Genetic polymorphism and gene expression of microsomal epoxide hydrolase in non-small cell lung cancer

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Abstract. Genetic polymorphisms of microsomal epoxide hydrolase (mEH) have been associated with increased risk of lung cancer. However, expression of mEH and its clinical significance in non-small cell lung cancer (NSCLC) have not been investigated. In this study we investigated the expression and genetic polymorphism of mEH in non-small cell lung cancer (NSCLC) patients. Genetic polymorphism was determined by restriction fragment length polymorphism of polymerase chain reaction (PCR) products. The allelic expression pattern as well as expression level of mEH were determined by reverse transcription-PCR (RT-PCR), cDNA sequencing, sequence alignment, immunoblotting and immunohistochemistry. Genotype distributions of mEH in Taiwan’s NSCLC patients were 44.4% of 340TAC/340TAC, 48.6% of 340TAC/340CAC, and 7.0% of 340CAC/340CAC in exon 3, and 80.6% of 418CAT/418CAT, 19.4% of 418CAT/418CGT and 0% of 418CGT/418CGT in exon 4. Of the 72 NSCLC biopsies analyzed, mEH was expressed in 60 (83%) surgical specimens, and the major allelic expression pattern was fast type (Tyr113) in exon 3 (90.3%) and slow type (His139) in exon 4 (100%). Immunohistochemical staining showed that mEH was expressed in 326 of 423 (77.0%) tumor (lung tissue) specimens and in 48 of 93 (51.6%) metastatic lymph nodes. A significant difference in patient survival was found when mEH expression and adriamycin-containing chemotherapy were used to group patients (p=0.0167). In conclusion, with the combination of fast type (Tyr113) and slow type (His139), the mEH enzyme expressed in most NSCLC patients may have intermediate activity. Our findings indicate that with respect to cancer risk and disease progression, the expression level of mEH is as important as genetic polymorphism. In addition, mEH expression in NSCLC could be involved in drug resistance and prognosis of patients.

Introduction

Epoxide hydrolase (EH) is a phase I biotransformation enzyme that catabolizes epoxides, which are converted from hydrophobic polycyclic aromatic hydrocarbons (PAH), into dihydrodiols (1-3). In contrast to highly reactive epoxides, dihydrodiols are mostly inert and can be excreted after conjugating to glutathione (4). However, EH may not always be protective. Hydrolysis of certain epoxides by EH may result in carcinogenic metabolites that can inauspiously form DNA adducts (1).

EH is most abundant in liver and kidney (5). Soluble and microsomal are two forms of EH which have been detected (6,7). Among them, microsomal epoxide hydrolase (mEH, EC 3.3.2.3) has been more broadly studied. The enzyme is encoded by a single gene (EPHX1) in chromosome 1p11-qter. Two polymorphic sites have been reported within the coding region of EPHX1. One is located in exon 3 at amino acid residue 113, in which tyrosine (Tyr113, TAC) or histidine (Hist113, CAC) is expressed, and the other is located in exon 4 at amino acid residue 139, in which histidine (His139) or arginine (Arg139) is expressed (1,8,9). The enzyme with Tyr113 (fast type) has 50% higher activity than that with His139 (slow type), whereas mEH with His139 (slow type) has only 25% activity of that with Arg139 (fast type) (8).

Previous studies indicate that the mEH enzyme with Tyr113 (fast type) is closely associated with the increased risk of lung, breast, laryngeal and colon cancers (10-15). In particular, when patients with higher mEH activity (fast type) were exposed frequently to tobacco smoking, cancer risk increased significantly (16,17). In addition, mEH is further suggested to have a role in suppressing tamoxifen response and causing poor prognosis in patients with primary breast cancer (12,18).

Several molecular methods, including restriction fragment length polymorphism (RFLP), single-stranded conformation polymorphism (SSCP), and polymerase chain reaction (PCR) followed by direct DNA sequencing, have been used to detect...
and genetic polymorphisms (11,19,20). Although data from epidemiological screening and extensive statistical analysis have indicated that certain allelic polymorphism is indeed correlated with higher risk of cancer, the allelic expression pattern and expression level of mEH, which may actually determine the predisposition of tumor development and possibly disease progression, on the other hand, have not been intensively investigated.

In this report, we used reverse transcription-polymerase chain reaction (RT-PCR) to assess expression of mEH mRNA, and to obtain mEH cDNA. Subsequently, allelic variants of mEH were determined by cDNA nucleotide sequencing. Moreover, we used an immunohistochemical method to detect mEH expression in surgical specimens of NSCLC, and mEH expression was confirmed by immunoblotting. Correlation between clinicopathological parameters and mEH expression as well as the prognostic significance of mEH in NSCLC patients were evaluated statistically.

Materials and methods

Patients and tissue samples. From September 1986 to September 2001, samples were collected from 452 patients who had been diagnosed as NSCLC. Stages of the disease were classified according to the new international staging system for lung cancer. The Medical Ethics Committee approved the protocol, and written informed consent was obtained from every patient before surgery. All patients had undergone surgical resection and radical N2 lymph node dissection. Tumor size, lymph node number, differentiation, vascular invasion and mitotic number were also evaluated. Patients with lymph node involvement and patients with locoregional recurrence received irradiation at the afflicted areas. Those with distant metastasis were treated with locoregional recurrence. Tumor recurrence and metastasis were identified when blood examination, followed every 3 to 6 months as outpatients. Tumor recurrence and metastasis were identified when blood examination, followed every 3 to 6 months as outpatients. Tumor recurrence and metastasis were identified when blood examination, followed every 3 to 6 months as outpatients.

Genotype and haplotype analyses of mEH. To examine exon 3, primer sequences were 5'-GATCGATAAGTTCCGTTTC ACC-3' (sense); and 5'-ATCCCTAGTCTTGAAGTGAG GAT-3' (antisense). The amplified products were treated with EcoRV, and resolved in a 3% agarose gel. The homozygous allele of His113 was identified if a 162-bp fragment appeared. If a 140-bp band appeared, the patient was identified as having homozygous Tyr113 allele, and if both 162-bp and140-bp fragments appeared, the patient was identified as heterozygous with His113/Tyr113. Although a 22-bp DNA fragment was expected in Tyr113 allele following PRISM, Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Following alignment of the reading frame (http:// www.ncbi.nlm.nih.gov/entrez, NM_000120), amino acid sequence in the mEH coding region was determined for the individual specimen.

Immunoblotting and immunological staining. The procedure for immunoblotting has been described previously (22). Briefly, proteins were separated in a 10% SDS-polyacrylamide gel with 4.5% stacking gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was then incubated with mEH specific antibodies (Union Biotech, Inc., Taipei, Taiwan, R.O.C.). The signal was amplified by biotin-labeled goat anti-mouse IgG, and peroxidase-conjugated streptavidin. The protein was visualized by exposing the membrane to an ECL film (Amersham, Buckinghamshire, UK) with enhanced chemiluminescent reagent (Pierce, Rockford, IL, USA). The same antibodies were used for immunohistochemical staining, which was performed by an immunoperoxidase method as previously described (23).

Statistical analysis. Relationships between mEH overexpression and clinicopathological parameters were analyzed by Chi-Square test. When the expected number of any analysis cell was smaller than or equal to five cases, Fisher’s exact test was used. To calculate the correlation of mEH overexpression with more than two factors, the Chi-square test for trend was used. Survival curves were plotted using the Kaplan-Meier method (24). Statistical difference in survival between the various groups was compared by the log rank test (25). Statistical significance was set at p-value less than 0.05.
Statistical analysis was performed using GraphPad Prism4 statistical software (San Diego, CA, USA).

Results

Characterization of antibodies to mEH and expression of mEH in NSCLC cells. Specificity of the mEH antibodies was confirmed by immunoblotting analysis which detected a single protein band (~53 kDa) present in the whole cell lysate of lung cancer cells (Fig. 1A). Moreover, overexpression of mEH protein was detected in eight of ten (80%) samples (Fig. 1B). When five pairs of NSCLC specimens were compared with non-tumor fractions, mEH protein expression, though with various degrees, was elevated in tumor fractions (Fig. 1C). Gene expression of mEH was verified by RT-PCR in ten of twelve (83%) surgical specimens (Fig. 2A). Among eleven pairs of NSCLC and non-tumor lung tissue that were assayed, gene expression of mEH was mainly detected in the tumor fraction (Fig. 2B). The nucleotide sequence of amplified cDNA fragments matched with the database of GenBank BC008291, Homo sapiens, epoxide hydrolase 1 and microsomal (xenobiotic).

Analyses of phenotype (allelic expression pattern), genotype and haplotype distributions. Following alignment of mRNA sequences, codon 139TAC that corresponds to Tyr113 (fast type, exon 3) was detected in 65 (90.3%) of 72 NSCLC biopsies. However, codon 418CAT of exon 4 (His139, slow type) was detected exclusively in the corresponding mRNA. No 418CGT (Arg139, fast type) was identified, despite that CAT/CGT heterogenotype was detected in 14 (19%) of 72 samples. In non-tumor fractions, mEH protein expression, though with various degrees, was elevated in tumor fractions (Fig. 1C). Gene expression of mEH was verified by RT-PCR in ten of twelve (83%) surgical specimens (Fig. 2A). Among eleven pairs of NSCLC and non-tumor lung tissue that were assayed, gene expression of mEH was mainly detected in the tumor fraction (Fig. 2B). The nucleotide sequence of amplified cDNA fragments matched with the database of GenBank BC008291, Homo sapiens, epoxide hydrolase 1 and microsomal (xenobiotic).

Table I. Distribution of mEH phenotypes in NSCLC patients as determined by mRNA sequences.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mEH polymorphism of codon 139 in exon 4</th>
<th>mEH polymorphism of codon 113 in exon 3&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>TAC/Tyr</td>
<td>CAC/His</td>
</tr>
<tr>
<td>Male</td>
<td>47</td>
<td>2</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Total percentage (%)</td>
<td>90.3</td>
<td>9.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>p-value of phenotype distribution was determined by Fisher's exact test (gender, p=0.03; smoking, p=0.428). <sup>b</sup>Odds ratio of phenotype distribution was determined by Fisher's exact test.
in order to eliminate the possibility of preferential selection of cDNA, sequences of PCR product and four other clones from these 14 samples were repeatedly determined, and the result remained negative for Arg139/CGT phenotype (Table I). Interestingly, when a smoking habit with a cut-off value of 20 pack-years (10), was included for evaluating the gene expression pattern, the predominant mEH phenotype expressed in patients with His/Arg139 in heterogentype was His139/Arg139 (Table II, p=0.035), and the male patients (96%) were more prone to express fast-type mEH than the female patients (78%) (p=0.03). The difference was not statistically significant when haplotype was used as a determinant. Distributions of phenotype (cDNA sequences), and genotype (genomic DNA sequences) are respectively summarized in Tables I and II.
Clinical significance of mEH overexpression in NSCLC patients. Among the 452 patients, 29 deaths were surgery-related (12 patients died of sepsis and 17 of cardiopulmonary failure). The median follow-up time for the remaining 423 patients was 26 months, ranging from 2.0 to 109 months. The mean age of the patients was 64.1 years, ranging from 27 to 87 years. In this study, 323 men and 100 women were enrolled, and 276 patients (65.2%) were smokers. Following surgery, 180 patients showed evidence of tumor recurrence. Positive mEH overexpression was identified when the tumor fraction expressed a higher level of mEH than the non-tumor fraction of resected lung tissue. As shown in Table III, no significant difference was found between mEH overexpression and patient gender, cell differentiation patterns, tumor type or histopathological features (mitotic index and evidence of lymphovascular invasion). Statistical differences however, were found between mEH overexpression and age (p<0.001), smoking habit (p<0.001), stages (p=0.001) and dihydrodiol dehydrogenase (DDH) expression (p<0.001). Interestingly, male patients also had a significantly higher incidence of mEH expression and tumor recurrence than female patients (p<0.005).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mEH overexpression</th>
<th>Odds ratio or likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.3±7.8</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=323)</td>
<td>254</td>
<td>0.168*</td>
</tr>
<tr>
<td>Female (n=100)</td>
<td>72</td>
<td>0.858 to 2.387*</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers (n=276)</td>
<td>244</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Non-smokers (n=174)</td>
<td>82</td>
<td>3.654 to 9.759*</td>
</tr>
<tr>
<td>Mitotic index (#/10 HPF)</td>
<td>5.5±4.3</td>
<td>0.32*</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCC (n=225)</td>
<td>162</td>
<td>10.222*</td>
</tr>
<tr>
<td>AD (n=147)</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>Mixed (n=51)</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (n=170)</td>
<td>118</td>
<td>0.001*</td>
</tr>
<tr>
<td>IIa (n=42)</td>
<td>31</td>
<td>20.342*</td>
</tr>
<tr>
<td>IIb (n=112)</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>IIIa (n=41)</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>IIIb (n=45)</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>IV (n=13)</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Cell differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well (n=63)</td>
<td>38</td>
<td>0.776*</td>
</tr>
<tr>
<td>Moderate (n=265)</td>
<td>153</td>
<td>0.507*</td>
</tr>
<tr>
<td>Poor (n=95)</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (n=253)</td>
<td>186</td>
<td>0.135*</td>
</tr>
<tr>
<td>Negative (n=170)</td>
<td>140</td>
<td>0.897 to 2.227*</td>
</tr>
<tr>
<td>DDH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (n=362)</td>
<td>297</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Negative (n=61)</td>
<td>29</td>
<td>2.852 to 8.914*</td>
</tr>
</tbody>
</table>

142 (43.6%) had tumor recurrences, and among 97 mEH-
negative (mEH-) patients, only 38 (39.1%) developed meta-
al disease-related. However, it may be difficult to obtain lung
tissue from normal population. It is interesting to note that
phenotype distribution between men and women was sig-
ificantly different (p=0.03), and most male patients expressed
Tyrl113.

As determined by immunohistochemistry, 326 (77.0%) of
423 patients overexpressed mEH (Fig. 3A and B), and 97
(23.0%) were mEH-negative (Fig. 3C and D). Overexpression
of mEH was also detected in 51.6% (48/93) of metastatic
lymph nodes. Among 326 mEH-positive (mEH+) patients,
142 (43.6%) had tumor recurrences, and among 97 mEH-
negative (mEH-) patients, only 38 (39.1%) developed meta-
estatic lesions. The difference however, was marginal (p=0.058).
When mEH overexpression was used to divide patient groups
(mEH+, n=97; and mEH-, n=326), a marginal difference was
found in total survival (p=0.061, Fig. 4A). When only patients
who had received adjuvant chemotherapy containing doxorubicin
were analyzed according to mEH expression (mEH-negative,
n=77; mEH-positive, n=192), the difference of cumulative survival
was significant (p=0.00167).

Discussion

The results presented above showed that the major form of
mEH expressed in NSCLC is the fast type (Tyrl113) in exon 3
(90.3%) and the slow type (His139) in exon 4 (100%). In
combination, these types constituted an mEH with intermediate
activity. Clinically, mEH overexpression in cancer cells was
correlated inversely with patient survival, in particular in
patients who received adjuvant chemotherapy containing
doxorubicin and cisplatin. Patients with mEH overexpression
had significantly poorer prognosis.

Previous studies by several groups provided invaluable
data on genotypic polymorphism in various cancers including
lung cancer, and indicated that a detection rate of 340TAC
allele (Tyrl113) in exon 3 ranged from 0.63 to 0.77, and that
of 418CAT (His139) in exon 4 ranged from 0.77 to 0.94 (10-15).
Our results agreed with their findings, and show that genotype
distributions of mEH in Taiwan's NSCLC patients were 44.4%
of 340TAC/340TAC, 48.6% of 340TAC/340CAC, and 7.0% of
340CAC/340CAC in exon 3, and 80.6% of 418CAT/ 418CAT,
19.4% of 418CAT/418CGT and 0% of 418CGT/418CGT in exon 4.
In contrast, phenotype distributions were 90.3% of intermediate
(Tyr113/His139) and 9.7% of slow (His113/His139) activity variants (Table I). Even in patients with
418CAT/418CGT heterogeneity, phenotype was mainly
His139. A parallel study on normal population would help to determine whether the imbalanced
allelic expression of mEH observed in NSCLC patients
is disease-related. However, it may be difficult to obtain lung
tissue from normal population. It is interesting to note that
phenotype distribution between men and women was sig-
ificantly different (p=0.03), and most male patients expressed
Tyrl113.

In a large population study, Zhou et al (10) raised a
controversial issue that although passive cigarette smoking
could increase lung cancer risk in non-smokers, cumulative
cigarette smoking, on the other hand, could fortuitously protect
heavy smokers from lung carcinogenesis. In a mutagenesis
study, Hasset et al (8) suggested that the protection effect
could be due to the ‘allelic selection’ of gene expression. By
showing that high activity mEH with Tyrl113/Arg139 was
barely detected in NSCLC patients, our results indicated
that selection pressure could be from air pollution, in particular,
cigarette smoking or heavy oil fumes from the traditional
Chinese cooking method (such as frying fish) (26). Several
studies supported such a point of view by demonstrating
that in addition to genetic predisposition, the activity and
stability of PAH metabolism-associated enzymes, e.g. mEH,
glutathione-S-transferase (GST), N-acetyltransferase-2, and
Cyp1A1, are also critical in cellular response to mutagen(s)
or carcinogen(s), which are closely associated with lung
cancer risk (11,27-32).

By measuring the conversion activity of the S-12 fraction
from lung peripheral parenchyma, Petruzzielli et al demon-
strated that normal lung epithelial cells were not capable of
metabolizing PAH (33). By immunohistochemical staining,
Coller et al indicated that the incapability to catabolize
PAH could result from the lack of GST, aryl hydrocarbon
hydroxylase and mEH (5). These two groups further suggested
that pulmonary carcinogenesis might be directly mediated
by PAH conjugates, e.g., benzo(a)pyrenated serum albumin,
instead of PAH per se, which had to be activated in other
organs, such as liver and kidney (5,6). Moreover, minute
particles of cigarette smoking could also induce mEH
expression and local pulmonary inflammation. The increased
mEH and PAH metabolites could aggravate the damage to
lungs epithelial, and activate regeneration of pulmonary epithelial cells to repair respiratory function. Growth factor(s) produced during repair might conversely facilitate disease progression of NSCLC.

Besides lung cancer, by examining antiestrogen binding activity, Fritz et al demonstrated that mEH expression could also be an important index for tamoxifen resistance, which was closely associated with poor prognosis, in primary breast cancer (18). By showing a similar correlation between survival and mEH expression in NSCLC patients, in particular, in those who had received regimens containing doxorubicin, our data not only supported their findings, but also suggested that involvement of mEH in drug resistance of cancer cells could be general. In this study, mEH overexpression in NSCLC patients was associated significantly with DDH expression (p<0.001) (Table III). It is worth noting that expression of DDH, an essential enzyme for catalyzing epoxide hydrolase-mediated formation of epoxide, was also associated with cisplatin-related drug resistance in ovarian cancer cells (34). The role of mEH as well as the involvement of DDH in drug resistance are currently being evaluated in an ongoing in vitro study.

In conclusion, our results showed that mEH overexpression was frequently detected in the pathologic specimens of NSCLC patients and correlated with tumor stages. The majority of mEH detected are the fast type (Tyr113) in exon 3 and the slow type (His139) in exon 4. With this combination, the enzyme has intermediate enzyme activity. Although the prognosis of patients with mEH overexpression in NSCLC cancer cells was generally poor, especially in those who had received regimens containing anthracycline and cisplatin, the clinical association of increased mEH expression with disease progression, however, remains to be determined.

Acknowledgments

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References


