

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 inauspiciously 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 (His113, 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 His113 (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

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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 chemotherapy (21). After treatment, patients were routinely followed every 3 to 6 months as outpatients. Tumor recurrence and metastasis were identified when blood examination, biochemical studies, chest radiography, abdominal sonography, whole body bone scan and computerized tomography scans of chest showed any suspected evidence of the disease.

RNA extraction and reverse transcription-polymerase chain reaction. Total RNA was isolated from lung cancer tissue by using a SNAP RNA column (Invitrogen, San Diego, CA). After measurement of RNA yield, cDNA was synthesized by random primers and AMV reverse transcriptase. An aliquot of cDNA was then subjected to 35 cycles of PCR. The reaction mixture contained 1X Taq buffer (BRL, Bethesda, MD), 1.5 mM MgCl₂, 2 μM dNTP, 0.25 μM of respective 3' and 5' primers, 1 U of Taq DNA polymerase, and 2 μl of cDNA. PCR was carried out in a standard procedure: denaturing at 94°C for 30 sec, hybridizing at 52°C for 45 sec, and elongating at 72°C for 2 min. The primer sequences for mEH were 5'-CATGTGGCTAGAAATCCTCC-3' (sense) and 5'-TCAT TGCCGCTCCAGCACCGACA-3' (antisense). The amplified products were analyzed in 1% agarose gel, and visualized by ethidium bromide staining. The mEH fragment was 1369 base pairs (bp). The cDNA was inserted into plasmid pCRII and a DNA sequence of five selected clones as well as PCR products were determined by an automatic DNA sequencer (ABI

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).

Slide evaluation. In each case, normal lung tissue served as the internal negative control. Slides were read by two independent pathologists without prior clinicopathological knowledge. A specimen was considered positive if more than 10% of cancer cells were positively stained and negative if less than 10% were positively stained (23).

Genotype and haplotype analyses of mEH. To examine exon 3, primer sequences were 5'-GATCGATAAGTTCCGTTTC ACC-3' (sense); and 5'-ATCCTTAGTCTTGAAGTGAG GAT-3' (antisense). The amplified products were treated with *EcoR* V, 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 and 140-bp fragments appeared, the patient was identified as heterozygous with His113/Tyr113. Although a 22-bp DNA fragment was expected in Tyr113 allele following *EcoR* V digestion, the primers frequently obscured the fragment. For exon 4, primers were 5'-GGGGTGCCAGAGCCTGACCGT-3' and 5'-AACACCGGGCCCACCCTTGGC-3'. The amplified products were digested with *Rsa* I before resolving in a 3% agarose gel. When 295- and 62-bp fragments appeared, the patient had homozygous allele of His139. When 174-, 121- and 62-bp bands appeared, the patient was identified as homozygous Arg139 allele, and when all four DNA fragments appeared, the patient was heterozygous with His139/Arg139 (1,8-15).

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

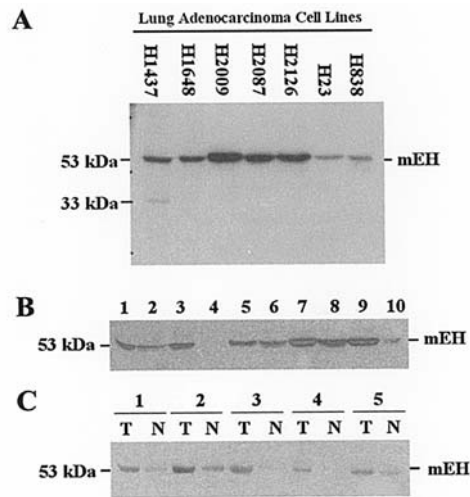


Figure 1. Overexpression of mEH in lung adenocarcinoma cell lines and NSCLC specimens as determined by immunoblotting. (A) Overexpression of mEH was detected in seven lung adenocarcinoma cell lines. The antibody mainly recognized a 53-kDa protein. The antibody also interacted with a 33-kDa protein (lane 1). The signal however, was much weaker. (B) Overexpression of 53-kDa mEH was identified in eight out of ten human NSCLC specimens. (C) When tumor and non-tumor lung tissue were compared, the enzyme was mainly expressed in the tumor fraction. N, non-tumor fraction of resected lung tissue; T, tumor fraction of surgical resections.

<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

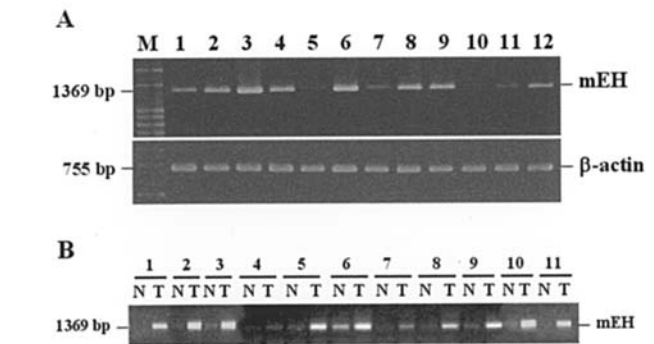


Figure 2. Expression of mEH in NSCLC detected by RT-PCR. (A) Expression of mEH mRNA (1369-bp RT-PCR product) was identified in ten out of twelve human NSCLC samples. M, DNA ladder marker. (B) When eleven pairs of NSCLC and non-tumor lung tissue were assayed, gene expression of mEH was mainly detected in the tumor fraction. N, non-tumor fraction of resected lung tissue; T, tumor fraction of surgical resections.

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 ³⁴⁰TAC 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

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

Parameter	mEH polymorphism of codon 139 in exon 4			mEH polymorphism of codon 113 in exon 3 ^a	
	TAC/Tyr	CAC/His	Odds ratio	CAT/His	CGT/Arg
Gender					
Male	47	2	6.53 ^b	49	0
Female	18	5		23	0
Smoking					
Smokers	40	3	2.13 ^b	43	0
Non-smokers	25	4		29	0
Total percentage (%)	90.3	9.7		100	0

^ap-value of phenotype distribution was determined by Fisher's exact test (gender, p=0.03; smoking, p=0.428). ^bOdds ratio of phenotype distribution was determined by Fisher's exact test.

Table II. Distribution of mEH genotypes in NSCLC patients as determined by restriction enzyme length polymorphism of PCR products.

Parameter	mEH polymorphism of codon 113 in exon 3 ^a			mEH polymorphism of codon 139 in exon 4 ^b		
	TAC/TAC	TAC/CAC	CAC/CAC	CAT/CAT	CAT/CGT	CGT/CGT
Gender						
Male	20	27	2	38	11	0
Female	8	12	3	20	3	0
Smoking						
Smokers	15	26	2	31	12	0
Non-smokers	13	13	3	27	2	0
Total percentage (%)	44.4	48.6	7.0	80.6	19.4	0

^ap-value of genotype distribution of codon 113 polymorphism was determined by the Pearson χ^2 test (gender, $p=0.371$; smoking, $p=0.510$).

^bp-value of genotype distribution of codon 139 polymorphism was determined by Fisher's exact test (gender, $p=0.525$; odds ratio =1.93; smoking, $p=0.035$; odds ratio = 5.22).

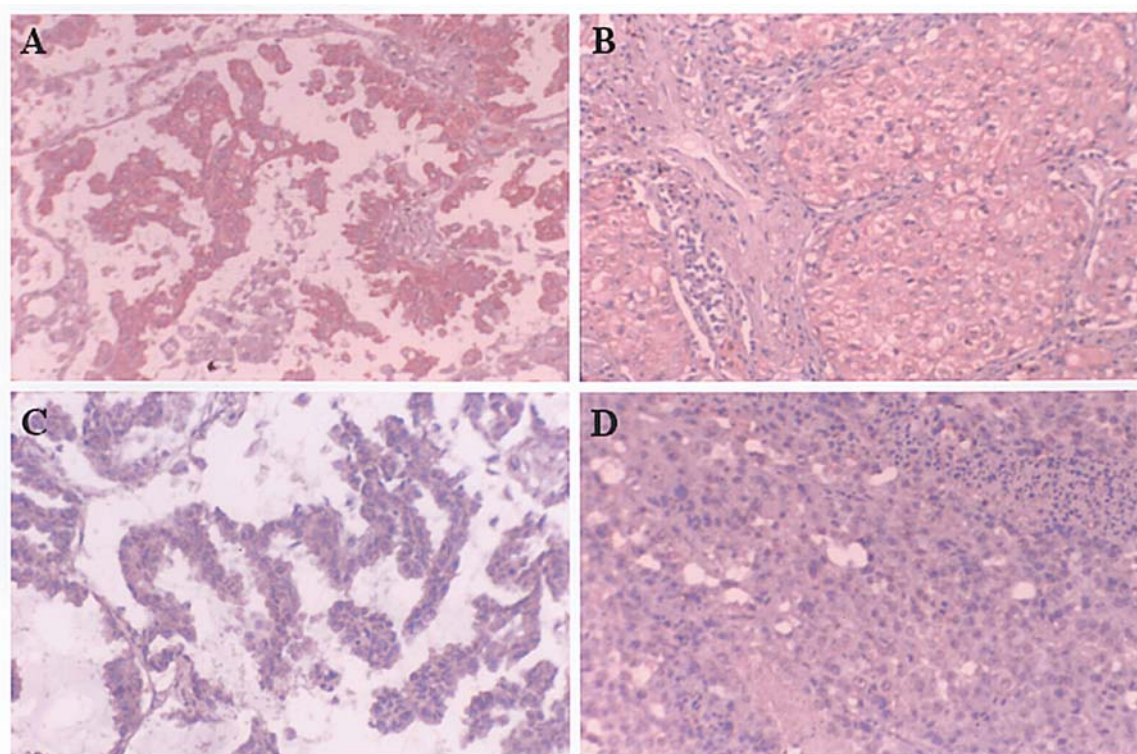


Figure 3. Representative examples of mEH overexpression. (A) Adenocarcinoma. (B) Squamous cell carcinoma of NSCLC detected by immunohistochemical staining. (C) Adenocarcinoma cells. (D) Squamous cell carcinoma of NSCLC that did not express mEH. Expression of mEH was not detected in the normal stroma (original magnification x200).

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/Arg^{exon 4} heterogenotype was His139^{exon 4} (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.

Table III. Comparison of clinicopathological parameters between patients with and without mEH overexpression.

Parameter	mEH overexpression		p-value	95% confidence interval	Odds ratio ^e or likelihood ratio ^f
	Positive (n=326)	Negative (n=97)			
Age (years)	66.3±7.8	56.8±9.2	<0.001 ^a		
Gender					
Male (n=323)	254	69	0.168 ^b	0.858 to 2.387 ^d	1.432 ^e
Female (n=100)	72	28			
Smoking					
Smokers (n=276)	244	32	<0.001 ^b	3.654 to 9.759 ^d	5.971 ^e
Non-smokers (n=147)	82	65			
Mitotic index (#/10 HPF)	5.5±4.3	5.1±4.7	0.32 ^a		
Tumor type					
SCC (n=225)	162	63	0.008 ^b		10.222 ^f
AD (n=147)	126	21			
Mixed (n=51)	38	13			
Stage					
I (n=170)	118	52	0.001 ^c		20.342 ^f
IIa (n=42)	31	11			
IIb (n=112)	88	24			
IIIa (n=41)	35	6			
IIIb (n=45)	41	4			
IV (n=13)	13	0			
Cell differentiation					
Well (n=63)	38	25	0.776 ^c		0.507 ^f
Moderate (n=265)	153	112			
Poor (n=95)	52	43			
Lymphovascular invasion					
Positive (n=253)	186	47	0.135 ^b	0.897 to 2.227 ^d	1.413 ^e
Negative (n=170)	140	50			
DDH					
Positive (n=362)	297	65	<0.001 ^b	2.852 to 8.914 ^d	5.043 ^e
Negative (n=61)	29	32			

^aTwo-sided p-value determined by t-test. ^bTwo-sided p-value determined by the Pearson χ^2 test. ^cChi-square test for trend. ^d95% confidence interval determined by χ^2 test. ^eTwo-sided odds ratio determined by χ^2 test. ^fTwo-sided likelihood ratio determined by χ^2 test. SCC, squamous cell carcinoma; AD, adenocarcinoma; HPF, high power field.

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 ($p<0.005$).

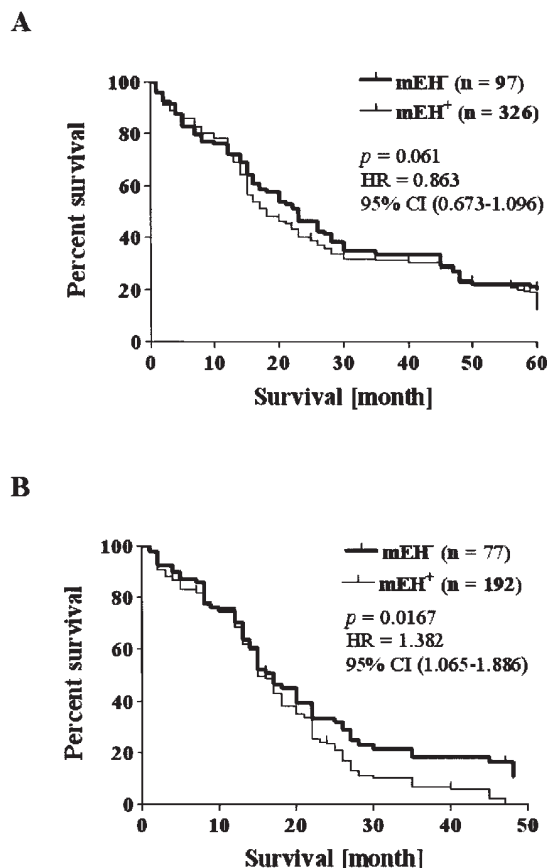


Figure 4. Cumulative survival curves in patients with NSCLC. Survival curves were plotted with the Kaplan and Meier method. Statistical difference of survival between two groups was compared by a log rank test. (A) A marginal difference in total survival rate was found between patients divided by mEH expression (mEH-negative, n=97; mEH-positive, n=326) ($p=0.061$). (B) However, when only patients who received chemotherapy containing doxorubicin were analyzed according to mEH expression (mEH-negative, n=77; mEH-positive, n=192), the difference of cumulative survival was significant ($p=0.0167$).

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 metastatic 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 investigated, the survival of mEH⁺ patients (n=192) was significantly worse than that of mEH⁻ patients (n=77) ($p=0.0167$, Fig. 4B).

Discussion

The results presented above showed that the major form of mEH expressed in NSCLC is the fast type (Tyr113) 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 (Tyr113) 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^{exon 3}/His139^{exon 4}) and 9.7% of low (His113^{exon 3}/His139^{exon 4}) activity variants (Table I). Even in patients with ⁴¹⁸CAT/⁴¹⁸CGT^{exon 4} heterogenotype, phenotype was mainly His139^{exon 4} (Tables I and II). 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 significantly different ($p=0.03$), and most male patients expressed Tyr113.

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, Hassett *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 Tyr113^{exon 3}/Arg139^{exon 4} 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, Petruzzelli *et al* demonstrated that normal lung epithelial cells were not capable of metabolizing PAH (33). By immunohistochemical staining, Collier *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

lung epithelium, 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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