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Identification of Potential Carcinogenic Biomarker Following Exposure to *N*-ethyl-*N*-nitrosourea in Mice

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Abstract

***N*-ethyl-*N*-nitrosourea (ENU), which is a toxin and a carcinogen, as well as a mutagen, has a variety of effects on mice. ENU induces point mutation in male germ cell. Number of mutant animals are developed with ENU treatment. However, potentiality ot ENU as a carcinogen is not fully understood, even though, mutagenicity of ENU is broadly studied, In the present study, the gene expression profiling and histopathological investigation of ENU treated mouse's liver and brain were investigated. Also, the expression patterns of cancer related genes in ENU-treated mouse were analyzed.**

Keywords: ENU, carcinogen, gene expression, biomarker

N-ethyl-*N*-nitrosourea (ENU) was discoverd to induce mutations with high efficiency in the proportion of mutants in phage treated in vitro[1]. ENU, a toxin and a carcinogen, as well as a mutagen, has a variety of effects on mice[2]. ENU induces point mutation in male germ cell of[3,4]. Number of mutant animals are developed with ENU treatment, and mutagenicity of ENU is broadly studied. ENU is the most potent chemical mutagen, yet discovered for the mouse and has the advantage that primarily generates point mutations. Mutations have been implicated in the etiology of cancer and are thought to be important in the aging process, as well as in the induction of a number of human diseases. ENU is an alkylating carcinogenic to various organs of several animal species[5].

However, potentiality of ENU as carcinogen is not fully understood. Microarray technology is a powerful tool that can be used to elucidate molecular mechanisms and identify markers of toxicity in laboratory animals and humans[6,7]. Moreover, gene expression patterns have recently shown predicted molecules and phenotypes successfully, with remarkable achievement in the field of carcinogenesis[8-10]. We applied microarray and real-time PCR (RT PCR) techniques for identification of potential biomarker after ENU administration. In this paper we sought to investigate the effect of the alkylating agent, ENU single administration on the gene expression. The main goal of this study was to identify changes in gene expression that would be predictive for this type of toxicity and to be considered as a potential biomarker.

cDNA Microarray and Real-time PCR

Mouse 10 K cDNA chip (GaiaGene, Seoul) was used for gene expression profiling study in low and high doses for 24 and 48 hrs on ENU treated mouse's liver. The low dose group did not show significant differences. In high dose group, 90 genes were down-regulated and 37 genes were up-regulated in 24 hrs after the treatment. Seventy-five genes were down-regulated and 32 genes were up-regulated in 48 hrs after the treatment. Significantly expressed gene lists are presented in Table 1 & 2.

We found several up-regulated cancer related genes, such as p53 related protein, metallothionein (MT) I & II. Growth hormone receptor (GHR) was down-regulated. Most of drug metabolizing enzymes (Cytochrome P450 family) were down-regulated with ENU-treatment, however cytochrome P450 4A was up-regulated with ENU treatment.

In order to get the information on induction or repression of the RT PCR has to be used to measure the data. The selected cancer related genes are p53, GHR and MT I & II. Livers and brains for RT PCR analysis were collected 4, 12, 24, or 48 hrs after ENU single administration. Primer sequences are indicated in Table 3. In order to validate the expression levels of these genes, RT PCR was performed at each time point. The gene expression reached a peak (15-20 times higher than control) at 4 hrs after the treatment.

The expression level was then gradually decreased but still higher or equal to the control. p53 related

Table 1. Genes whose expression is significantly down-regulated by intraperitoneal treatment of ENU in the liver

Gene bank no.	Gene name	High dose liver 24 hr	High dose liver 48 hr
AA122925	Carbonic anhydrase 2	−3.6	−2.0
AA268120	Cytochrome P450, family 3, subfamily a, polypeptide 11	−2.3	−1.5
AA109900	Hemoglobin alpha, adult chain 1	−2.0	−4.2
BG072812	Growth hormone receptor	−1.6	−1.1
AA212435	Cytochrome P450, family 2, subfamily j, polypeptide 5	−1.3	−0.4
W36511	Arachidonate 12-lipoxygenase, 12R type	−1.1	−0.3
BG073184	Vimentin	−1.1	−0.3
AI322933	Interleukin 4 receptor, alpha	−1.0	−0.3
BG076	Interferon gamma induced GTPase	−0.9	−1.5

Table 2. Genes whose expression is significantly up-regulated by intraperitoneal treatment of ENU in the liver

Gene bank no.	Gene name	High dose liver 24 hr	High dose liver 48 hr
AA221141	alcohol dehydrogenase 1	0.6	1.0
AA051654	metallothionein 1	0.8	1.2
AI851449	carnitine acetyltransferase	1.1	1.2
BG087383	cathepsin D	1.2	0.8
AI835817	p53 related protein (rp)	1.4	
AA060595	cytochrome P450, family 4A	1.6	1.7
BG063925	metallothionein 2	2.0	2.5
BG065049	procollagen, type II, alpha 1	2.9	2.9
AA097421	apolipoprotein A-IV	3.8	3.5

Table 3. Primers used for validation of selected gene expression changes

Gene name	Forward primer sequence	Reverse primer sequence
GH receptor	GAA TGG AAA GAA TGC CCT GA	GGT TGC CAA CTC ACT TGG AT
MT 1	CTC CGT AGC TCC AGC TTC AC	GTT CGT CAC ATC AGG CAC AG
MT 2	ACT TGT CGG AAG CCT CTT TG	CCG ATC TCT CGT CGA TCT TC
p 53	ACT TAC CAC AGC AGG CGA GT	AAC CCT GTG CTC TAG CCT CA

protein and MT are well known tumor related molecular markers. Enhanced expression of p53 and MT at the 4 hrs of ENU treatment showed that certain carcinogenetic actions occurred in the early time. Interestingly, p53, MT and GHR showed similar patterns in their gene expression. The gene expression of liver tissues (Fig. 1A-D) reached a peak (15-20 times higher than control) at 4 hrs after the treatment. The expression level was then gradually decreased, but still higher or equal to its control. The gene expression of the brain tissues (Fig. 2A-D) showed similar pattern as the liver. Histopatholgically, there were no significant changes in ENU-treated livers and brains (data not shown).

Potential Biomarker for Carcinogenesis

We found several genes (GHR, p53, MT I&II) that are related with cancer after ENU single administration. Firstly, we checked expression level of GHR at the various time points.

Growth hormone (GH) is a protein hormone mainly produced in the anterior pituitary, but also in other tissues to a lower extent[11,12]. GH actions are exerted over a wide number of tissues, and apart from promoting body growth; GH has important effects on body metabolism. GH actions depend on the activation of specific receptors located on the cell surface of the target cells. The GHR is a single transmembrane domain protein, which belongs to the family of cytokine receptors. Although the original suggestion of GHR expression was restricted to the liver, both GH binding sites and GHR mRNA are widely distributed throughout the body[13]. In our study, GHR is down-regulated at 24 and 48 hrs after the ENU treatment. However, GHR is up-regulated during the early time (especially 4 hrs) after ENU treatment (Fig. 1A).

Secondly, we focused on p53 tumor suppressor gene, which p53 is the most commonly mutated gene in cancer that reflects its crucial tumor suppression activity[14]. p53 acts to obstruct tumorigenesis by

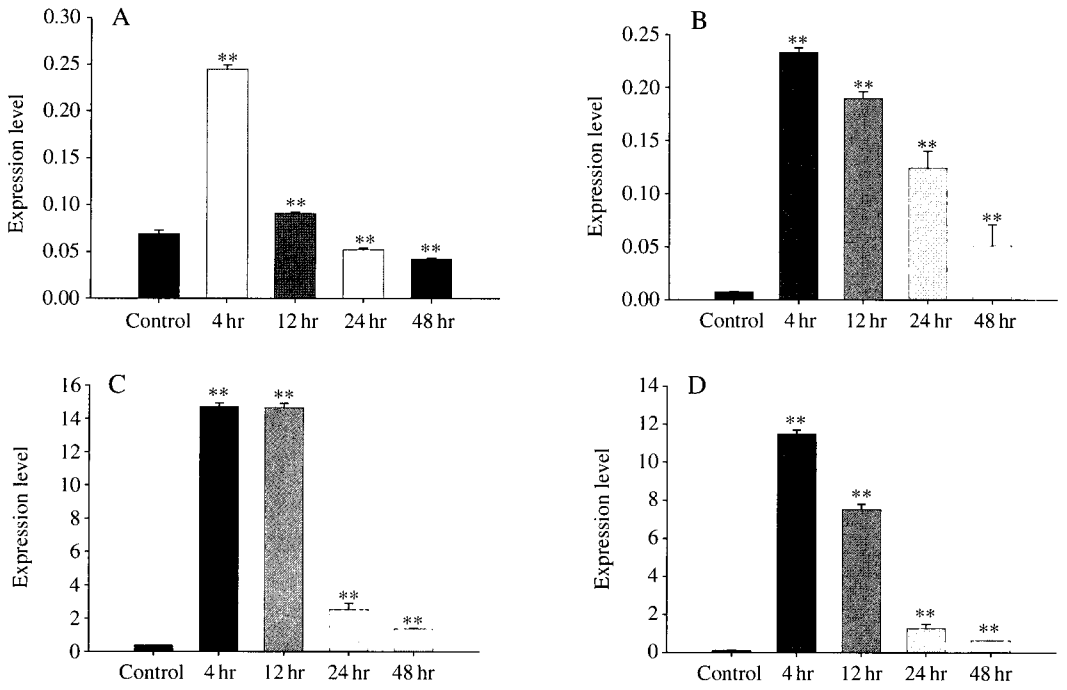

Fig. 1. Validation of expression levels for selected genes significantly changed cancer related genes in liver. Expression levels of GH receptor, p53 and MT I, MT II genes were determined by Real-time PCR at 4, 12, 24, or 48 hrs after injected with 250 mg/kg of ENU. (A) GH receptor, (B) p53, (C) MT I, (D) MT II, $^{**}p < 0.01$, vs control.

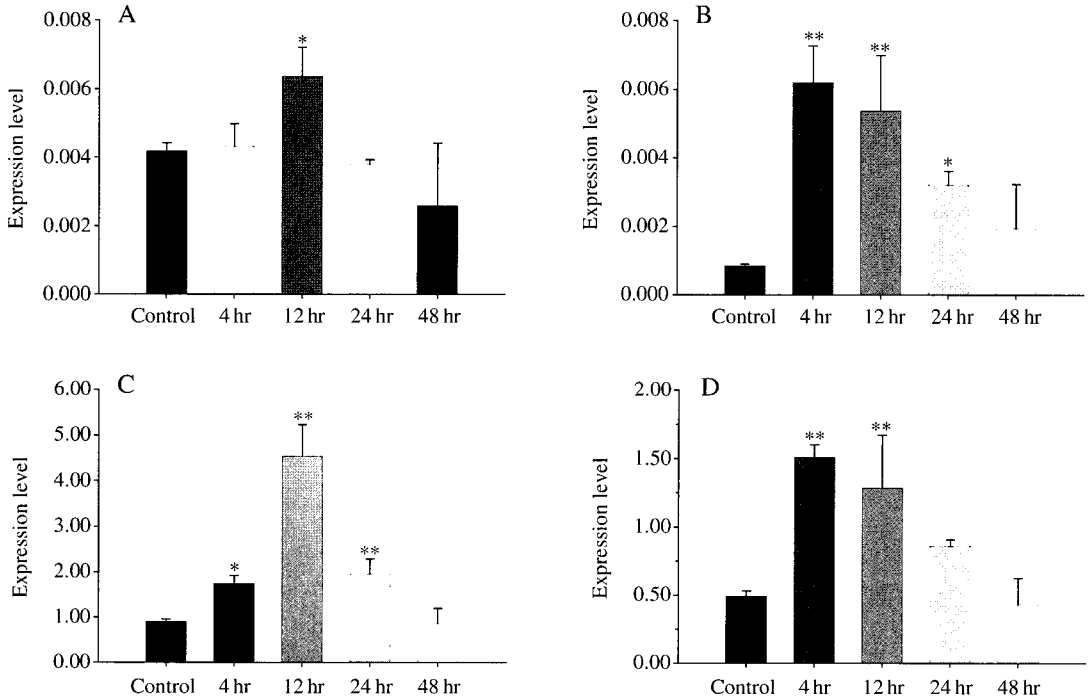

Fig. 2. Validation of expression levels for selected genes significantly changed cancer related genes in brain. Expression levels of GH receptor, p53 and MT I, MT II genes were determined by Real-time PCR at 4, 12, 24, or 48 hrs after injected with 250 mg/kg of ENU. (A) GH receptor, (B) p53, (C) MT I, (D) MT II, $^{*}p < 0.05$, $^{**}p < 0.01$, vs control.

serving as a cellular stress and DNA damage. In response to DNA damage, hypoxia, or proliferative signals, p53 protein becomes stabilized, causing cells to undergo either cell cycle arrest or apoptosis[15]. Also, ENU effect on the carcinogenic susceptibility of p53 (+/−) mice was reported by Mitsumori *et al.*[16]. According to ENU mechanism of action, it is certain that p53 is an essential safeguard against tumorigenesis.

Finally, MT expression is another good indicator for ENU induced carcinogenesis. The expression and induction of MTs have been associated with protection against DNA damage, oxidative stress and apoptosis. In addition, MT may potentially activate certain transcriptional factors. MT I and MT II isoforms are usually expressed in low levels, but inducible by a variety of metal ions, hormones, inflammatory cytokines and xenobiotics[17,18]. MT expression deficiency implicated in carcinogenesis and possible relation of MT over expression and resistance of tumors to the anti-cancer therapy has provided evidence of the importance of MT expression in cancer[19,20]. Although the number of biological functions have been proposed for MT, most of numbers are related to metal-binding property of MT. Thus MT may protect against certain metal toxicity. Also, MT may protect against oxidative stress due to its high cysteine content. The relationship between MT and the p53 tumor suppressor gene has been investigated in many human cancers. A significant correlation between MT expression and p53 expression has been reported in carcinoma[21].

We found significantly changed gene expression of GHR, p53 and MT I & II in ENU treated liver are correlated with time, which means the expression is time dependent. Some of them are well known cancer relate gene, and they were highly expressed in the mouse brain. Increased expression of p53 and MT at the 4 hrs ENU-treatment showed that certain carcinogenic actions occurred in the early time. MT is closely related to the tumor grade and proliferative activity. Additional experimental data, on these genes in carcinogenesis, are needed to elucidate the biological functions during ENU-induced hepato- and neuro -carcinogenesis, along with other cancer molecular markers.

Methods

Animals

We obtained mice from Charles River Japan. All mice have been maintained in the barrier system under the specific-pathogen free (SPF) condition with regulated lightning (12 h/12 h day/night cycle), temperature (24 ± 0.5°C), relative humidity (55 ± 5%) and with free access to pellet rodent chow (Purina, Korea) and water. Animals were acclimated to this environment for two weeks prior to the start of the study. The care and treatment of animals were given in accordance to the *Guide for the Care and Use of Laboratory Animals* (NIH).

Chemicals

N-ethyl-*N*-nitrosourea (ENU) was purchased from Sigma Chemical Company. Trizol, RNeasy MiniElute Cleanup kit (QIAGEN, Germany), Reverse transcription system (Promega, U.S.A.), 2X QuantiTect SYBR RT-PCR, sense and anti-sense primer (0.5 μM), 0.5 μl QuanTect RT mix)) were used conventional molecular biology work.

Animal Treatment and Sample Collection

Phosphate buffered saline, ENU 50 mg/kg (low dose), 250 mg/kg (high dose) were injected to the intraperitoneal of BALB/c male mice, respectively. Liver and brain samples were taken at 4, 12, 24, or 48 hrs. Animals were taken to a deep plain of anesthesia diethyl ether. A cross section of the left lateral lobe of the liver and brain were collected in RNase-free tubes and snap frozen in liquid nitrogen. Frozen tissues were stored at −70°C until RNA extraction process. Samples for the histopathology were collected in 10% neutral buffered formalin.

RNA Extraction

Liver tissues were lysed with Trizol according to the manufacture's protocol and total RNA were extracted and stored −80°C. The concentration and purity of total RNA were measured with NanoDrop (Nanodrop technologies, U.S.A.) and the 260/280 ratio of RNA was 1.8 to 2.0. The quality were checked by Agilent 2100 bioanalyzer (Agilent, Germany).

cDNA Synthesis and Identification

cDNA was synthesized from Reverse transcription system (Promega, U.S.A.) according to the manufacture's protocol. Reverse transcription reaction was performed in a 20 μl reaction containing 1 μl RNA template, 4 μl $MgCl_2$, 2 μl reverse transcription 10X buffer, 2 μl dNTP mix (10 mM each), 1 μl oligo (dt) primer (0.5 μg), 0.5 μl AMV Reverse Transcriptase, 0.5 μl Recombinant RNasin Ribonuclease inhibitor and nuclease-free water. After mixture was reacted at 42°C for 15mins, the mixture was heated at 95°C for 5 mins, then cool the sample for 5 mins at 4°C.

Analysis of DNA Chips

Total RNA was purified by using the RNeasy MiniElute Cleanup Kit (QIAGEN, Germany). Individual

RNA was generated by pooling same time and the use of same dose of samples. Mouse 10 K cDNA chip (Gaia gene, Korea) was used for gene expression profiling experiments. Cy5- and Cy3-labeled probes were combined and hybridized to microarrays (10 K) for the overnight at 42°C. Microarrays were washed, dried and scanned using an Axon Scanner (Axon Instrument Inc., U.S.A.). Fluorescence intensities of the Cy3 and Cy5 channels were quantitated using GenePix software (Axon Instrument Inc.). Gene expression data were analyzed as follows: the Cy3/Cy5 ratios of each gene's replicates were averaged and normalized to the ratio of total fluorescence across the slide. Normalized ratios were log base-2 transformed. Hierarchical clustering was performed for data analysis.

Real-time PCR

Real-time PCR performed using the Rotor gene 3000 (Corbett research, Australia). Master mix solution prepared with 25 μl 2X Quantitect SYBR Green RT-PCR, 1 μl sense and antisense primer (0.5 mM), 0.5 μl QuanTect RT mix and 22.5 μl RNase-free water. Master mix solution was aliquot to the tubes. Template RNA ($\leq$500 ng/reaction) was added in the master mix tube and incubated for 10 mins in ice. Amplification reactions were carried out using the following temperature profile: 50°C, 30 mins; 95°C, 15 secs; 55°C, 30 secs; 72°C, 30secs for 40 cycles. Fluorescence emission was detected for each PCR cycle and the threshold cycle (C_T) values were determined. The C_T value was defined as the actual PCR cycle when the fluorescence signal was increased above the background threshold.

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