RESEARCH COMMUNICATION

Low Microsomal Epoxide Hydrolase Expression is Associated with Bladder Carcinogenesis and Recurrence

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Abstract

   Microsomal epoxide hydrolase (mEH) plays a significant role in the metabolism of numerous xenobiotics and is associated with several forms of cancer. Here, we investigated the role of mEH expression in bladder carcinogenesis, subsequent progression and recurrence. The expression of mEH was analyzed by Western blot in 50 bladder urothelial carcinoma and 20 normal epithelial tissues. There was a significantly higher mEH expression in the normal epithelium ($P<0.05$) and mEH expression was lower in high stage than in low stage tumors ($P<0.05$). Further, immunohistochmistry in 106 bladder urothelial carcinoma demonstrated mEH expression to be negatively correlated with histological grade, pT stage and recurrence ($P<0.05$). These findings suggest the important role of mEH in bladder carcinogenesis, cancer development and recurrence, providing support for efforts to develop mEH-based gene therapy.

Keywords: mEH - bladder carcinoma - histopathological grade - clinical stage - recurrence

Introduction

   The bladder is a common site for cancer development in the urinary tract. Urinary bladder cancer ranks ninth in worldwide cancer incidence; it is the seventh most common malignancy in men and 17th in women (Lotan et al., 2008). In the United States and Western Europe, the lifetime risk is about 1 in 25 and 1 in 80 for white males and females, respectively (Rothman et al., 2010). Furthermore, approximately 145,000 patients die from this disease worldwide per year (Crockett et al., 2011). Majority of bladder tumors originate from transitional epithelium or urothelium, a multi-layered epithelium without squamous cells, which covers the inside of this organ.

   Microsomal epoxide hydrolase (mEH), a phase II metabolic enzyme, catalyses the hydrolysis of arene, alkene and aliphatic epoxides from polycyclic aromatic hydrocarbons and aromatic amines. Although this hydrolysis is generally a detoxification reaction because less reactive and more watersoluble trans-dihydrodiols are produced (Abdel-Rahman et al., 2005), in the case of some hydrocarbons such as benzo(a) pyrene, present in tobacco smoke, more highly reactive and mutagenic compounds for example the 7, 8-diol-9, 10 epoxide are generated in the metabolic process (Mukherjee et al., 2011). Thus, mEH exhibits a dual role of procarcinogen detoxification and activation (Shou et al., 1996) and may be considered a cancer risk factor as well as a protective factor, depending on the carcinogens. Putative low mEH enzyme activity may have a potential protective effect on tobacco-related carcinogenesis of lung and UADT cancers (Rosenberger et al., 2008; Li et al., 2011). Various reports have associated genetic polymorphism in mEH with increased risk of ovarian cancer, hepatocellular carcinoma, and other disease syndromes (Pande et al., 2008; Kiran et al., 2009; Goode et al., 2011). In addition to genetically encoded structural differences in biotransforming enzymes, interindividual differences in transcriptional controls and in tissue-specific regulation are likely contributors to disease risk resulting from chemical exposures. These findings imply that the loss of mEH protein is associated with tumorigenesis. This can be the basis for the gene therapy using mEH in cancer patients.

   Taken together, it is suggested that mEH protein might have a negative regulation of tumor progression. In this study, to the roles of mEH expression in the bladder carcinogenesis and subsequent progression, we examined the expression of mEH protein in bladder cancer and the adjacent normal tissues. Additionally, its expression in tissues was compared to clinicopathological parameters of carcinomas. It suggests the important role of mEH in bladder carcinogenesis, cancer development and recurrence, which support the effort to additionally investigate a mEH -based gene therapy.

Materials and Methods

Patients, Recurrence and Follow up Analysis
   From 2000 to 2011, at the Department of Urology,

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the First Affiliated Hospital of China Medical University in China, a total of 106 consecutive patients with bladder urothelial cell carcinoma underwent transurethral resection, partial cystectomy and radical cystectomy. Furthermore, 20 normal urothelial specimens were obtained by biopsy or from cystectomy, which were used as normal controls. The study was conducted according to an institutional review board-approved protocol, and written informed consent was obtained from each patient for surgery and for research purposes. A total of 106 cases were identified, including 83 men and 23 women with a median age of 58 years old (range, 35–76 years old). The series included 66 cases with transurethral resection, 23 cases with partial cystectomy, and 17 cases with radical cystectomy. The tumor size ranged from 0.5 to 9.6 cm in greatest dimension (median size: 3.5 cm). Histologically, the tumors were classified according to the 2004 World Health Organization histologic classification of the urinary tract tumors (Gupta et al., 2008), including 4 papillary urothelial neoplasms of low malignant potential (PUNLMP), 54 low grade papillary urothelial carcinoma, and 48 high grade papillary urothelial carcinoma. Tumors were staged by the American Joint Committee on Cancer system of 2002 (Daneshmand et al., 2004), including 50 urothelial carcinoma without invasion (<pT1), and 56 invasive urothelial carcinoma (≥pT1). No cancer patients received adjuvant chemotherapy or radiation therapy before surgery. All patients with noninvasive bladder carcinomas (Ta-T1) were treated with intravesical chemotherapy after transurethral resection, whereas all patients with invasive disease (T2-T4) were treated with chemotherapy or radiotherapy after cystectomy. 89 patients with bladder reservation received routine urine examination, chest X-ray, abdominal and pelvic ultrasonography, cystoscopy, and cytology every 3 months. In this series, the recurrence was defined as a new urothelial carcinoma recurring in the bladder and/ or posterior urethra. During the follow-up period, tumor recurrences and metastasis were observed in 68 and 33 patients, respectively. The median follow-up time was 72 months for patients still alive at the time of analysis, ranging from 3 to 132. 41of 106 patients were dead in the follow-up period. Among the 41 patients who died, 29 died of bladder cancer and the remaining 12 died of other causes without evidence of tumor metastasis.

Immunohistochemistry and evaluation

Formalin-fixed, paraffin-embedded tissue blocks were cut into 4-μm sections and subjected to immunohistochemistry with mouse monoclonal antibody for human mEH (1:1000 dilution; Santa Cruz, America). Immunohistochemistry was carried out using the streptavidin-peroxidase -conjugated method. Negative controls were prepared by substituting PBS substituting for primary antibody. Immunohistochemical staining was performed according to previously published methods with minor modification. All the immunoreactions were separately evaluated by two senior pathologists. The immunoreactivity for mEH was considered positive when brown particles appeared in cytoplasm. The intensity of mEH immunostaining (1=weak, 2=moderate, and 3=intense) and the percentage of positive tumor cells (0% = negative, 1–50% = 1, 51–75% = 2, ≥76% = 3) were assessed in at least 5 high power fields (×400 magnification). The scores of each tumorous sample were multiplied to give a final score of 0, 1, 2, 3, 4, 6 or 9, and the tumors were finally determined as negative: score 0; lower expression: score ≤4; or higher expression: score ≥6.

Western blotting

Frozen tissues (including tumor and non-tumorous portion) or cells were washed twice with ice-cold phosphate-buffered saline (PBS), homogenized on ice in 10 volumes (w/v) of lysis buffer containing 20mM Tris–HCl, 1mM EDTA, 50mM NaCl, 50mM NaF, 1mM Na_3VO_4, 1% Triton-X100, 1mM PMSF and phosphatase inhibitor using a homogenizer (Heidoph, DLA×900). The homogenate was centrifuged at 12000g for 30min at 4°C. The supernatant was collected and stored at -70°C. Protein content was determined by the BCA assay (BCA protein assay kit, Pierce Biotechnology, USA). From each sample preparation, 80μg of total protein was separated by 8% SDS-PAGE and then transferred to PVDF blotting membranes.

The total protein extracts were analyzed by immunoblotting with indicated antibodies following SDS-PAGE analysis. Immunoblots were performed using mouse monoclonal primary antibodies specific for mEH and mouse monoclonal antibody for β-actin (Abcam, Hongkong, a housekeeping protein used as a loading control to assure equal amounts of protein in all lanes). After blocking nonspecific binding with 5% BSA in TBS (pH 7.5) containing 0.05% Tween-20 (TBST), primary antibodies were incubated on the membranes for mEH (1:300) and β-actin (1:200) overnight at 4°C in TBST. Following three times washes in TBST, the membranes were incubated for 2 hours at 37°C with goat polyclonal secondary antibody to mouse IgG (1:5000, Abcam, Hongkong) labeled with horseradish peroxidase. The proteins were detected using an ECL detection system (Pierce, Rockford, IL, USA), as directed by the manufacturer. Specific bands for mEH and β-actin were identified by prestained protein molecular weight marker (MBI Fermentas, USA). The EC3 Imaging System (UVP Inc.) was used to catch up the specific bands, and the optical density of each band was measured using Image J software. The ratio between the optical densities of mEH and β-actin of the same sample was calculated as relative content and expressed graphically.

Statistical analysis

SPSS 13.0 was used to perform data analysis. The T test was used to analyze the data from western blot in the tissues. The χ^2 Test was used to evaluate the association between mEH expression and clinicopathologic variables. The significance of mEH expression for tumor recurrence was analyzed by the Kaplan-Meier method, and the differences were evaluated by the log-rank test. Multivariable recurrence-free survival analyses were performed with the Cox proportional hazards model. P values<0.05 were considered statistically significant.
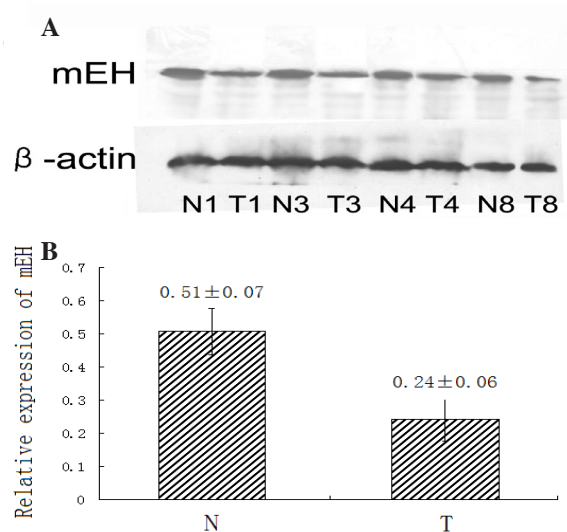
A

Figure 1. Expression of mEH by Western Blotting in 4 Bladder Cancer Tissues and 4 Normal Bladder Tissues. (A) Band intensities indicate significant down-regulation in bladder cancer tissues (T1, T3, T4 and T8) in comparison with the normal bladder tissues (N1, N3, N4 and N8). β-actin was used as a loading control to assure equal amounts of protein in all lanes. (B) The ratio between the optical density of mEH andβ-actin of the same tissue was calculated and expressed graphically. Significant differences of mEH expression between tumor (T) and normal tissues (N) were analyzed statistically and mEH expression was obviously lower in bladder cancer tissues (P<0.05). The data are representative of three individual experiments

Results

Expression of mEH in bladder tissues

Western blot analysis was used to evaluate mEH expression in 50 bladder urothelial carcinoma tissues and 20 normal bladder epithelial tissues. It showed that the decreasing mEH expression could be detected in bladder cancer samples in comparison with the normal bladder samples by statistical analysis (P<0.05). The western blotting of eight samples was shown in Figure 1A, and the optical density of the tumorous (T) and normal (N) tissues was measured and expressed graphically (Figure 1B).

Association between mEH expression and clinicopathologic parameters

To evaluate the significance of low mEH expression in bladder urothelial carcinoma, we investigated the relationship between mEH immunostaining and clinicopathologic features (Table 1). Overall, there was no significant relationship between mEH expression and gender (P=0.324), age (P=0.862), and Tumor size (P=0.623). However, significant correlations were found between mEH expression and Histologic grade (P<0.05), pT stage (P<0.05), Recurrence (P<0.05). Therefore, mEH expression status was closely correlated with important histopathologic characteristics (grades and stages) and the recurrence of bladder urothelial carcinomas.

Relation to recurrence and prognosis

In the follow-up period, 72.0% (59 of 82) of tumors

Table 1. Association of mEH Expression with Clinicopathologic Characteristics of the Bladder Cancer Patients

| Parameters	Group	No. of cases (%)	Cases with low mEH expression (%)	X^2	P
Gender	Male	83(78.3%)	67(80.7%)	1.666	0.197
	Female	23(21.7%)	15(65.2%)		
Age(years)	<58	65(61.3%)	54(83.1%)	2.35	0.125
	≥58	41(38.7%)	28(68.3%)		
Histologic	PUNLMP	4(3.8%)	0(0%)		
grade	Low grade	54(50.9%)	38(70.1%)	6.023	0.014
	High grade	48(45.3%)	44(91.7%)		
pT	<T1	50(47.2%)	30(60.0%)	14.46	0
	≥T1	56(52.8%)	52(92.3%)		
Tumor size	<3.5cm	66(62.3%)	51(77.3%)	0.045	0.832
	≥3.5cm	40(37.7%)	31(77.5%)		
Recurrence	Yes	68(65%)	59(86.8%)	8.142	0.004
	No	38(35%)	23(60.5%)		
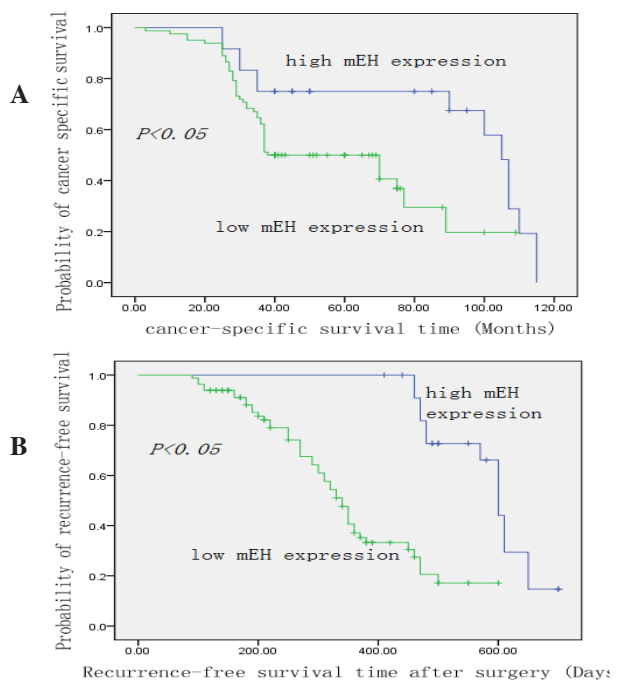
Figure 2. Cancer-Specific Survival and Recurrence-Free Survival Grouped by mEH Expression, and Calculated by the Kaplan-Meier Method. (A) The patients with intense mEH expression had significantly higher cancer-specific survival than those with weak mEH expression through log-rank univariate analysis (n =106, P<0.05). (B) The patients with intense mEH expression had significantly higher cancer-specific survival than those with weak mEH expression through log-rank univariate analysis (n =106, P<0.05)

with low mEH expression recurred compared with 37.5% (9 of 24) of tumors with high mEH expression having recurrence (P<0.05). Therefore, low expression of mEH was positively associated with the incidence of recurrence of bladder urothelial carcinomas.

Kaplan-Meier plots and log-rank tests showed that the patients with low mEH expression in their tumor tissues had statistically significant shorter cancer-specific survival and recurrence-free survival rate, compared with those whose tumors had high mEH expression through log-rank univariate analysis (P<0.05; Figure 2A and 2B).

Table 2. The Association Among mEH Expression, pT Stage and Histologic Grade with Poor Prognosis in Cox Proportional Hazards Model

Parameters	Ward	P	95%CI
Histologic grade	0.003	0.96	0.584–1.668
pT stage	7.758	0.005	1.329–5.126
mEH	7.473	0.006	1.387–7.260

Log-rank univariate analysis also showed that pT stage (P<0.05), histological grade (P<0.05) and the expression of mEH (P<0.05) were significant predictors of the recurrence of bladder tumor. In contrast, there was no significant association between tumor recurrence and other clinicopathologic factors such as gender, age and size of tumor. However, when the parameters with significant prognostic impact in univariate analysis were introduced as covariates in Cox proportional hazards model, the expression of mEH and pT stage had statistically significant independent association with poor prognosis (Table 2). Therefore, low mEH expression in bladder urothelial carcinomas was associated with poor prognosis of the patients.

Discussion

The mEH enzyme is an important Phase II biotransformation enzyme, and it is highly expressed in several human tissues including the bladder, where it catalyzes the hydrolysis of various epoxides and reactive epoxide intermediates into less reactive and more water soluble dihydrodiols, which are then excreted from the body (Hassett et al., 1994). Hence, mEH is a protective enzyme involved in general oxidative defenses against a number of environmental chemicals and pollutants (Harrison et al., 1999). However, mEH is also involved in the xenobiotic activation of tobacco carcinogens.

Bladder cancer is one of the leading cause of cancers death among both men and women in China. Although smoking, radon, asbestos, diet, non-neoplastic bladder disease, and other environmental/occupational carcinogenic exposures have been reported to be associated with the development of bladder cancer (Kyrgidis et al., 2010), the precise genes that affect individual susceptibility to the carcinogenic effects of these agents remain undetermined. Hsu Li et al. (2008) observations implied that impaired metabolism of carcinogenic exposure as well as impaired DNA repair function play an important role in arsenic-related urinary transitional cell carcinogenesis (Hsu et al., 2008). polymorphic phase II enzymes such as glutathione S-transferases (GSTs) and microsomal epoxide hydrolase (mEH) were reported to be associated with bladder cancer (Brockmoller et al., 1996; Okkels et al., 1997; Engel et al., 2002; Garcia-Closas et al., 2005). In this study, we explored the relationship between mEH expression and the carcinogenesis and recurrence of bladder cancer in 106 patients. Our results indicate that there was a significantly higher mEH expression in normal epithelium than those in bladder urothelial carcinoma (P<0.05) and there was lower mEH level in more higher histological grade, higher pT stage and more likely to

relapse (P<0.05). These data were the same with previous reports about mEH was related with carcinoma (Kang et al., 2004; Nock et al., 2007; Hsu et al., 2008; Khedhaier et al., 2008; Lacko et al., 2009; Erkisi et al., 2010; Ihsan et al., 2010). These findings suggest the important role of mEH in bladder carcinogenesis, cancer development and recurrence, which support the effort to additionally investigate a mEH -based gene therapy.

In conclusion, we propose that mEH expression status is closely correlated with important histopathologic characteristics (grades and stages) and the recurrence of bladder urothelial carcinomas. mEH can be a potential target for the treatment of bladder carcinoma. More efforts to investigate the mechanism of mEH involved in bladder cancer development and metastasis would be valuable.

Acknowledgements

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