Lactate dehydrogenase (LD; EC 1.1.1.27) isoenzymes are formed by random combinations of two different subunits encoded by structurally distinct genes, LDHA and LDHB (1). Expression of mammalian LDHA and LDHB is regulated during development and is tissue specific; therefore, alterations in the serum LD isoenzyme pattern serve as indicators of pathologic conditions and cancer development (2). Different phenotypes of LD isoenzyme patterns in cancer patients may originate from changes in expression of LDHA or LDHB caused by other regulatory genes or promoter methylation or from mutations involving deletions, duplications, or increased copy numbers. We previously observed in a retinoblastoma cell line a high proportion of LD1 with an extra band that migrated above LD2 and LD3. The unique LD isoenzyme pattern was attributable in part to transcriptional silencing by hypermethylation of the LDHA promoter (3). We also found that the increased concentrations of electrophoretically slow-moving LD isoenzymes in many gastric cancer cell lines are attributable to transcriptional silencing of LDHB expression by aberrant promoter methylation (4).

We recently encountered a male patient with mediastinal germ cell tumor who showed high serum LD activity and high LD1 isoenzyme activity. In germ cell tumors, increased LD1 concentrations often correlates with total copy number for the short arm of chromosome 12, which is where LDHB is located (5), but not all germ cell tumors show this increased copy number for chromosome 12. We therefore hypothesized that the high LD1 isoenzyme activity could be caused by aberrant methylation of the LDHA promoter.

The patient, a 21-year-old man, was admitted to our hospital because of fever and abnormal chest x-ray results. Laboratory tests revealed increased serum LD activity (299 U/L; reference interval, 101–193 U/L). Serum a-fetoprotein was increased to 2535 μg/L (reference interval <10 μg/L). The LD profiles included increased LD1 (49%) and a high LD1/LD2 ratio (2.2; Table 1). Chest x-ray examination revealed a mediastinal tumor. A pathology examination revealed that the tumor consisted of a combined malignant germ cell tumor (yolk sac tumor plus embryonal carcinoma plus dysgerminoma) with a focal rhabdomyosarcoma component arising from a mature cystic teratoma of the mediastinum (size, 8 × 8 × 4.8 cm). After surgical resection of the pulmonary metastasis, total LD activity decreased, but the LD isoenzyme pattern with high LD1 activity remained. After surgical resection of the primary tumor followed by high-dose chemotherapy, both the total LD activity and the LD isoenzyme pattern returned to normal (Table 1).

LD activity was measured by spectrophotometric assay on a Hitachi 7350 automated biochemistry analyzer (Hitachi High Technologies). Assay conditions were based on the method recommended by the Japanese Society of Clinical Chemistry (6). LD isoenzymes in serum were separated electrophoretically with Titan III support medium (Helena Laboratory).

Genomic DNA was extracted from the formalin-fixed, paraffin-embedded specimens of the surgically resected pulmonary metastasis and the primary mediastinal tumor with DNA isolation reagents (DNeasy Tissue Kit; Qiagen). For methylation analysis, we used methylation-specific PCR (MSP) (7). The sequences of primers specific for the methylated allele of LDHA were 5′-CGATTTTCGTAGTTTTAGTACGGC-3′ (forward) and 5′-CGCCATCCCCCTCAACCCGTACG-3′ (reverse); those for the unmethylated allele were 5′-TATTTAGTACGGGTGAAGTTTGACCT-3′ (forward) and 5′-ACCTAAAAATTTACACCAACGG-3′ (reverse; see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vo150/issue10/). The MSP-amplified products were confirmed by single-strand DNA conformation polymorphism (SSCP) analysis (8) and DNA sequencing analysis. MSP-SSCP analysis was performed with 15% nondenatur-

### Table 1. Patient’s lactate dehydrogenase profile and a-fetoprotein concentrations on various dates during ????.

<table>
<thead>
<tr>
<th>Test</th>
<th>Reference Interval</th>
<th>Jan. 16</th>
<th>Feb. 11*</th>
<th>Feb. 27</th>
<th>March 18</th>
<th>June 12</th>
<th>Aug. 5*</th>
<th>Oct. 1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD, U/L</td>
<td>101–193</td>
<td>299</td>
<td>307</td>
<td>166</td>
<td>143</td>
<td>177</td>
<td>161</td>
<td>110</td>
</tr>
<tr>
<td>LD1, %</td>
<td>20.0–31.0</td>
<td>48.6</td>
<td>78.6</td>
<td>22.9</td>
<td>39.8</td>
<td>32.7</td>
<td>25.3</td>
<td>23.2</td>
</tr>
<tr>
<td>LD2, %</td>
<td>28.8–37.0</td>
<td>22.0</td>
<td>23.5</td>
<td>17.5</td>
<td>16.0</td>
<td>35.2</td>
<td>9.1</td>
<td>7.2</td>
</tr>
<tr>
<td>LD3, %</td>
<td>21.5–27.6</td>
<td>13.8</td>
<td>23.1</td>
<td>13.0</td>
<td>4.8</td>
<td>25.3</td>
<td>9.1</td>
<td>7.2</td>
</tr>
<tr>
<td>LD4, %</td>
<td>6.3–12.4</td>
<td>7.8</td>
<td>7.8</td>
<td>10.0</td>
<td>6.8</td>
<td>7.2</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>LD5, %</td>
<td>5.4–13.2</td>
<td>7.8</td>
<td>7.8</td>
<td>10.0</td>
<td>6.8</td>
<td>7.2</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>LD1/LD2</td>
<td>0.60–0.93</td>
<td>2.2</td>
<td>2.2</td>
<td>1.0</td>
<td>1.2</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>AFP, μg/L</td>
<td>&lt;10</td>
<td>2535</td>
<td>372</td>
<td>43</td>
<td>229</td>
<td>92</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*After resection of pulmonary metastasis of the tumor and chemotherapy.
†After high-dose chemotherapy.
‡After surgical resection of the primary tumor.
§AFP, a-fetoprotein.
ing polyacrylamide gels and silver-staining detection (Daiichi Pure Chemicals). DNA sequencing analysis was performed with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and a PRISM 310 Genetic Analyzer (PE Applied Biosystems).

For mutation analysis, all seven exons of the LDHA and LDHB genes were investigated by PCR-SSCP and DNA sequencing analysis to detect mutations (9, 10). To examine gene amplification, we used two pairs of PCR primers to amplify short fragments of the genomic DNAs of LDHA and LDHB. These primers, the same ones used for the mutation analysis described above, amplified exon 2 (9, 10). We performed 25, 30, and 35 cycles of PCR and analyzed the LDHA and LDHB genes separately. PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining and ultraviolet transillumination. Stained bands were analyzed densitometrically with Cool Saver imaging software (ATTO Corp.).

Methylation of the LDHA promoter was detected by MSP in three representative tumor regions (1T, 2T, and 3T), with trace bands of unmethylated allele. In the nontumorous region (nontumor control), however, only the unmethylated promoter was observed (Fig. 1A). Products amplified from tumor regions with methylated allel-specific primers showed SSCP patterns similar to the pattern from control methylated DNA with several additional bands or slight smearing. The differences probably originated from partially methylated DNAs that were methylated to various degrees at individual CpG sites. Products amplified from the nontumor control and tumor regions showed the same SSCP pattern as control unmethylated DNA (Fig. 1B). Sequence analysis revealed methylation of CpGs in the LDHA promoter region of methylated allele-specific PCR products from tumor regions. No methylation was observed in the unmethylated allele-specific PCR products from the nontumor control region (data not shown).

We did not detect any missense or nonsense mutations in the protein-coding exons and exon-intron boundaries of both the LDHA and LDHB genes in either the nontumor control or tumor specimens (data not shown). At different cycles of amplification for genomic DNAs of LDHA and LDHB, the LDHA/LDHB ratios in the nontumor control samples and the tumor region and the nontumor/tumor ratios in LDHA and LDHB products did not differ significantly (see Fig. 2 in the online Data Supplement). Therefore, amplification of LDHB and deletion of LDHA were possibly contradicted.

In mammals, DNA methylation usually occurs at CpG dinucleotides. Methylation is known to play a role in regulation of gene expression during cell development, X chromosome inactivation, genomic imprinting, and carcinogenesis (11, 12). In neoplastic cells, some usually unmethylated CpG islands in the promoter region become aberrantly methylated, and this leads to transcriptional silencing of various genes (13). The human LDHA and LDHB genes both have CpG-rich regions in their promoters (14).

In the patient described here, we observed that the promoter region around exon a of LDHA was aberrantly methylated. This might silence expression of the somatic LDHA gene, possibly leading to relative increases in LDHB protein concentrations and LD1 activity. We also found that increased concentrations of electrophoretically fast-moving LD isoenzymes in some types of cancer are the result of transcriptional silencing of LDHA expression as result of aberrant methylation of the LDHA promoter. Thus, as reported previously, enzyme abnormalities in tumors occasionally originate from aberrant methylation. Significant increases in LD1 and LD5 have been reported previously (15, 16). The LD isoenzyme patterns in these patients could be the result of aberrant LDHA or LDHB methylation in cancer cells.

Human testicular germ-cell tumors are typically characterized by overrepresentation of 12p. These tumors were shown to contain striking amplification of a restricted region of 12p that included the K-ras protooncogene. Seminomas with this 12p amplification do not undergo apoptosis, and the tumor cells showed prolonged in vitro survival, as do seminoma cells with a mutated ras gene (17). Indeed, high concentrations of LD1 may yield a better prognostic predictor for the patients with testicular germ cell tumors (5, 18). Amplification of 12p is associated with poor prognosis, whereas methylation of LDHA may indicate a good prognosis. This issue

Fig. 1. Methylation analysis.

(A), results of bisulfite treatment and MSP analysis of the LDHA promoter in three tumor tissue sections (T) and one nontumor tissue section (N). (B), results of SSCP analysis of the above MSP-amplified products. SSCP was performed at 15 °C with 15% polyacrylamide gels. U, unmethylated control DNA; M, methylated control DNA.
should be addressed by investigating a large sample of patients with high LD1 attributable to amplification of LDHB or methylation of LDHA.

To our knowledge, this is the first reported case in which the specific LD isoenzyme pattern in serum was linked directly to promoter methylation. The next issue is whether methylation of the LDHA promoter is a common mechanism underlying increased LD1 concentrations in germ-cell tumors.

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