Overexpression of Dihydrodiol Dehydrogenase as a Prognostic Marker of Non-Small Cell Lung Cancer

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ABSTRACT

By using mRNA differential display to examine specimens of non-small cell lung cancer (NSCLC), we have identified overexpression of dihydrodiol dehydrogenase (DDH) that was not detected in the corresponding normal lung tissue. Normally DDH is associated with catalysis of polycyclic aromatic hydrocarbons (PAHs) in the liver; in NSCLC cells, DDH expression would implicate an association with disease progression. In this study we investigated the prognostic significance of DDH expression in patients with NSCLC. Using a immunohistochemistry, we measured DDH expression in 381 patients with NSCLC. The relationship between DDH expression and clinicopathological parameters (age, gender, smoking history, mitotic index, histological type, stage; cell differentiation, and lymphovascular invasion) was analyzed by χ² analysis. Survival curves were plotted with the method of Kaplan-Meier, and statistical difference of survival between different groups was compared by a log-rank test. Our results showed that DDH overexpression could be detected in 317 (83.2%) of 381 pathological sections and in 77.9% (60 of 77) of metastatic lymph nodes. Expression of DDH was confirmed by immunoblotting. Compared with patients with DDH overexpression in tumors, patients with low DDH expression had significantly lower incidence of early tumor recurrence and distant organ metastasis (46.7 versus 29.7%; P = 0.045). Interestingly, survival was also significantly better in patients with low DDH expression than in those with DDH overexpression (P = 0.0017). Using univariate analysis, we correlated three important factors, DDH overexpression, tumor stages, and gender, with poor prognosis for NSCLC patients. Nevertheless, biological function and involvement of DDH in the disease progression of NSCLC require additional studies.

INTRODUCTION

Lung cancer is one of the leading causes of cancer death worldwide. It is anticipated that lung cancer would remain being a major cause of cancer death in the next century if conditions of air pollution are not effectively improved and the smoking population keeps increasing, especially in Eastern Europe and the developing countries (1, 2). In Taiwan, the annual death rate from lung cancer is more than 5000 people (3). Most of the patients who die are at the late stage of the disease when they are diagnosed (4). However, some patients who are diagnosed at the early stage and undergo adequate surgical resection still die of cancer because of the early recurrence and metastasis (5).

Advances in molecular biology have indicated that oncogene expression, such as c-myc, c-mdm, Ki-ras, c-erbB-1 (6–9) and HER-2/neu (10), are frequently associated with lung cancer. Although the oncogenic consequences are yet to be determined, risk factors in life-style (e.g., smoking habits) as well as in environment (e.g., exposure to asbestos, radon, and air pollutants) are implicated in the activation of oncogene expressions. The consensus has come to a conclusion that development of cancer is a result of cumulative multiple genetic alterations. However, disease progression could be affected by the sequential and coordinated pathophysiological dys-regulations, which would augment oncogene expressions and the aberrant cell growth (11). A pressing problem, nonetheless, is how to identify and characterize the related genes that may form a malicious cycle for the cancer progression, so that the nature of the disease could be comprehended more thoroughly and treatment regimens could be devised more rationally (12).

Application of differential display had distinguished various gene expressions in two or more cell populations (12–14). We have detected overexpressions of several pertinent genes in NSCLC (3) by using this approach (15). Among these, expression of DDH is most prominent in both primary NSCLC and lung cancer cell lines. DDH is a member of the aldo-keto reductase superfamily (16, 17), which changes the aldehyde or ketone moiety to a corresponding alcohol by using NADH or NADPH as a cofactor. In liver, the enzyme is abundantly located in the cytoplasm as a monomeric Mr 34,000–36,000 protein (18, 19). Interestingly, by differential display, Shen et al. (20) also showed that overexpression of DDH could be identified in ethacrynic acid-induced drug-resistant human colon cancer cells. Detection of DDH overexpression in drug-resistant human stomach cancer cells, which were selected by the gradual adaptation to daunorubicin, further suggested that DDH might be associated with the drug-resistance in cancer cells (21). Therefore, it is reasonable to ask whether DDH expression could have any prognostic significance in patients with NSCLC.

In this study, we used an immunohistochemical method to determine DDH expression in surgical specimens from patients with lung cancer. Overexpression of DDH was confirmed by immunoblotting and ISH. Correlation between clinicopathological parameters and DDH expression and the prognostic significance of DDH expression in patients with NSCLC were evaluated.

MATERIALS AND METHODS

Patients and Tissue Samples. From September 1986 to December 1999, samples were collected from 398 patients in whom NSCLC was diagnosed. Stage of the disease was classified according to the new international staging system (22) for lung cancer. All patients had undergone surgical resection and radical N2 lymph nodes dissection. Tumor size, lymph node number, differentiation, vascular invasion, and mitotic number were also evaluated. Patient with lymph node involvement and patients with locoregional recurrence were irradiated with 22 Gy at the afflicted areas. Those with distant metastasis were treated with chemotherapy (23). After treatment, all of the patients were routinely followed every 3 to 6 months in the Out-Patient Department. Tumor recurrence and metastasis were identified when blood examination, biochemical studies, chest radiography, abdominal sonography, whole body bone scan, and computerized tomography scans of the chest showed any suspected evidence of the disease.

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3 The abbreviations used are: NSCLC, non-small cell lung cancer; DDH, dihydrodiol dehydrogenase; ISH, in situ hybridization; PAH, polycyclic aromatic hydrocarbon.
RNA Extraction, Differential Display, Gene Identification, and Preparation of Polyclonal Antibodies. Total RNA was isolated from lung cancer tissue by using SNAP RNA column (Invitrogen, San Diego, CA). After measurement of RNA yield, cDNA was synthesized by oligo (dT) primer and Avian Myeloblastosis virus reverse transcriptase. An aliquot of cDNA was then subjected to 35 cycles of PCR by using [α-35S]dATP and a combination of specific 3’ anchored oligo (dT) primers and arbitrary 5’ primers H-AP (GenHunter Co., Nashville, TN). PCR mix contained 1× buffer (Life Technologies, Inc., Rockville, MD), 1.5 mM MgCl2, 2 μM dNTP, 0.25 μM 3’ and 5’ primers, 1 unit of Taq DNA polymerase, 2 μl of cDNA and 1 μl of [α-35S]dATP (1060 Ci/mmol; NEN, Boston, MA). PCR was carried out in a standard procedure denaturing at 94°C for 30 s, hybridizing at 40°C for 2 min, and elongating at 72°C for 30 s. PCR products were separated by electrophoresis in a 6% polyacrylamide gel. The gel was dried at 80°C for 1 h and exposed to an X-ray film for 2–3 days. After differential comparison of PCR products, the overexpressed gene fragments were subjected to sequence analysis (ABI Prism; Perkin-Elmer, Foster City, CA), and the nucleotide sequences were matched with the database listed in GenBank4 (15). DDH isoform DD1 (ARK1C1)5 was identified. The complete coding region was 972 bp.

The full-length cDNA was then cloned into the expression vector pET-29b+. Bacterial colony-containing plasmid with designated DD1 gene was selected, and induced to mass-produce DD1. DD1 was purified by affinity column, and the composition of recombinant protein was determined by amino acid sequencing. Affinity-purified DD1 was then used to immunize BALB/c mice, and sensitivity of antiserum (A110 nm > 0.1 at 1:3125 dilution) was determined by ELISA.

Immunoblotting and Immunological Staining. Procedure for immunoblotting has been described previously (24). Briefly, proteins were separated in a 10% polyacrylamide gel with 4.5% stacking gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was then probed with DDH-specific antibodies. The signal was amplified by biotin-labeled goat antimouse IgG, and peroxidase-conjugated streptavidin. Immunological staining was performed by an immunoperoxidase (Eastman Kodak, Rochester, NY) with enhanced chemiluminescent reagent biotin-labeled goat antimouse IgG, and peroxidase-conjugated streptavidin. Then probed with DDH-specific antibodies. The signal was amplified by electrophoresis in a 6% polyacrylamide gel. The gel was dried at 80°C for 1 h and exposed to an X-ray film for 2–3 days. After differential comparison of PCR products, the overexpressed gene fragments were subjected to sequence analysis (ABI Prism; Perkin-Elmer, Foster City, CA), and the nucleotide sequences were matched with the database listed in GenBank4 (15). DDH isoform DD1 (ARK1C1)5 was identified. The complete coding region was 972 bp.

RESULTS

Two overexpressed DNA fragments, DDa and DDb (Fig. 1), were detected by differential display. After sequence analysis, nucleotide sequences of both DNA fragments from eight surgical resections of the lung cancer matched with that of DD1: GenBank accession no. D26124 HUMDD1, Homo sapiens mRNA for DDH isoform DD1. The homology is more than 99% (335/336). The results were confirmed in these eight samples by immunoblot analysis (Fig. 2).

Among the 398 patients, 17 deaths were surgery related (12 patients died of cardiopulmonary failure and 5 died of sepsis). The median follow-up time for the remaining 381 patients was 37 months, ranging from 2.0 to 109 months. The mean age of the patients was 64.4 years (range, 32–87 years). In this study, 304 men and 77 women were enrolled, and 244 patients (58.8%) were smokers. After surgery, 167 patients had evidence of tumor recurrence. As shown in Table 1, no significant difference was found between patients with and those without metastasis according to the following clinicopathological parameters: age, smoking habits, tumor type, cell differentiation pattern, and histopathological characters (mitotic index and evidence of lymphovascular invasion). Nevertheless, statistical differences were found with respect to the tumor stages (P < 0.005) and patients’ gender. Male patients had a significantly higher incidence of tumor recurrence than female patients (P = 0.0093).

As determined by immunohistochemistry, 317 patients (83.2%) were positive for DDH expression (Fig. 3, A and B). DDH was also detected in 77.9% (60 of 77) of metastatic lymph nodes, and DDH mRNA in surgical specimens was verified by ISH (Fig. 3, C and D). Expression of cytochrome P450 was detected in 80 specimens (21%), expression of glutathione S-transferase was identified in 141 surgical samples (37%), and expression of mdr-1 was in 43 pathological sections (11.3%). In terms of clinicopathological parameters, however, no significant differences were found between patient groups that were divided according to DDH expression.

Interestingly, among the 317 patients who had DDH overexpression, 148 (46.7%) patients had tumor recurrences. All of the 148 patients had evidence of tumor recurrence. As shown in Table 2, no significant difference was found between patients with and those without metastasis according to the following clinicopathological parameters: age, smoking habits, tumor type, cell differentiation pattern, and histopathological characters (mitotic index and evidence of lymphovascular invasion). Nevertheless, statistical differences were found with respect to the tumor stages (P < 0.005) and patients’ gender. Male patients had a significantly higher incidence of tumor recurrence than female patients (P = 0.0093).

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Interestingly, among the 317 patients who had DDH overexpression, 148 (46.7%) patients had tumor recurrences. All of the 148 patients with tumor recurrence developed tumor within 24 months.
after the operation. On the other hand, among the 64 patients who had low DDH expression, only 19 (29.7%) patients had metastatic lesions. The difference was significant ($P = 0.045$; Table 1). The survival rate of patients who overexpressed DDH was significantly poorer than that of patients who had low DDH expression (Fig. 4). The differences in cumulative survival were significant ($P = 0.0017$). Using univariate analysis, we found that tumor stage, DDH overexpression, and gender were three important factors correlated with poor prognosis for patients with NSCLC.

**DISCUSSION**

The results presented above demonstrate that overexpression of DDH in NSCLC correlated with tumor recurrence, metastasis, and patients’ survival. Patients with DDH overexpression in lung cancer cells have significantly higher incidence of the early tumor recurrences that are frequently associated with poor prognosis.

Normally, DDH converts mutagenic PAH into catechol in the liver (16, 18). Further oxidation of catechol could form PAH $\alpha$-quinones that can rapidly conjugate with glutathione (19, 29, 30). However, DDH is not regularly expressed in the human lung. Even if DDH expression were detected in the normal counterpart of the lung cancer, DDH content is only 0.01–0.001 of that in human liver. The lack of detoxification system for PAH in the lung could then provide some explanation to the fact that the lung is more susceptible than the liver to PAH-related carcinogenesis (20, 31–33).

In addition to PAH metabolism, DDH could also be involved in drug detoxification (19, 24, 29, 31). Shen et al. (20) has shown that

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**Table 1.** Comparison of parameters between patients with and without metastasis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Metastasis $(n = 167)$</th>
<th>No metastasis $(n = 214)$</th>
<th>$P$</th>
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<tr>
<td>Age (yr)</td>
<td>64.1 ± 5.8</td>
<td>64.7 ± 6.2</td>
<td>0.17</td>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male $(n = 304)$</td>
<td>144</td>
<td>160</td>
<td>0.0091</td>
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<tr>
<td>Female $(n = 77)$</td>
<td>23</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Smokers $(n = 244)$</td>
<td>114</td>
<td>130</td>
<td>0.111</td>
</tr>
<tr>
<td>Nonsmokers $(n = 137)$</td>
<td>53</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Mitotic index $(n/10 HPF^c$</td>
<td>5.2 ± 2.8</td>
<td>4.8 ± 2.5</td>
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<td>DDH expression</td>
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<td></td>
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<tr>
<td>Overexpression</td>
<td>148 (46.7%)</td>
<td>169 (53.3%)</td>
<td>0.045</td>
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<tr>
<td>Low</td>
<td>19 (29.7%)</td>
<td>45 (70.3%)</td>
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</tr>
<tr>
<td>Tumor type</td>
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<tr>
<td>SCC</td>
<td>86</td>
<td>104</td>
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</tr>
<tr>
<td>AD</td>
<td>60</td>
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<tr>
<td>I</td>
<td>186</td>
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<td>IIIa</td>
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<td>IV</td>
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<td>Cell differentiation</td>
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<td>Well</td>
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<td>Poor</td>
<td>41</td>
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<td>Lymphovascular invasion</td>
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<td>114</td>
<td>0.068</td>
</tr>
<tr>
<td>Negative</td>
<td>63</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*a* Two-sided $P$ determined by $t$ test.

*b* Two-sided $P$ determined by the $\chi^2$ test.

*c* HPF, high-power field; SCC, squamous cell carcinoma; AD, adenocarcinoma.

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Fig. 3. Representative examples of DDH expression in (A) squamous cell carcinoma and (B) adenocarcinoma of NSCLC detected by immunohistochemical staining. Expression of DDH mRNA in (C) squamous cell carcinoma and (D) adenocarcinoma of NSCLC was further identified by ISH. DDH expression was not detected in the normal stroma ($\times$200).
express a high level of DDH. An elegant study by Ax et al. (21) further demonstrated that anthracycline resistance in human stomach cancer cells could be mediated via DDH by altering daunorubicin into a less toxic daunorubicinol. Although intracellular events between DDH and drug function are yet to be elucidated, a variety of evidence suggests that DDH expression could be responsible for the drug inactivation. In particular, the chemical structures among anticancer drugs, e.g., Adriamycin, etoposide, mitoxantron, and PAH-derivatives that are highly similar further indicate the possibility. Our results elucidated not only the refractory mechanism of daunorubicin in lung cancer chemotherapy (34, 35) but also the clinical association of DDH expression as a prognostic marker in lung cancer cells that correlated with disease progression and survival of patients with NSCLC.

For NSCLC, the poorly differentiated cell type has been frequently found in patients at the late-stage. Moreover, the evident lymphovascular invasion and the increased number of lymph node involvement are also associated with early recurrence and unfavorable outcome of the treatment. It was indicated that early tumor recurrence and disease progression were correlated with the rapid growth of tumor cells and the overexpression of oncoproteins. Nonetheless, results of several studies that emphasized the relationship between prognosis and expressions of Ki-67, p53, CEA, HER2/neu, and bcl-2 were not conclusive (6–10, 36–38). The discrepancy could be attributable, in part, to the marked heterogeneity of tumor cell proliferation and oncone gene expression. In part, the method used may not be able to determine the difference quantitatively. It is, therefore, crucial to identify correctly the cells that express these genes as true cancer cells, and that the differential expression of these genes could be measured among different patients, especially patients with the advanced diseases, in whom the pathophysiological variables would be far more than one gene product.

Harpole et al. (39) has examined the question in detail, and their results of stage I NSCLC studies showed that male sex, tumor size, poor cell differentiation, vascular invasion, HER-2/neu expression, p53 expression and high Ki-67 index could be independent prognostic factors. They skillfully proposed that the outcome of the disease could be a “dose response” of the additive effects of the above parameters.

However, without confining the designated parameters dose response of the additive effect might not be applicable (38, 39). Further work is required to establish the cause of early cancer death and the mechanisms for early relapse and metastasis.

In conclusion, our results demonstrated that tumor stages, male gender, and DDH expression in tumor cells are three important parameters to assess the aggressiveness of NSCLC. The impact of DDH on drug resistance of lung cancer cells, however, remains to be clarified if this is the basis of drug inactivation. It should be noted that other explanations are possible (40–42). At the present time, our data showed that DDH was frequently detected in the pathological sections of NSCLC, and was associated with poor prognosis. Although there is not yet a clear explanation for the clinical correlation among male gender (38, 39, 43), DDH overexpression, and the disease progression, our results provide a focus for future studies to elucidate the mechanism by which drug resistance in NSCLC could be mediated by DDH overexpression.

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Fig. 4. Cumulative survival curves in patients with NSCLC. Survival curves were plotted with the method of Kaplan and Meier. Statistical difference of survival between two groups was compared by the log-rank test ($P = 0.0017$).
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