Original

Analysis of Gene Expression Profile on Uterine Tumorigenesis Initiated with *N*-ethyl-*N*-nitrosourea and Inhibited by Ethinylestradiol in *rasH2* Mice

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Abstract: In order to clarify the possible mechanisms underlying the inhibition of uterine tumorigenesis in *rasH2* mice carrying a human prototype *c-Ha-ras* gene when treated with ethinylestradiol (EE), mice were given a single intraperitoneal injection of 120 mg/kg *N*-ethyl-*N*-nitrosourea (ENU), followed by 2.5 ppm EE for 6 weeks. Many genes involved in estrogen responses and cell proliferation in the uterus of *rasH2* mice are activated by treatment with ENU followed by EE. These include genes associated with receptors such as estrogen receptor (ER) alpha, cell cycles such as cyclin E, cyclin dependent kinase (CDK) 4, CDK inhibitors, ubiquitin and smad3, and growth factors such as epidermal growth factor (EGF) receptor, transforming growth factor (TGF) beta, TGF beta receptor and insulin-like growth factor (IGF). In this study, the ENU+EE treated *rasH2* mice demonstrated acceleration of estrogen decomposition and down-regulation of the expression of ER alpha. TGF beta, serine/threonine kinase and smad3, which are downstream of the TGF beta signaling pathway, were also down-regulated, indicating signal values such as CDK4 were down-regulated in the ENU+EE treated *rasH2* mice. Ubiquitin which indicates CDK inhibitory metabolism was also down-regulated. Finally, several genes involved in growth factors such as EGF receptor, TGF beta receptor and IGF were also down-regulated. On the other hand, the ENU+EE treated ICR mice showed deceleration of estrogen decomposition and up-regulation of the expression of ER alpha, down-regulation of TGF beta, serine/threonine kinase, smad3, CDK4, ubiquitin, EGF receptor, TGF beta receptor and IGF, indicating that these genes appear to act as stimulants of cell proliferation and carcinogenesis. Therefore, we consider that these genes are key genes which are strongly involved in the inhibition of uterine carcinogenesis in ENU-initiated *rasH2* mice treated with EE.

(J Toxicol Pathol 2004; 17: 155–164)

Key words: mechanism, *rasH2* mice, inhibited uterine tumorigenesis, gene expression profile

Introduction

Estrogen has potent tumor promoting effects in the uterus, liver, and kidney of experimental animals^{1–3}. In the rodent uteri *in vitro*, estrogen stimulates DNA synthesis and cell proliferation in the luminal and glandular epithelia. Although, to date, several approaches to help decipher the signaling pathways involved in uterine carcinogenesis have

been introduced for rats and mice, links between hormonal carcinogenesis promoted by estrogens and overexpression of related oncogenes have yet to be definitively demonstrated. In our previous studies in which *N*-ethyl-*N*-nitrosourea (ENU) was intraperitoneally given to transgenic mice carrying a human prototype *c-Ha-ras* gene (*rasH2* mice), the incidences of uterine adenocarcinomas, atypical hyperplasias and endometrial hyperplasias were significantly increased, compared to those in non-transgenic mice initiated with ENU⁴. In contrast, the incidences of these uterine proliferative lesions in ENU-initiated *rasH2* mice followed by dietary treatment of 2.5 ppm ethinylestradiol (EE) for 26 weeks were completely inhibited, although significantly high incidences of these proliferating lesions were observed in ENU-initiated ICR mice followed by dietary treatment of 2.5 ppm EE for 26

Received: 29 March 2004, Accepted: 24 June 2004

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weeks⁵. Proliferating cell nuclear antigen (PCNA) positive indices for uterine adenocarcinomas and atypical hyperplasias in ENU+EE treated ICR mice showed high values, but those in rasH2 mice given ENU alone were comparable to the intact uterine epithelium⁶. Moreover, the immunohistochemical expression of the estrogen receptor (ER) alpha in the uterine luminal and glandular epithelia of the ENU + EE treated ICR mice group was moderate to marked in intensity, compared to that in the ENU alone group (slight in intensity), and there was no consistent difference in ER alpha expression in the uterine luminal and glandular epithelia between the ENU + EE and ENU alone groups of rasH2 mice. The purpose of the present study was to clarify the response to EE in the uterus of rasH2 mice in which proliferative lesions do not develop as a preliminary study to investigate the mechanisms underlying the inhibition of uterine carcinogenesis. The gene expression patterns from uterine tissues in rasH2 mice given EE for 6 weeks after ENU initiation were examined using a high density gene chip array and compared with those in ICR mice subjected to the same treatment.

Materials and Methods

Animals and housing

A total of 6 female, 6-week-old rasH2 mice were obtained from the Central Institute for Experimental Animals (CIEA: Kawasaki, Japan), and 6 female ICR mice of the same age were obtained from Japan SLC, Inc. (Shizuoka, Japan). Throughout the acclimatization and experimental periods, animals were housed at a maximum of 3 per polycarbonate cage with absorbent hardwood bedding (White Flakes, Charles River Inc, Tokyo, Japan) in an air-conditioned animal room (room temperature, $24 \pm 2^\circ\text{C}$; relative humidity of $60 \pm 10\%$; and 12 hours light/12 hours dark lighting cycle). All animals were transferred to clean cages with fresh bedding twice weekly. The mice were quarantined for 2 weeks in the animal room assigned for the study, and only those without any abnormal findings at the end of this acclimatization period were selected for the experimental period. This study was carried out in accordance with the Guide for Animal Experimentation in the National Institute of Health Sciences of Japan.

Chemicals

N-ethyl-*N*-nitrosourea (CAS No. 759-73-9) and ethinylestradiol (CAS No. 57-63-6) were purchased from Nacalai Tesque, Inc (Kyoto, Japan).

Experimental design

After the acclimatization period, all female ICR and rasH2 mice were each divided into 2 groups consisting of 3 animals each (groups 1 and 2: ICR mice; groups 3 and 4: rasH2 mice). Groups 1 and 2 received a single intraperitoneal injection of 120 mg/kg ENU, followed by 0 and 2.5 ppm EE for 6 weeks, respectively (group 1: ENU alone; and group 2: ENU+2.5 ppm EE). The same

administration and doses were used for the rasH2 mice (group 3: ENU alone; and group 4: ENU+2.5 ppm EE). All surviving animals were checked for estrous cycles by vaginal smear at 2 weeks before the final sacrifice at week 6, when they were exsanguinated from the posterior vena cava under ether anesthesia and subjected to autopsy. After measuring the uterine weights, the uteri were frozen in liquid nitrogen for RNA extraction and subjected to the microarray analysis.

RNA isolation

Total RNA was isolated from the frozen tissue using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions, and purified using RNeasy Maxi Kit (Quiagen, Valencia, CA). Total RNA was prepared from each uterus of ICR or rasH2 mice administered either ENU alone or in combination with 2.5 ppm EE, and equal amounts of total RNA were pooled from each preparation. The former was used as a control.

High density oligonucleotide microarray analysis

Murine Genome U74AV.2 Arrays, which contained 6,000 probe sets of known genes and 6,500 probe sets of expressed sequence tags (ESTs) were purchased from Affymetrix (Santa Clara, CA). For microarray probing, reverse transcription, second strand synthesis, and probe generation were all accomplished by the standard Affymetrix protocol (Affymetrix)⁷. Briefly, from 10 μg of total RNA, the first strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Groningen, the Netherlands) and a T7-(dT)24 primer (Amersham Bioscience, Buckinghamshire, UK), and then the double strand cDNA was synthesized with *Escherichia coli* RNase H, *E. coli* DNA polymerase I and *E. coli* DNA ligase (Invitrogen). From the double strand cDNA, biotin-labeled cRNA was prepared using the BioArray HighYield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). Twenty micrograms of labeled cRNA was fragmented, and the MGU74AV.2 arrays were hybridized as described in the Gene Chip Expression Analysis Technical Manual (Affymetrix). The arrays were stained with R-Phycoerythrin Streptavidin (Molecular Probes, Eugene, OR, USA), and the fluorescence was intensified by the antibody amplification method. The arrays were scanned with a GeneArray scanner (Agilent Technologies, Palo Alto, CA, USA). The derived signal value was globally normalized and targeted to all probe sets equal to 100 before comparative analysis.

Microarray data analysis

To examine the gene expression differences between ENU alone treatment and ENU+EE treatment, we performed comparison analysis using the Affymetrix data suite system, MAS 5.0. The genes (probe sets) showing greater than a two-fold change in value with a change of the increase (I) or decrease (D) were designated as changed genes.

Fig. 1. Pie charts showing gene classification of >2-fold up-regulated 1140 genes in the ENU+EE treated rasH2 mice.

Fig. 2. Pie charts showing gene classification of <-2-fold down-regulated 1628 genes in the ENU+EE treated rasH2 mice.

Results

No animals died during the experimental period. Body weight gains of the ENU+EE group in each strain were depressed throughout the study, although no significant differences in food consumption were observed between the ENU+EE and ENU alone groups. The absolute and relative uterine weights in the ENU+EE group of rasH2 mice were significantly higher than those in the ENU alone group. All mice of both strains in the ENU+EE group showed persistent estrus in the examination of vaginal smears.

Gene expression profiles of uterine tissues in mice treated with ENU+EE are summarized in Figs. 1–4. The genes with unknown function, which were 34–40% in the expression profiles, were excluded from Figs. 1–4. In the ENU+EE treated rasH2 mice group, the numbers of >2-fold up-regulated genes, as compared to the samples from uterine tissues of rasH2 mice of the ENU alone treated group, were 1140, whereas the numbers of <-2-fold down-regulated genes were 1628 (Figs. 1 and 2). When the fold-change criteria was increased to >3-fold, 570 and 1965 genes were found to be up-regulated in rasH2 mice and ICR mice, respectively, 775 and 207 genes being down-regulated in rasH2 mice and ICR mice, respectively (data not shown). Specifically, the up-regulated genes in the ENU+EE treated

rasH2 mice group included several genes involved in metabolism such as 11beta-hydroxysteroid dehydrogenase/carbonyl reductase (average fold change: 2.0), cell growth and maintenance such as Ca^{2+} dependent activator protein for secretion (3.3-fold), and transcription such as max dimerization protein (8.1-fold) (Table 1). The down-regulated genes in the ENU+EE treated rasH2 mice group also included several genes involved in metabolism such as hydroxysteroid sulfotransferase (mSTa2) (–2.8-fold), cell growth and maintenance such as inhibition alpha subunit (–3.5-fold) (Table 2). In contrast, in the ENU+EE treated ICR mice group, 6567 genes were >2-fold up-regulated, and 409 genes were <-2-fold down-regulated as compared to the ENU alone treated ICR mice (Figs. 3 and 4). In detail, the up-regulated genes in the ENU+EE treated ICR mice group included several genes involved in metabolism such as hydroxysteroid dehydrogenase 3 (2.9-fold), cell growth and maintenance such as ros proto-oncogene (13.6-fold), and transcription such as homeo box D1 (10.8-fold) (Table 3). The down-regulated genes in the ENU+EE treated ICR mice group also included several genes involved in metabolism such as hydroxysteroid 17-beta dehydrogenase II (–3.6-fold), cell growth and maintenance such as mitogen-activated protein kinase kinase (MKK7) (–4.0-fold), and transcription such as IFN-response binding factor 1 (–6.4-

fold) (Table 4).

As the second step selecting the key genes that are related to the enhanced uterine carcinogenesis in the ENU+EE treated ICR mice and depressed uterine

carcinogenesis in the ENU+EE treated rasH2 mice, we selected the candidate genes up-regulated in rasH2 mice and down-regulated in ICR mice or the candidate genes down-regulated in rasH2 mice and up-regulated in ICR mice, based

Fig. 3. Pie charts showing gene classification of >2-fold up-regulated 6567 genes in the ENU+EE treated ICR mice.

Table 1. Expression Profiles Categorization Based on the Biological Process (up-regulated gene in ENU+EE rasH2 mice)

Biological process	Gene name	Average fold change	Accession number
Metabolism			
Steroid metabolism	11beta-hydroxysteroid dehydrogenase/carbonyl reductase	2.0	X83202
	17-beta-hydroxysteroid dehydrogenase type 1	3.3	AF039299
Alcohol metabolism	Aldehyde dehydrogenase II	4.7	M74570
Lipid metabolism	Hepatic lipase	3.8	X58426
Fatty acid metabolism	Long chain fatty acyl CoA synthetase	2.4	U15977
Electron transport	Cytochrome P450, 2d11	3.5	AI595772
	Cytochrome P450 monooxygenase (CYP2J6)	2.9	U62295
Cell growth and maintenance			
Cell growth and maintenance	Ca ²⁺ dependent activator protein for secretion	3.3	D86214
Regulation of cell cycle	EGF-like growth factor receptor ErbB4 intracellular domain	2.0	AF059177
Ion transport	Potassium channel, subfamily K, member 1	3.6	AF033017
Cation transport	Plasma membrane Ca ²⁺ -ATPase 2	3.5	AF053471
Cell proliferation	Protein tyrosine phosphatase, non-receptor type 18	2.0	U49853
Cell adhesion			
Cell adhesion	CD6 antigen	12.3	U37543
	Vascular cell adhesion molecule-1 truncated form T-VCAM-1	2.9	U12884
	Type 2 desmoglein	2.7	AJ000328
	Procollagen, type XVIII, alpha 1	2.3	L22545
Signal transduction			
G-protein coupled receptor signaling	Cannabinoid receptor 2 (macrophage)	6.0	X86405
Intercellular signaling cascade	Natriuretic peptide receptor A	3.8	L31932
Immunity and defence			
Immune response	Small inducible cytokine A2	3.4	M19681
Complement activation	Complement factor H-related protein	2.3	M29007
Transcription			
Regulation of transcription	Max dimerization protein	8.1	X83106
	Cyclin dependent kinase inhibitor 2B	4.3	AF059567
Morphogenesis			
Skeletal development	Homeo box D12	2.5	X58849
Neurogenesis	mammalian achaete scute homolog 2	3.2	U77628
Cell death			
Apoptosis	Amyloid beta (A4) precursor-like protein 1	2.3	L04538

Fig. 4. Pie charts showing gene classification of ≤ -2-fold down-regulated 409 genes in the ENU+EE treated ICR mice.

Table 2. Expression Profiles Categorization Based on the Biological Process (down-regulated gene in ENU+EE rasH2 mice)

Biological process	Gene name	Average fold change	Accession number
Metabolism			
Steroid metabolism	Hydroxysteroid sulfotransferase (mSTa2)	-2.8	L27121
Lipid metabolism	Monoglyceride lipase	-2.6	AJ001118
Amino acid metabolism	Glutamic acid decarboxylase 1	-3.1	Z49976
Electron transport	Cytochrome P450, 26 retinoic acid	-9.8	Y12657
	CYP 1-b-1	-7.6	X78445
Cell growth and maintenance			
Cell growth and maintenance	Inhibin alpha subunit	-3.5	X55957
Regulation of cell cycle	Insulin like growth factor binding protein 4	-3.1	X76066
	Cyclin B1	-2.9	X64713
	Cyclin B2	-2.3	X66032
	Cyclin E	-2.0	X75888
Regulation of cell growth	Insulin-like growth factor binding protein 5	-3.4	L12447
Ion transport	Calcium channel, P/Q type, alpha 1A	-12.1	U76716
	Glutamate receptor, ionotropic, NMDA2B (epsilon 2)	-4.7	D10651
Cation transport	Potassium channel Kv4.2	-22.8	AF107780
Cell proliferation	Fibroblast growth factor 6	-9.9	M92416
	Hepatocyte growth factor	-5.3	X72307
Cell cycle	Growth arrest specific 2	-11.0	M21828
	Cell division cycle 7-like 1	-2.8	AB019388
Mitosis	Cell division cycle control protein 2a	-4.0	M38724
	Cyclin A2	-2.5	X75483
Cell adhesion			
Cell communication	Connexin-36	-2.0	Y14331
Cell adhesion	Procollagen, type XI alpha 1	-19.8	D38162
	Procollagen, type XIII, alpha 1	-15.8	U30292
	Procollagen, typeXVIII, alpha 1	-6.4	U03715
	Procollagen, typeXV	-4.0	D17546
	Cadherin 16	-6.5	AF016271
	Laminin, alpha 2	-4.1	U12147
Signal transduction			
G-protein coupled receptor signaling	G-protein coupled receptor (EBI1)	-5.5	L31580
	G-protein signaling 9 (RGS9)	-3.2	AF011358
Intercellular signaling cascade	Megakaryocyte-associated tyrosine kinase	-5.6	D45243
	Growth factor receptor bound protein 10	-4.4	U18996
Immunity and defence			
Immune response	Interleukin 10	-5.1	M37897
Morphogenesis			
Skeletal development	Mesenchyme homeobox 2	-15.0	Z16406
	Homeo box D1	-14.0	M87802
	Homeo box A10	-4.5	U08757
Muscle development	Myosin heavy chain, skeletal muscle	-2.4	M12289
Cell death			
Apoptosis	Bcl-x gene, promoter region	-2.8	AF088904

Table 3. Expression Profiles Categorization Based on the Biological Process (up-regulated gene in ENU+EE ICR mice)

Biological process	Gene name	Average fold change	Accession number
Metabolism			
Steroid metabolism	Hydroxysteroid dehydrogenase-3	2.9	M77015
	Hydroxysteroid dehydrogenase-4	4.0	L16919
	17-beta-hydroxysteroid dehydrogenase type 7	6.6	Y15733
	17-beta-hydroxysteroid dehydrogenase type 3	5.0	U66827
Lipid metabolism	Sterol-C5-desaturase	2.7	AB016248
Amino acid metabolism	Glutamate decarboxylase	7.5	D42051
	glycoprotein-associated amino acid transporter	3.4	AJ012754
Electron transport	Cytochrome P450, 3a16	5.0	D26137
Carbohydrate metabolism	Glycoprotein 39	9.3	X93035
	N-acetyl galactosaminidase, alpha	2.6	AJ223966
Cell growth and maintenance			
Regulation of cell cycle	Ros proto-oncogene	13.6	X81650
	Interleukin 1-alpha	4.2	M14639
	Interleukin 1-beta	9.3	M15131
	Transforming growth factor, beta2	3.8	X57413
Regulation of cell growth	Insulin-like growth factor binding protein 2	16.0	X81580
	Insulin-like growth factor binding protein 10	3.5	M32490
	Fibroblast growth factor	5.2	AF065903
	Fibroblast growth factor 5	7.5	M37823
	Fibroblast growth factor 6	3.9	M92416
	Fibroblast growth factor 9	2.3	U33535
Ion transport	Potassium voltage-gated channel	8.0	X60475
	K ⁺ channel beta-1	5.6	AF033003
	Chloride channel protein 3	3.9	AF029347
Cation transport	Transient receptor protein 1	17.3	U73625
Lipid transport	Fatty acid transport protein 4	2.9	AF072759
Cell cycle	Cyclin-dependent kinase inhibitor p27kip 1	5.7	U09968
	Cyclin-dependent kinase inhibitor 1A (p21)	5.0	U09507
	Cyclin-dependent kinase 5	2.7	D29678
	Cyclin D2	3.0	M83749
	Cyclin G2	2.1	U95826
Cell adhesion			
Cell communication	Gap junction membrane channel protein beta 2	5.0	M81445
	Gap junction membrane channel protein beta 6	15.6	Z70023
Cell adhesion	Integrin alpha 6	5.9	X69902
	Laminin, beta 3	5.2	U43298
	Collagen IV alpha 4 chain	4.6	Z35167
Cell-cell adhesion	Vascular cell adhesion molecule 1	5.1	M84487
	Intercellular adhesion molecule 2	4.4	X65493
Signal transduction			
G-protein coupled receptor signaling	Regulator of G-protein signaling 9	7.0	AF011358
	G-protein coupled receptor	4.0	D21062
Intercellular signaling cascade	Protein kinase C epsilon	5.1	AF028009
Immunity and defence			
Immune response	Fc receptor, IgG, alpha chain transporter	13.3	L17022
	Interleukin 10	3.6	M37897
Complement activation	Complement component 4 binding protein	26.9	M17122
	Complement factor H-related protein	5.4	M29009
Transcription			
Regulation of transcription	Homeo box D1	10.8	M87802
Morphogenesis			
Cellular morphogenesis	Type 1 epidermal keratin	4.4	M13805
Cell death			
Apoptosis	Tnf-receptor-associated factor 1	18.5	L35302
	Apoptosis inhibitor 1	4.8	U88908
	Bcl-2-binding protein	2.9	AF022223
	Caspase 7	2.4	U67321

Table 4. Expression Profiles Categorization Based on the Biological Process (down-regulated gene in ENU+EE ICR mice)

Biological process	Gene name	Average fold change	Accession number
Metabolism			
Steroid metabolism	Hydroxysteroid 17-beta dehydrogenase II	-3.6	Y09517
Electron transport	Cytochrome P450, steroid inducible 3a11	-27.4	X60452
	Cytochrome P450III A25	-4.1	Y11995
Cell growth and maintenance			
Regulation of cell cycle	Mitogen-activated protein kinase kinase (MKK7)	-4.0	AF026216
Ion transport	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 6	-3.2	X51986
Lipid transport	Apolipoprotein A-I gene	-11.7	U79573
	Very-long-chain acyl-CoA synthetase related protein	-4.1	AJ223959
Cell cycle	P38 delta MAP kinase	-4.9	U81823
Mitosis	Cell division cycle 25A	-3.8	U27323
Cell adhesion			
Cell adhesion	Cadherin 16	-7.7	AF016271
	Procollagen, type XIII, alpha 1	-3.4	U30292
	Laminin, gamma 1	-3.3	J03484
Signal transduction			
G-protein coupled receptor signaling	Orphan G protein-coupled receptor FEX	-3.7	AF110818
Intercellular signaling cascade	Nitric oxide synthase 1, neuronal	-2.8	D14552
Immunity and defence			
Immune response	Macrophage interferon inducible protein 10	-2.4	M33266
Complement activation	Complement receptor 2	-5.8	M29281
Transcription			
Regulation of transcription	IFN-response binding factor 1	-6.4	M55290
	Mesenchyme homeobox 2	-4.8	Z16406

Table 5. Representative Common Genes that Fluctuated in Opposite Directions between ENU+EE rasH2 and ENU+EE ICR mice

Gene name	Average fold change		Accession number
	rasH2	ICR	
Estrogen decomposition			
Hydroxysteroid 17-beta dehydrogenase II	7.1	-3.6	Y09517
Estrogen receptor			
Estrogen receptor alpha	-1.4	2.8	M38651
TGF beta/smad signaling pathway			
Transforming growth factor beta 1 induced transcript 4	-1.2	2.2	X62940
Transforming growth factor beta 1 induced factor 2	-1.3	2.8	AF075717
Transforming growth factor beta type 1 receptor	-1.8	2.0	L15436
Serine/threonine kinase (nek2)	-1.9	1.2	AF013166
Smad 3	-1.4	3.3	AB008192
Cell cycle			
Cyclin E	-2.0	1.2	X75888
Cyclin dependent kinase 4	-1.7	1.7	L01640
Cdk4 and Cdk6 inhibitor p18 protein	-1.6	1.7	U19596
Cdk4 and Cdk6 inhibitor p19 protein	-1.3	2.2	U19597
Cyclin-dependent kinase inhibitor 1C (p57)	-1.7	1.7	U22399
Ubiquitin-conjugating enzyme	-1.1	2.0	AJ130961
mutant p53	-1.3	2.1	AB021961
Growth factor			
Epidermal growth factor receptor (EGFR)	-3.6	1.9	L06864
Transforming growth factor-beta type 1 receptor	-1.8	2.0	L15436
Insulin-like growth factor 1	-4.3	1.5	X04480
Insulin-like growth factor 2	-2.6	1.9	X71922
Insulin-like growth factor binding protein 3	-3.0	1.9	X81581
Insulin like growth factor binding protein 4	-3.1	2.3	X76066

on the data in Tables 1 to 4. There were no candidate genes other than hydroxysteroid 17-beta dehydrogenase II among the genes that fluctuated more than 2-fold. Therefore, we extended the analysis to the genes in which fold changes were smaller than 2. In the ENU+EE treated *rasH2* mice, one gene involved in estrogen metabolism, hydroxysteroid 17-beta dehydrogenase II (7.1-fold), was up-regulated, whereas estrogen receptor such as ER alpha (−1.4-fold) was down-regulated (Table 5). Many genes related to transforming growth factor (TGF) beta/smad signaling pathway such as TGF beta 1 induced transcript 4 (−1.2-fold), TGF beta 1 induced factor 2 (−1.3-fold), TGF beta type 1 receptor (−1.8-fold), serine/threonine kinase (nek2) (−1.9-fold) and smad 3 (−1.4-fold) were down-regulated in *rasH2* mice, as shown in Table 5. Other down-regulated genes observed were involved in cell cycles such as cyclin E (−2.0-fold), cyclin dependent kinase (CDK) 4 (−1.7-fold), CDK4 and CDK6 inhibitor-p18 protein, -p19 protein (−1.6, −1.3-fold), CDK inhibitor 1C (p57) (−1.7-fold), ubiquitin-conjugating enzyme (−1.1-fold) and mutant p53 (−1.3-fold). Several genes involved in growth factor such as epidermal growth factor receptor (EGFR) (−3.6-fold), transforming growth factor-beta type 1 receptor (−1.8-fold), insulin-like growth factor (IGF) -1, -2 (−4.3, −2.6-fold), IGF binding protein-3, -4 (−3.0, −3.1-fold) were down-regulated genes in *rasH2* mice. In contrast, in the ENU+EE treated ICR mice, average fold changes of these genes showed fluctuations opposite to those in *rasH2* mice (Table 5).

Discussion

In the present study, we analyzed mRNA expression of some molecules involved in estrogen metabolism, TGF beta/smad signaling pathway, cell cycle and growth factor. With regards to estrogen metabolism, hydroxysteroid 17-beta dehydrogenase II (which is known to be involved in the decomposition of estradiol (E2) to estrone) was up-regulated in the ENU+EE treated *rasH2* mice compared with the ENU alone treated *rasH2* mice, indicating that the decomposition rate of EE is relatively rapid in *rasH2* mice. The findings that ER alpha and several transcription factors were down-regulated in the ENU+EE treated *rasH2* mice, suggest that it is difficult to progress in the direction of transcription and growth stimulation. In the TGF beta/smad signaling pathway, TGF beta (which acts as a suppressor of tumor formation in early stages of carcinogenesis and stimulates invasiveness and metastasis of carcinoma cells in late stages^{8,9}) was down-regulated in the ENU+EE treated *rasH2* mice. An alteration in the effects of TGF beta is caused by changes in TGF beta receptor levels at the cell surface or through epigenetic mechanisms^{8,9}. Therefore, it is considered that TGF beta is not involved in the inhibition of uterine tumorigenesis in the ENU+EE treated *rasH2* mice especially at an early stage. Serine/threonine kinase and smad3, which are downstream of TGF beta signaling pathway, were also down-regulated in the ENU+EE treated *rasH2* mice, indicating that it is difficult to progress in the

direction of transcription. In contrast, in the ENU+EE treated ICR mice, the genes described above were up-regulated, indicating that advance in the direction for transcription was accelerated and that these genes were involved in the enhanced uterine tumorigenesis.

It is known that estrogens (E2) and estrone (E1) are metabolized via two major pathways: formation of the 2- and 4-catechol estrogens and 16 alpha-hydroxylation^{10,11}. CYP1B1 which is the major enzyme catalyzing the synthesis of 4-catechol estrogen^{11,12} was down-regulated in both strains of the ENU+EE treated group (data not shown). However, the signal value was larger in the ENU+EE treated *rasH2* mice (approximately 1/7-fold) than that in the ENU+EE treated ICR mice (approximately 1/2-fold), indicating the difficulty of generating malignant tumors in the ENU+EE treated *rasH2* mice. With regard to the genes involved in cell cycle processes (cyclin E, cyclin dependent kinase such as CDK4, and CDK inhibitor such as p16INK4A, p18INK4C, p19ARF, and p57KIP2), these were down-regulated in the ENU+EE treated *rasH2* mice, whereas these genes were up-regulated in the ENU+EE treated ICR mice. It is known that four important pathways, the p21WAF1-p27KIP1-cyclin E-CDK2, β -catenin-Tcf-myc-telomerase, ARF-MDM2-p53, and pRb1-cyclin D1-CDK4/6-p16INK4A, are mediated through the appropriate functioning of cell-cycle mechanisms^{13,14}. In the pRb1-pathway, each of these components plays either a positive or a negative role in cell-cycle control mechanisms^{15,16}. This pathway in uterine endometrial carcinogenesis participates in the regulation of the cellular processes at the transition of G1/S phases of the cell-cycle in humans¹⁷. The phosphorylation of pRb1 is generally achieved by cyclin D1 complex with cyclin-dependent kinases (4 and 6) at the G1/S checkpoint. Conversely, expression of cyclin D1 is also dependent on the phosphorylation status of pRb1¹⁸. The potential inhibitors of CDKs (p15INK4B, p16INK4A, p18INK4C and p19ARF), inhibit CDK4 and, in particular, CDK6, whereas p21WAF1/p27KIP1/p57KIP2 inhibitors halt the broad spectrum of various cyclins (including cyclin E) and cyclin-dependent kinases (CDK and CDK4/6)¹⁹. In addition, ubiquitin, by which CDK inhibitor p27 is regulated²⁰, was also down-regulated in the ENU+EE treated *rasH2* mice, whereas it was up-regulated in the ENU+EE treated ICR mice. Based on the results in the present study, we concluded that there is a tendency to slow down the cell cycle at the G1 to S transition phase in the ENU+EE treated *rasH2* mice, whereas there is a strong tendency to accelerate the cell cycle in the ENU+EE treated ICR mice. Moreover, regarding the genes related to growth factors, in the ENU+EE treated *rasH2* mice, several genes involved in growth factors such as EGF receptor, TGF beta receptor, and IGF were down-regulated, while these genes acted as stimulants of cell proliferation and carcinogenesis in the ENU+EE treated ICR mice.

In our previous report in which *rasH2* and ICR mice were initiated with ENU followed by 2.5 ppm EE for 24 weeks, the PCNA positive indices for endometrial

hyperplasias, atypical hyperplasias and adenocarcinomas in ICR mice were higher than those for luminal and endometrial glandular epithelia in which no proliferative lesions were developed in ICR mice, while the values for adenocarcinomas and atypical hyperplasias in the ENU alone group were comparable to those of the intact luminal and endometrial glandular epithelium in rasH2 mice⁶. These data may suggest a possible functional disorder of the cell cycle only in the uterine epithelium of rasH2 mice. However, with regard to the enhanced carcinogenesis in various organs of rasH2 mice treated with genotoxic carcinogens, it has been reported that somatic mutations of codons 12, 13 and 61 of the c-Ha-ras transgene were detected in spontaneous or chemically induced tumors in rasH2 mice²¹⁻²⁷. On the other hand, the incidences of point mutations of the transgene appeared to vary in tumors of rasH2 mice, and some carcinogens induced extremely low incidences of transgene mutations^{21,28,29}. Thus, it can be considered that some factors other than the transgene mutation might be related to the tumor enhancement in rasH2 mice. It has been reported that c-Ha-ras mRNA gene expression in papillomas/squamous cell carcinomas of the forestomach induced by *N*-methyl-*N*-nitrosourea and lung adenomas induced by urethane in rasH2 mice were quantitated by means of the real-time RT-PCR, and overexpression of c-Ha-ras gene in the forestomach and lung tumors was found³⁰. No mutations of endogenous Ha-ras, Ki-ras and p53 genes in mice were detected in rasH2 mice, indicating that mutations of endogenous genes do not make a contribution toward carcinogenesis. Thus, it was proposed that overexpression of the transgene is the first important factor of the carcinogenesis in rasH2 mice rather than the transgene mutation. This explanation of the enhanced carcinogenesis in rasH2 mice is contradictory to the results of the present study and our previous study⁶.

In conclusion, we consider that the accelerated estrogen decomposition and the down-regulation of genes such as ER alpha, TGF beta, serine/threonine kinase and smad3, ubiquitin, EGF receptor, TGF beta receptor and IGF observed in the present study are strongly involved in the inhibition of uterine carcinogenesis in ENU-initiated rasH2 mice treated with EE. Since this is only a preliminary study, further analyses are needed to quantify the expression of these genes and identify additional genes involved in the inhibition of uterine tumorigenesis in ENU+EE treated rasH2 mice.

Acknowledgments: This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan.

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