isozymes with more of M-chain than H, can catalyze the conversion of pyruvate to lactate more efficiently. Increase in H-chain favors the conversion of pyruvate to acetyl coenzyme A that enters the Citric acid cycle. Previous studies have reported differential expression of various LDH isoforms in specific cancers. For example, LDH1 (LDH B) was found significantly up-regulated in lung cancer [10], while LDH5 (LDH A) is recently shown to be involved in both tumor initiation as well as for its maintenance [7]. LDH 5 or LDH A is found to be related with colorectal cancer metastasis and prostate cancer [11, 12]. The increased expression of LDH 5 in various cancers, melanoma, leukemia, testicular cancer and certain solid tumors, led to suggest it to be a useful prognostic marker in these pathogenesis [13–16].

In view of the characteristics of LDH and/or its isoforms as indicators of tissue damage and the widely suggested usefulness as prognostic marker in various diseases, including cancer as mentioned above, we thought to evaluate the diagnostic and prognostic value of LDH and its various isoforms alone, or in combination with other biochemical or clinical parameters in the progression of GBC as well as other gall bladder diseases, e.g., cholelithiasis and chronic cholecystitis. To the best of our knowledge, no such study has yet been carried out in GBC or any other gall bladder diseases. Our study revealed that the level of LDH-3 and 4 isoforms are elevated in gallbladder cancer. This can be further investigated for its usefulness in the prognostic management of GBC during disease progression because LDH, in general, or its specific isoforms, increases with the extent of tissue damage in malignancy.

Materials and Methods

Subject Selection and Sample Collection

The GBC patients referred at the Cancer Hospital and Research Institute, Gwalior [Regional Cancer Centre, Code-08, Ministry of Health and Family Welfare (MOHFW), Government of India] during the period 2007–2009 were taken for the study. The study was approved by the Institutional Ethics Committee and an informed consent was taken from each participant. Blood sample was collected on diagnosis when the patient was admitted in the hospital and before the start of chemotherapy. The patients with metabolic disorders, cardiovascular diseases, respiratory problems and with any kind of infections were excluded in the study. Only histopathologically confirmed cases of GBC with stones, non cancerous gallstone and chronic cholecystitis were included. Gallstones with inflammatory diseases were also excluded. The reports of CT scan, MRI, USG, X-ray and FNAC were analyzed by the pathologist and surgeon on-site. The surgically removed tissue samples collected were then classified into different stages of GBC (I, II, III and IV) according to the American Joint Committee on Cancer, 2007 (AJCC) following histopatholgical investigations. For the study of LDH, 50 histopathologically well identified GBC, 15 cholelithiasis and 15 chronic cholecystitis patients and 50 age and sex matched healthy individuals as controls were selected. All the disease cases included in the present study were primarily admitted as suspected GBC cases, but after histo-pathological investigation, they were categorized into either cholelithiasis, chronic cholecystitis or GBC. The demographic profile of the subjects is given in Table 1. About 5 ml of blood samples were collected from each participant in a sterilized vial and allowed to clot, followed by centrifugation at 10,000 rpm. The serum was separated and stored at −20°C until further use.

Quantitative Estimation of Total LDH (Colorimetry Method)

The method of detection of LDH in blood serum was same as described by Bach et al. and Sharma et al. [17–19]. 50 µl of serum was mixed in phosphate buffer (pH 7.5) in a clean and sterile test tube. 80 µl of NADH (1 µg/µl) and 0.75 mM sodium pyruvate were added to the above. The solution was incubated at 37°C for 30 min. 80 µl of 2,4-dinitro-phenyl hydrazine was then added to the above solution and left at room temperature for another 15 min. Finally, the reaction was stopped by adding 40 µl of 3.5 M NaOH solution. The spectrophotometric absorbance reading was taken at 450 nm three times with a gap of 1 min each per reading per sample. An average of three readings was taken for the final calculation.

Analysis of LDH Isoforms by Native PAGE and Densitometric Analysis

An equivalent amount of serum (50 IU or 250 µg of LDH) was electrophoresed on 6% native polyacrylamide gel at 4°C. The activity staining of lactate dehydrogenase (LDH) on the gel was carried out by adding sodium lactate, PMS and NBT as substrates, onto the gel followed by incubation at 37°C till dark brown bands appeared [18]. The bands representing different isoforms were densitometrically analyzed using the software, UVI band provided with the UVI-tech gel documentation system (UK).

Table 1 Comparative demographic profile of gallbladder disease cases and control subjects

S. no.	Demographic parameters	Clinical status			
		GBC ($n = 50$)	Cholelithiasis ($n = 15$)	Chronic cholecystitis ($n = 15$)	Controls ($n = 50$)
1.	Age group (years)				
	31–40	10	2	3	14
	41–50	15	8	7	19
	51–60	13	2	4	10
	61–70	12	3	1	7
	Mean (± SD)	52.74 (±11.6)	45.47 (±15.9)	46.67 (±13.4)	47.44 (±11.3)
2.	Sex				
	Male (%)	13 (26)	5 (33.33)	3 (20)	17 (34)
	Female (%)	37 (74)	10 (66.67)	12 (80)	33 (66)
	Total (%)	50 (100)	15 (100)	15 (100)	50 (100)
3.	Habitat				
	Urban (%)	16 (32)	6 (40)	7 (46.67)	22 (44)
	Rural (%)	34 (68)	9 (60)	8 (53.33)	28 (56)
	Total (%)	50 (100)	15 (100)	15 (100)	50 (100)
4.	Food habit				
	Vegetarian (%)	34 (68)	10 (66.67)	9 (60)	26 (52)
	Non-vegetarian (%)	16 (32)	05 (33.33)	6 (40)	24 (48)
	Total (%)	50 (100)	15 (100)	15 (100)	50 (100)
5.	Occupation				
	Farmer (%)	22 (44)	4 (26.66)	6 (40)	15 (30)
	Govt. employee (%)	5 (10)	2 (13.33)	3 (20)	4 (8)
	Business (%)	5 (10)	5 (33.33)	1 (6.66)	7 (14)
	Housewives (%)	18 (36)	4 (26.66)	5 (33.33)	24 (48)
	Total (%)	50 (100)	15 (100)	15 (100)	50 (100)
6.	Education				
	Illiterate (%)	23 (46)	3 (20)	5 (33.33)	15 (30)
	Primary (%)	11 (22)	6 (40)	6 (40)	10 (20)
	Secondary (%)	9 (18)	2 (13.33)	2 (13.33)	13 (26)
	College (%)	7 (14)	4 (26.66)	2 (13.33)	12 (24)
	Total (%)	50 (100)	15 (100)	15 (100)	50 (100)
7.	Smoking habit				
	Smokers (%)	19 (38)	6 (40)	5 (33.33)	27 (54)
	Non-smokers (%)	31 (62)	9 (60)	10 (66.67)	23 (46)
	Total (%)	50 (100)	15 (100)	15 (100)	50 (100)

Liver Function Test Enzyme (LFT) Analysis

Liver function tests were performed using Bayer's Express Plus fully auto analyzer. The enzymes Alkaline Phosphatase (ALP), Serum Glutamate Pyruvic Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT) and Total Bilirubin (TB) were estimated following modified methods (as per kit manufacture's protocol) of King and Armstrong [20], Reitman and Frankel [21] and Malloy and Evelyn [22], respectively.

Statistical Analysis

The *P* values were calculated by comparing the mean value of each clinical parameter followed by paired *t*-test (two-tailed). The concentrations of ALP, SGPT, SGOT, TB and LDH (IU/l) were presented as mean, median, 50th percentile and 70th percentile. The statistical comparisons of GBC, cholelithiasis and chronic cholecystitis with normal subjects were made using Mann–Whitney's U test. The *P*-values less than or equal to 0.05, after Boneferroni correction, were considered statistically significant. Statistical

calculations were carried out using Graph Pad Prism 5 (San Diego California USA, and www.graphpad.com) [23].

Results

Histopathological Classification

The post surgical histological investigations were carried out at the hospital on 50 GBC cases. These were differentiated into various stages of adenocarcinomas according to TNM-AJCC classifications, USA, as Stage I-10% (05 of 50); Stage II-30% (15 of 50); Stage III-40% (24 of 50) and Stage IV-12% (06 of 50).

Blood Biochemistry

As compared to normal controls, a progressive increase in the serum level of liver function enzymes was observed in gallbladder cancer. The average content of ALP estimated in GBC stages I, II, III and IV were 465.33, 488.20, 670 and 918.50 IU/l, respectively. The total bilirubin was found to be 7.0, 9.6, 10.8 and 14.8 mg/dl, respectively in the four progressive stages (I–IV) of GBC. The mean values of ALP in cholelithiasis and chronic cholecystitis were 378.75 and 363.33 IU/l, while that of TB were 7 and 4 mg/dl, respectively. The average contents of other liver function test enzymes and LDH in GBC, cholelithiasis and chronic cholecystitis is shown in Table 2.

LDH Assay

Spectrophotometric assay showed significant variation in total LDH content (IU/l) in the patients' blood sera. Among GBCs, those at stage IV, showed an aggressive LDH level than those at early stages. Stage IV patients showed a value of 890.56 ± 45.22 (IU/l) (mean ± SD) ($P = 0.05$), while stages III, II and I showed a gradient of decrease in the values, from 624.50 ± 51.50 (IU/l) (mean ± SD) ($P = 0.15$), 530.25 ± 30.33 (IU/l) (mean ± SD) ($P = 0.25$) to 460.33 ± 25.10 (IU/l) (mean ± SD) ($P = 0.05$), respectively. The levels of mean LDH were recorded comparatively lower in cholelithiasis [$(484.55 \pm 10.45$ (IU/l) (mean ± SD) ($P = 0.05$)] and chronic cholecystitis [(475.63 ± 12.25) (IU/l) (mean ± SD) ($P = 0.05$)]. The average value of LDH in healthy controls was found to be significantly lower [(197.33 ± 12.5) (IU/l) (mean ± SD) ($P = 0.012$)] (see Table 2).

Native PAGE and Densitometric Analysis of the LDH Isoforms

Out of the five isoforms, the levels of LDH 3 and 4 were found elevated among the patients, more in the GBC than in cholelithiasis or chronic cholecystitis. The expression of isoforms 1, 2 and 5 were comparatively low (Fig. 1). The ALP and TB levels were consistently elevated along with LDH in GBC and cholelithiasis ($P = 0.05$) as compared to chronic cholecystitis. From the correlation study a positive association was observed between the increased levels of total LDH, ALP and TB in both GBC and cholelithiasis ($P = 0.05$) (Figs. 2, 3, 4, 5).

The densitometry analysis (using UVI-band software) of different LDH isoforms (or bands) revealed the elevated values (in arbitrary units) of LDH 3 and LDH 4 consistently in GBC (Fig. 2a, b), as compared to Cholelithiasis (Fig. 3a, b), chronic choelcystitis (Fig. 4a, b) and healthy controls (Fig. 5a, b). A comparative expression profile of LDH, ALP, SGPT and SGOT in the three clinical conditions showed the higher expression of LDH and ALP at the advanced stages of GBC than cholelithiasis, chronic cholecystitis and healthy controls (Fig. 6).

Discussion

Gallbladder cancer is diagnosed late and surgical resection is the only means to rescue. The survival rate is less than 5 years after surgical removal in advanced cases. During GBC progression the malignant cells secrete various enzymes, including LDH, both from cancerous as well as adjacent non-cancerous cells, resulting from inflammatory reactions. The total LDH or the specific LDH isoform/s, which are released in the blood stream draining from the gall bladder tissue, is likely to vary in concentration in the blood sera during different clinical stages or progression of GBC. Careful qualitative and quantitative analysis of this enzyme during different clinical conditions of gall bladder, including cholelithiasis, chronic cholecystitis and GBC, may be useful as an indicator of severity of the disease, and can also help in their prognostic management.

The molecular mechanisms, which play significant role in transforming normal gall bladder tissue into tumor, may be similar to other solid tumors as well. Thus, in GBC too, the tendency for conversion of pyruvate into lactate by LDH should be favored similarly as reported in endometrial cancer [24]. In liver and skeletal muscle, LDH 4 and 5 were observed significantly expressed as compared to LDH 1, 2 and 3. The LDH isoenzymes (or its isoforms) are internally synthesized and are released to extracellular microenvironment en route to circulation through damaged cell membrane in the tissues or through inflamed cells [25]. As the intensity of cellular stress increases in cholelithiasis, chronic cholecystitis or gall bladder carcinogenesis, the level of secretion of the cytoplasmic LDH isoenzymes may also go up. Due to poor oxygen availability the affected cells may generate at least the minimal required energy

Table 2 Comparative statistical values of LDH, ALP, SGPT, SGOT and TB in clinical stages of all Gallbladder diseases (i.e., GBC stages I, II, III, IV, Cholelithiasis and Chronic Cholecystitis)

Statistical analysis	Controls	GBC				Cholelithiasis	Chronic cholecystitis
		I	II	III	IV		
(A) Lactate dehydrogenase							
Mean	197.33	460.33	530.25	624.50	890.56	484.55	475.63
SD (±)	12.5	25.10	30.33	51.50	45.22	35.12	46.50
Median	210.25	445.15	520.17	600.10	850.30	440.50	474.50
50th percentile	220.50	450.25	522.50	600.25	855.25	450.00	478.50
70th percentile	325.25	658.12	630.12	720.01	950.45	560.12	590.24
Mann–Whitney mean rank test	102.50	225.03	250.65	300.23	405.45	239.50	250.50
P value (after boneforreni correction)	0.05	0.05	0.25	0.15	0.05	0.05	0.05
(B) Alkaline phosphatase							
Mean		465.3	488.20	670.00	918.50	378.75	363.33
SD (±)		35.33	37.50	37.23	35.29	25.34	30.25
Median		451.25	480.50	660.23	920.00	350.00	358.50
50th percentile		457.35	478.50	650.25	910.50	352.50	360.00
70th percentile		576.50	545.00	850.00	1450.00	450.00	480.25
Mann–Whitney mean rank test		295.23	250.25	320.50	450.25	165.00	135.50
P value (after boneforreni correction)		0.025	0.05	0.06	0.05	0.05	0.05
(C) Serum glutamic pyruvic transaminase							
Mean		178.20	210.00	236.33	257.00	152.50	142.25
SD (±)		38.56	37.56	33.00	31.50	24.13	23.95
Median		170.25	205.50	240.50	250.25	150.35	145.35
50th percentile		170.75	201.50	245.00	255.12	151.35	148.56
70th percentile		275.13	295.23	350.00	345.15	265.00	250.50
Mann–Whitney mean rank test		120.25	158.80	110.50	120.55	80.12	70.95
P value (after boneforreni correction)		0.085	0.38	0.43	0.28	0.38	0.439
(D) Serum glutamic oxaloacetic transaminase							
Mean		110.00	125.32	130.50	155.52	137.33	145.23
SD (±)		42.00	29.00	25.00	20.00	21.25	21.25
Median		107.25	120.20	128.33	158.33	140.50	140.30
50th percentile		105.25	125.25	130.50	156.33	142.00	142.12
70th percentile		204.95	205.17	235.00	270.25	250.75	255.50
Mann–Whitney mean rank test		105.5	65.50	75.00	81.50	75.25	78.45
P value (after boneforreni correction)		0.05	0.25	0.28	0.05	0.23	0.12
(E) Total bilirubin							
Mean		07.00	9.6	10.8	14.8	07.00	4.00
SD (±)		2.25	2.1	2.33	2.5	1.5	1.2
Median		07.50	9.0	10.00	12.5	8.50	5.5
50th percentile		07.00	9.5	10.50	12.00	8.00	6.0
70th percentile		10.50	18.2	18.25	19.56	15.80	12.56
Mann–Whitney mean rank test		07.50	4.3	5.20	6.50	5.50	3.60
P value (after boneforreni correction)		0.05	0.05	0.05	0.05	0.05	0.05

(ATP) to maintain cellular metabolism through anaerobic glycolysis (Warburg effect) [26]. The rapidly proliferating populations of neoplastic cells depend largely upon the glycolytic mechanisms for deriving the energy with high requirement of LDH to generate NAD+ and ATP for glycolysis. The factors involved in influencing the quantity of tumor enzymes produced or released by tumour cells may be considered as the likely risk factors, increasing the cellular burden of tumors at a given time [15]. Our findings provide strong support to this. But, the underlying factor

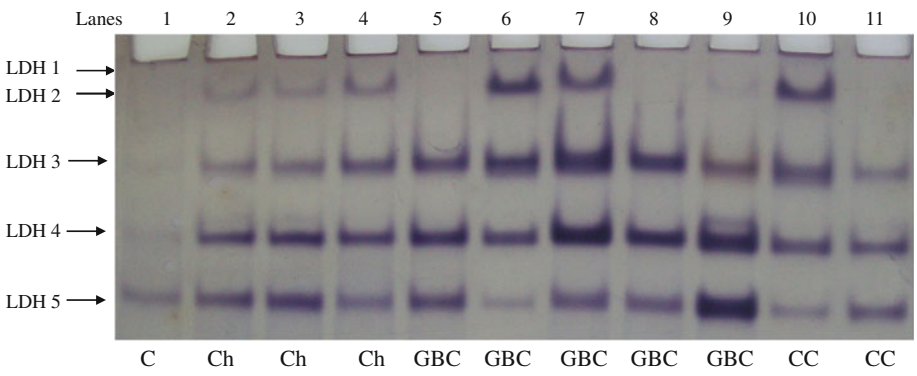
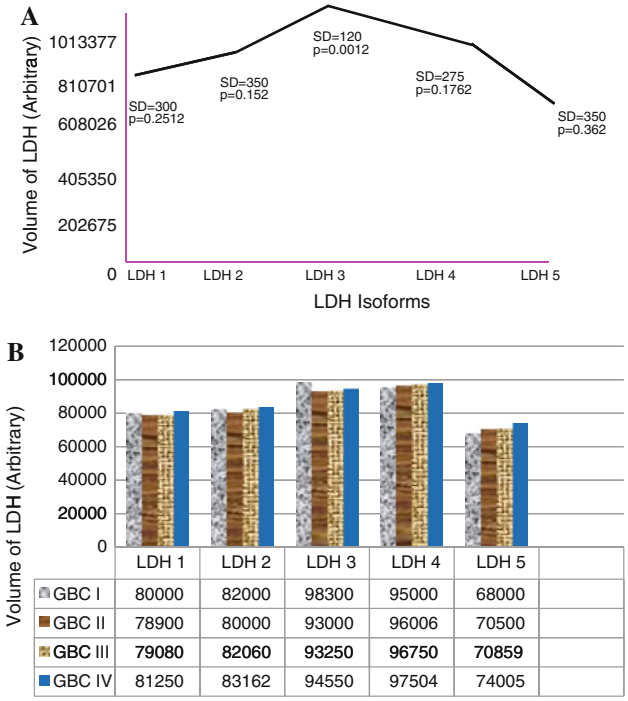


Fig. 1 Expression pattern of LDH isoforms in 6% native PAGE. *Lane 1* represents control (*C*), *Lanes 2–4*, cholelithiasis (*Ch*), *Lanes 5–9*, gallbladder cancer (*GBC*) and *Lanes 10–11*, chronic cholecystitis (*CC*)



	LDH 1	LDH 2	LDH 3	LDH 4	LDH 5
GBC I	80000	82000	98300	95000	68000
GBC II	78900	80000	93000	96006	70500
GBC III	**79080**	**82060**	**93250**	**96750**	**70859**
GBC IV	81250	83162	94550	97504	74005

Fig. 2 Densitometry of LDH isoforms in gallbladder cancer (in arbitrary unit). Line graph in **a** represents relative band density of each isoform (volume); histogram in **b** shows comparison of LDH isoforms in different stages

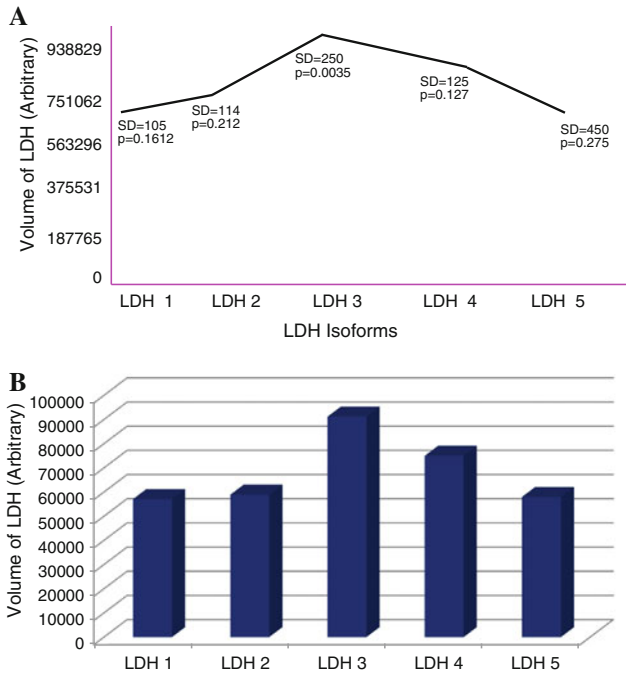


Fig. 3 Densitometry of LDH isoforms in cholelithiasis (in arbitrary unit). Line graph in **a** represent relative band density of each isoform (volume); histogram in **b** shows comparison of LDH isoforms

inducing the release of these enzymes still remains to be fully elucidated.

While total LDH content may not be very specific to a particular tissue type, the differential expression pattern of its isoforms is more useful in determining the cellular metabolic status of the diseased tissue. In different types of cancers, increased expression of different LDH isoforms, for example, LDH 1(LDHB) in lung cancer [10] and LDH 5 in colorectal, melanoma, breast cancer and endometrial cancers [11, 24, 27, 28], are found correlated with the progressive stages of malignancies. The present observation has shown significantly elevated expression of serum LDH 3 and 4 in GBC as compared to other isoforms. It appears interesting as these isoforms have earlier been shown specific to lung tissues (LDH 3) or liver (LDH 4) [8, 18, 19]. In GBC, cholelithiasis or chronic cholecystitis, the increased expression of LDH 3 and 4 may be a consequence of the obstructions in the release of bile juice or disturbance in the cellular functions of inflamed tissue and are not specific to only lungs or liver as reported earlier [8, 18, 19]. So far, no similar or related observation has yet been reported in the context of gall bladder diseases. The generally accepted major risk factors of gall bladder cancer include cholelithiasis and chronic cholecystitis. The epidemiological studies from our lab and others also suggest cholelithiasis as a major risk factor of gall bladder cancer [3, 4]. The presence of stone in cholelithiatic and inflammation in chronic cholecystitic gall bladder might potentially cause disturbance in the cellular glycolytic pathway.

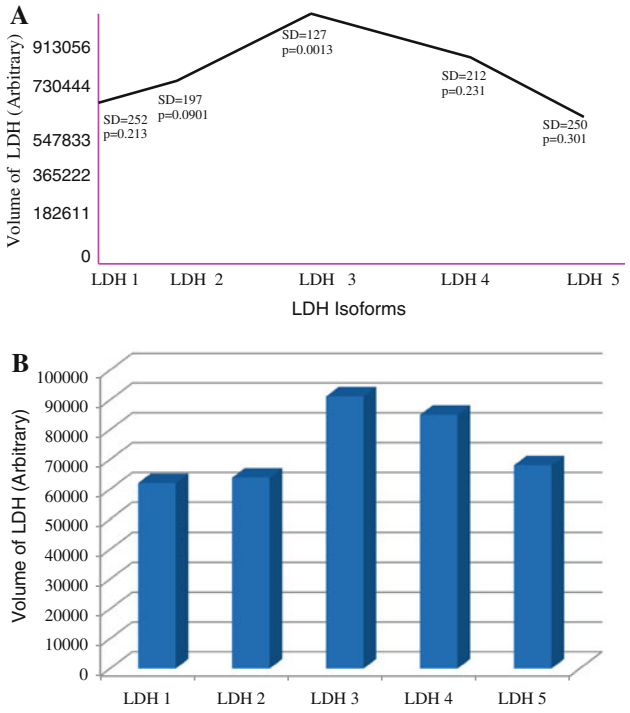


Fig. 4 Densitometry of LDH isoforms in chronic cholecystitis (in arbitrary unit). Line graph in **a** represent relative band density of each isoform (volume); histogram in **b** shows comparison of LDH isoforms

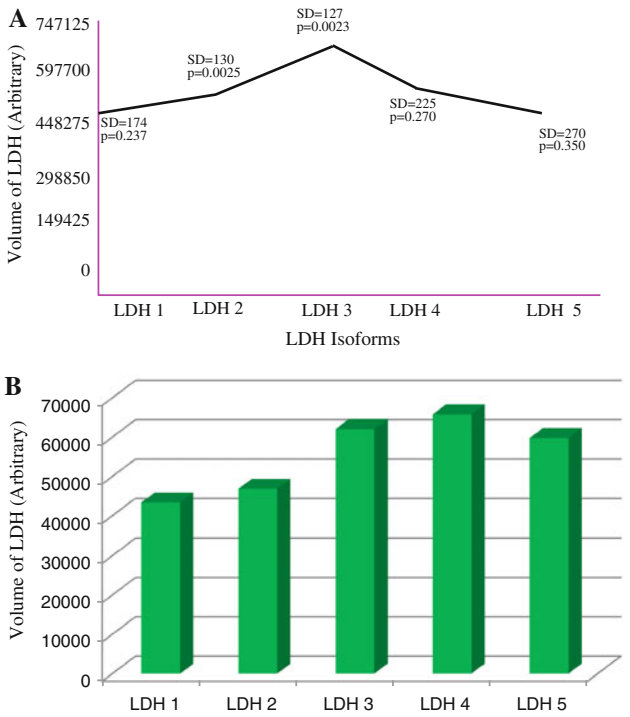


Fig. 5 Densitometry of LDH isoforms in controls (in arbitrary unit). Line graph in **a** represent relative band density of each isoform (volume); histogram in **b** shows comparison of LDH isoforms

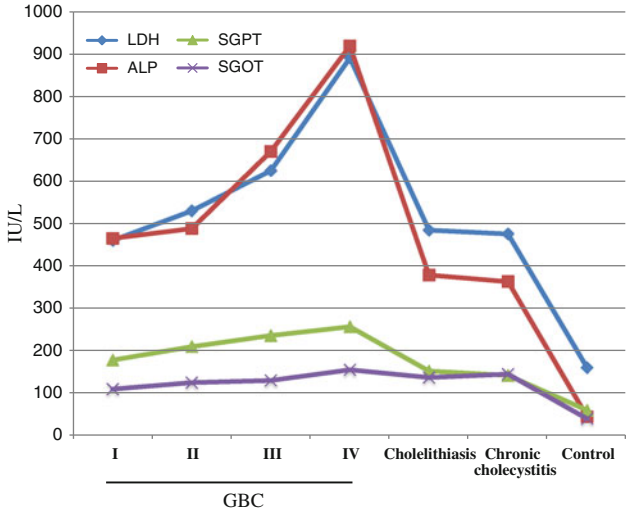


Fig. 6 Line graph showing comparative expression of LDH, ALP, SGPT and SGOT in gallbladder cancer (stages I, II, III and IV) cholelithiasis, chronic cholecystitis and controls

The relatively lower values of these isoforms in cholelithiasis or chronic cholecystitis (but significantly more than that of controls) as compared to GBC may be considered as an indication of more severe condition likely to come or a possible step towards gall bladder carcinogenesis. Thus, the significantly higher levels of LDH in gall bladder cancer and significantly lower level in cholelithiasis patients as compared to chronic cholecystitis and controls may have direct or indirect association with the advancement of pathogenesis or tumorgenesis. The conversion of pyruvate to lactate may likely be at lower rate in benign or cholelithiatic or chronic cholecystitic cells under the hypoxic environment, as compared to that in the advanced stages of gall bladder cancer. The increased cellular stress due to inflammatory reactions in chronic cholecystitic tissues is expected to raise the level of cellular LDH. We, therefore, suggest that the assessment of LDH 3 and 4 expression pattern alone or in association with other markers in the blood sera of gall bladder cancer, cholelithiasis and chronic cholecystitis may serve as diagnostic or prognostic marker in gall bladder cancer. The present observation is a pilot study and part of an ongoing study to search for an early diagnostic marker in GBC. Increasing the sample size of each clinical state may provide further support to the present observation. Interestingly, a recent study by Le et al. [7] demonstrated that LDH A (LDH5) is needed for tumor progression, however, selective inhibition of metabolic activities of cancer cells, including expression of LDH 5, using small drugs like molecules, such as FX 11 [3-dydroxy-6-methyl-7(phenylmethyl)-4propylnaphthalene-1-carboxylic acid], may control the tumor growth. In this context, our observation is significant and provides first

hand information for further research on the role of LDH-3 and 4 in gallbladder cancer pathogenesis.

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