Melphalan Modulates the Expression of *E7*-Specific Biomarkers in *E7*-Tg Mice

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Abstract. *HPV oncoproteins are selectively retained and expressed in HPV infected carcinoma cells. The E7 oncoprotein interacts with the tumour suppressor Rb, and leads to the progression of oncogenesis. In a previous study, E7 biomarkers were identified in E7 Tg mice. In this study, in order to investigate whether a genotoxic carcinogen would modulate carcinogenesis in the E7-Tg mice, an anticancer drug, melphalan, was intraperitoneally injected into E7-Tg mice for eight weeks at two-day intervals and then genes and proteins were analysed using Omics approaches and RT-qPCR. RT-qPCR was performed to confirm whether E7 biomarkers would be modulated by melphalan treatment in E7-Tg mice, revealing that up-regulated E7 markers such as cyclin B1, CD166, and actin α1 were down-regulated, whereas expression of down-regulated E7 markers such as vimentin was restored by melphalan treatment. These results suggest that melphalan inhibits carcinogenesis via modulating E7-specific genes and proteins expressed in the lung tissues of E7 Tg mice.*

Human papillomavirus (HPV) is a small circular double-stranded DNA virus that has been identified in cervical tumours associated with genital malignancies. It has been reported that many kinds of human malignancies could be related to HPV viruses (1-3). The key oncogenic role of HPV is to interfere with cell cycle and tumour suppressive functions of the cell through its oncoproteins such as E6 and E7. The E7 oncoprotein binds and inhibits the function of the retinoblastoma protein (pRb) that is a major regulator of the cell cycle. E7 protein is primarily localised in the nucleus, associates with hypophosphorylated pRb, and prevents its binding with E2F, thereby promoting cell cycle progression (4-6). Lung cancer is the major cause of cancer-associated death worldwide (7, 8). The possible role of HPV in the bronchial squamous cell lesions has been suggested and several studies reported that HPV has been found with variable frequency in lung cancer cases (1, 9, 10). The HPV virus is found in association with about 20% of lung cancer cases, with *E6* and *E7* genes of HPV16 and HPV18 as the most frequently found oncogenic viral genes (1). In a previous study using Omics approaches, it was confirmed that various transcripts and proteins including those of the cell cycle, migration, and adhesion, were modulated by the *E7* oncogene in the lung tissue of E7-Tg mice (11). These genes, related to cancer development, were proposed as novel prognostic biomarkers for lung cancer mediated by E7. In the present study, it was elucidated whether melphalan, a well-known anticancer drug, would affect these genes modulated by E7 and lung carcinogenesis. Melphalan hydrochloride, *p*-dichloroethyl amino-L-phenylalanine, is a chemotherapy agent that is used as a treatment for some types of cancer, including malignant melanoma, multiple myeloma (bone-marrow cancer), breast cancer, and leukemia (12, 13), and is known as a human carcinogen. Melphalan inhibits DNA and RNA synthesis by alkylating DNA, and induces DNA inter-strand cross linkages which are associated with apoptosis of cancer cells (14). There is much evidence for the carcinogenicity of melphalan in animal models. When administered by intraperitoneal injection, melphalan caused lymphosarcoma, lung tumours, and peritoneal sarcoma in animals (15-17). In this study, it was investigated whether

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Table I. *Primer pairs used for real-time qPCR analysis of gene expression.*

Gene	Forward primer	Reverse primer
β-Actin	CTGGGACGATATGGAGAAGA	AGAGGCATACAGGGACAACA
E7	ATGCATGGAGATACACCTACATTG	TTATGGTTTCTGAGAACAGATGGG
CDC46	TGGTGGGGTTGTCTGTATTG	GAGCAGCGAGAGTTCAAGGT
CD166	ATGGCATCTAAGGTGTCCCCT	AGACGGCAAGGCATGACAA
Gelsolin	TATCAGTGGTGTGGCTCTGG	CTTCTGGCTCTCCTCCCTCT
Actin alpha 1	ATGGCATCTAAGGTGTCCCCT	AGACGGCAAGGCATGACAA
Calnexin	TGGTTACTGTGTTTGCTGCTG	AGGAGTGCTGGCATCTGATTT
Cyclin B1	GGGTCACTAGGAACACGAAAA	CCAATGTCTCCAAGAGCAGTT
PDI 3	ATGCGCTTCAGCTGCCTA	AGAACTCGATAGCATGAGCC
Destrin	AGTTCAGGTTGCGGATGAAGT	AGAACTCGATAGCATGAGCC
Vimentin	ATTTCTCTGCCTCTGCCAAC	CCTGTCCATCTCTGGTCTCAA
Filamin	CCCAAACTCAACCCAAAGAA	CCTTCTGGGTCCTCAACAAA
Serpinb 1	AAATCCCAAACCTGCTACCC	CGAGTTCACACGGAAAGGAT
XIAP-1	GCTTAGCAATCTGGATGTCC	ATTCTCTTCCCTTCCTCAGG
2'-5'ODS	TCTGTTGCACGACTGTAGGC	CATCTTTCTGATGGGGCTGT
Check-point kinase 1	TGTGGAAGACTGGGATTTGGT	TCTATGGCCCGCTTCATGT
Sarcolipin	AATACTGAGGGGCCATGCTA	GGTGTGTCAGGCATTGTGAG
Cofilin 1	GACGACCCCTACACCACTTTT	TCCTCCTTCTTGCTCTCCTT
Histone H1	AGGTCGGTGGCTTTCAAGAA	GTGGCTTTGGGTTTCTTGC
Nucleolar protein 3	CAAACAGTGCGCATGCCAGA	ACATGTGGTCCCTGAACTGG

melphalan would exert an anticancer or a genotoxic carcinogenic effect in lung tissues of *E7* transgenic mice, and *E7* specific biomarkers would be modulated by melphalan in lung tissue.

Materials and Methods

E7 transgenic mice and melphalan treatment. *E7* transgenic mice were established by DNA microinjection of 20 μg *E7* DNA into embryos of BDF1 mice as previously described (11). These mice were obtained for *E7* oncogene modulator analysis in a mouse model. Male and female *E7*-Tg mice and non-Tg (12-week-old) littermates were injected intraperitoneally with 0.6 mg/kg of melphalan (Sigma, St. Louis, MO, USA) for eight weeks at two-day intervals. After two months of treatment, both *E7*-Tg and non-Tg mice in the injection groups, as well as both *E7*-Tg and non-Tg mice of the control groups were sacrificed and used for analysis of *E7* biomarkers. Experi-mental treatments were carried out according to the guidelines for animal experimentation at the Faculty of Laboratory Animal Research Center, Konkuk University (Seoul, Korea).

Histopathological examination. The lung tissue samples from the vehicle-treated controls or the melphalan-treated mice were fixed in 10% neutral buffered formalin immediately after organ harvest. The tissues were subsequently embedded in paraffin and sectioned into 3-4 μm slices, which were stained with haematoxylin and eosin (H&E) according to standard methods. The histopathological diagnoses were based on the criteria described by Frith and Ward (18).

Genomics analysis. Gene expression analysis was conducted on RNA samples from the lung tissue of *E7*-Tg mice from the groups treated with melphalan and the control group. Total RNA was prepared from mouse lung using TRIzol reagent (Invitrogen, NY, USA). For the microarray experiment, 10 μg of total lung RNAs from mice was used for cDNA synthesis as previously described (19). Briefly, RNAs were pooled because pilot experiments with Affymetrix chips at Pfizer indicated that the inter-animal variability in gene expression, as well as variability between repeated hybridizations of the same pooled RNA sample, was statistically insignificant. Labelling and hybridization were performed using the Affymetrix 430 2.0 Array Kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. Fluorimetric data were processed by Affymetrix Gene Chip3.1 software. Genes that had an average difference value above the threshold of 100 and at least 2-fold average difference values between melphalan-treated group and control group of *E7*-Tg mice were selected.

Proteomics analysis. Lung tissues of each mouse were homogenised and lysed in RIPA lysis buffer containing 150 mM NaCl, 10 mM Tris (pH7.4), 0.1% SDS, 1% TritonX-100, 0.1% SDC, 5m M EDTA, and 0.1 M Na-orthovanadates. Total proteins (100 μg) were loaded on 12% SDS-polyacrylamide gel. The gel fragments were excised in 10 bands according to molecular weight and digested with sequencing grade modified trypsin (Promega, Madison, WI, USA) at 12.5 ng/μl in 50 mM NH_4HCO_3 buffer (pH 8.0) at 37˚C overnight. Following digestion, tryptic peptides were extracted with 5% formic acid in 50% ACN solution at room temperature for 20 min. The samples were purified and concentrated using C18 ZipTips (Millipore, Billerica, MA, USA) before mass spectroscopy (MS) analysis. The tryptic peptides were loaded onto a fused silica microcapillary column (15 cm×75 μm) packed with C18 (5 μm, 200Å) reversed-phase resin and were separated by liquid chromatography using a linear gradient of 5-50% buffer A in 65 min followed by 50-90% buffer B in 5 min (buffer A: 0.1% formic acid in H_2O, buffer B: 0.1% formic acid in acetonitrile) at a flow rate of 250 nl/min. The column was connected directly to LTQ ion-trap

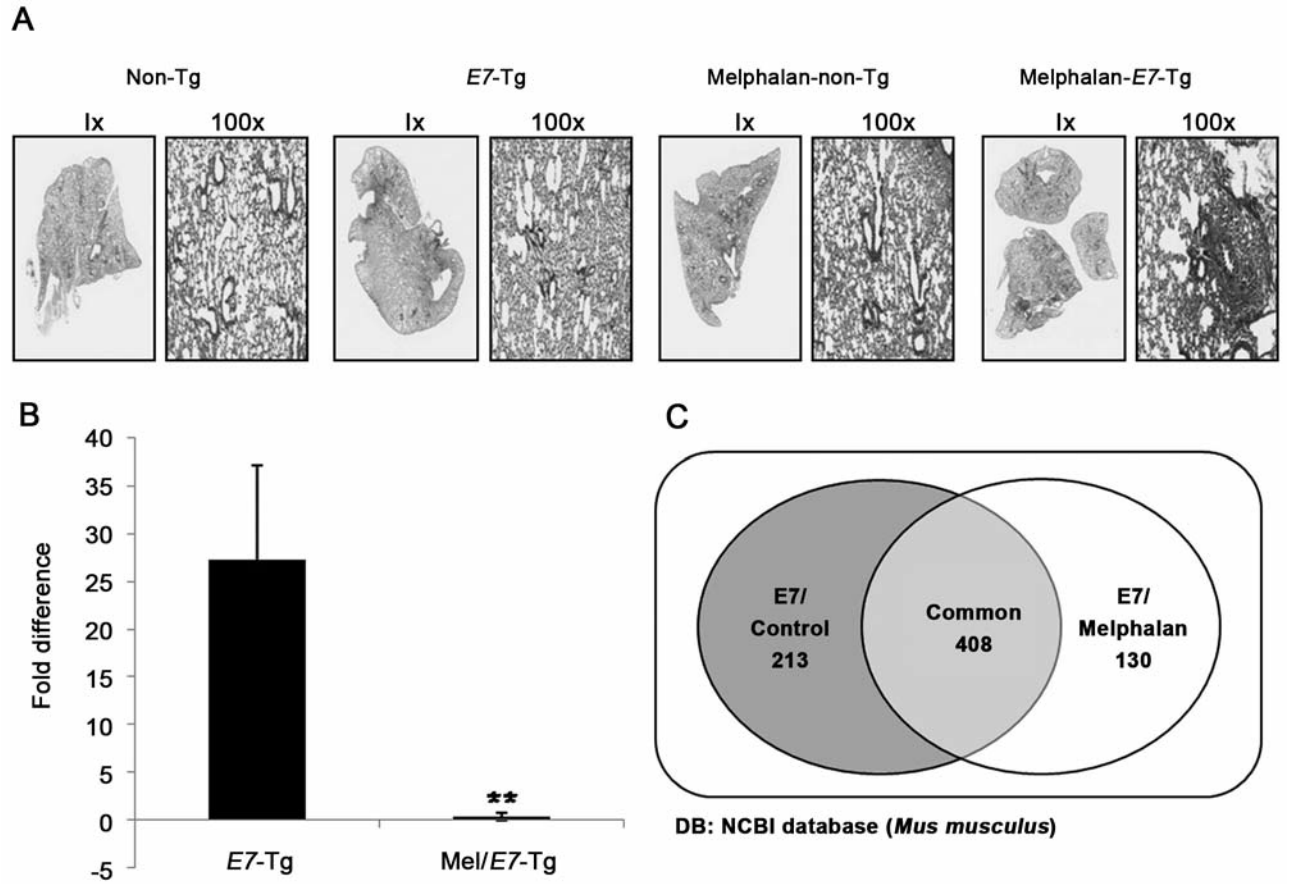

Figure 1. *A: Haematoxylin/eosin staining of lung tissues of non-Tg, melphalan-treated non-Tg, E7-Tg, and melphalan-treated E7-Tg mouse. B: Real-time qPCR analysis of E7 oncogene expression in E7-Tg mice and melphalan-treated E7-Tg mice. C: Proteomics analysis of protein profiling of lung proteins modulated by melphalan in E7-Tg mice. The number of proteins changed significantly in lung tissues from E7-Tg mice treated with melphalan.*

mass spectrometry (Thermo Finnigan, San Jose, CA, USA) equipped with a nano-electrospray ion source. The electrospray voltage was set at 2.0 kV, and the threshold for switching from MS to MS/MS was 500. The normalised collision energy for MS/MS was 35% of main RF amplitude and the duration of activation was 30 ms. All spectra were acquired in data-dependent scan mode. Each full MS scan was followed by nine MS/MS scans corresponding from the most intense to the ninth intense peaks of full MS scan. Repeat count of peak for dynamic exclusion was 1, and its repeat duration was 30 s. The dynamic exclusion duration was set for 180 s and exclusion mass width was ±1.5 Da. The list size of dynamic exclusion was 50. Data were calculated as previously described (11).

Quantitative real-time PCR. Total RNAs were isolated from the lung tissue of mice to measure the quantity of the various genes expression level by melphalan; 25×-diluted cDNA product was used as template. Real-time PCR was performed with relative quantification protocol on a Chromo 4 Real-Time PCR system (Bio-Rad, Hercules, CA, USA), using iQ™ SYBR® Green Supermix (Bio-Rad) for amplification detection. All target genes were normalised to the housekeeping gene, *β-actin*, and compared to normal mouse of the appropriate strain for relative expression values. Each sample was run in triplicate and fold changes represent the ratio of expression in *E7*-Tg mice to control non-Tg mice. The expression ratio of melphalan-treated non-Tg mice to control non-Tg mice and that of melphalan-treated *E7*-Tg mice to *E7*-Tg mice were also represented as fold difference. However, data from melphalan-treated *E7* -Tg mice were ultimately revealed as the ratio of melphalan-treated *E7* to *E7* in graphs and total data were plotted on the basis of control (non-Tg mice) expression values. The sequences of the primers used in this experiment are listed in Table I.

Statistical analysis. Results are presented as the mean±SD. Comparisons between *E7*-Tg and melphalan-treated *E7*-Tg groups were performed using Student's *t*-test. *P*-values <0.05 were considered significant.

Results

Histological examination and expression of E7 by melphalan. *E7*-Tg and non-Tg mice were treated with or without melphalan for eight weeks to assess the

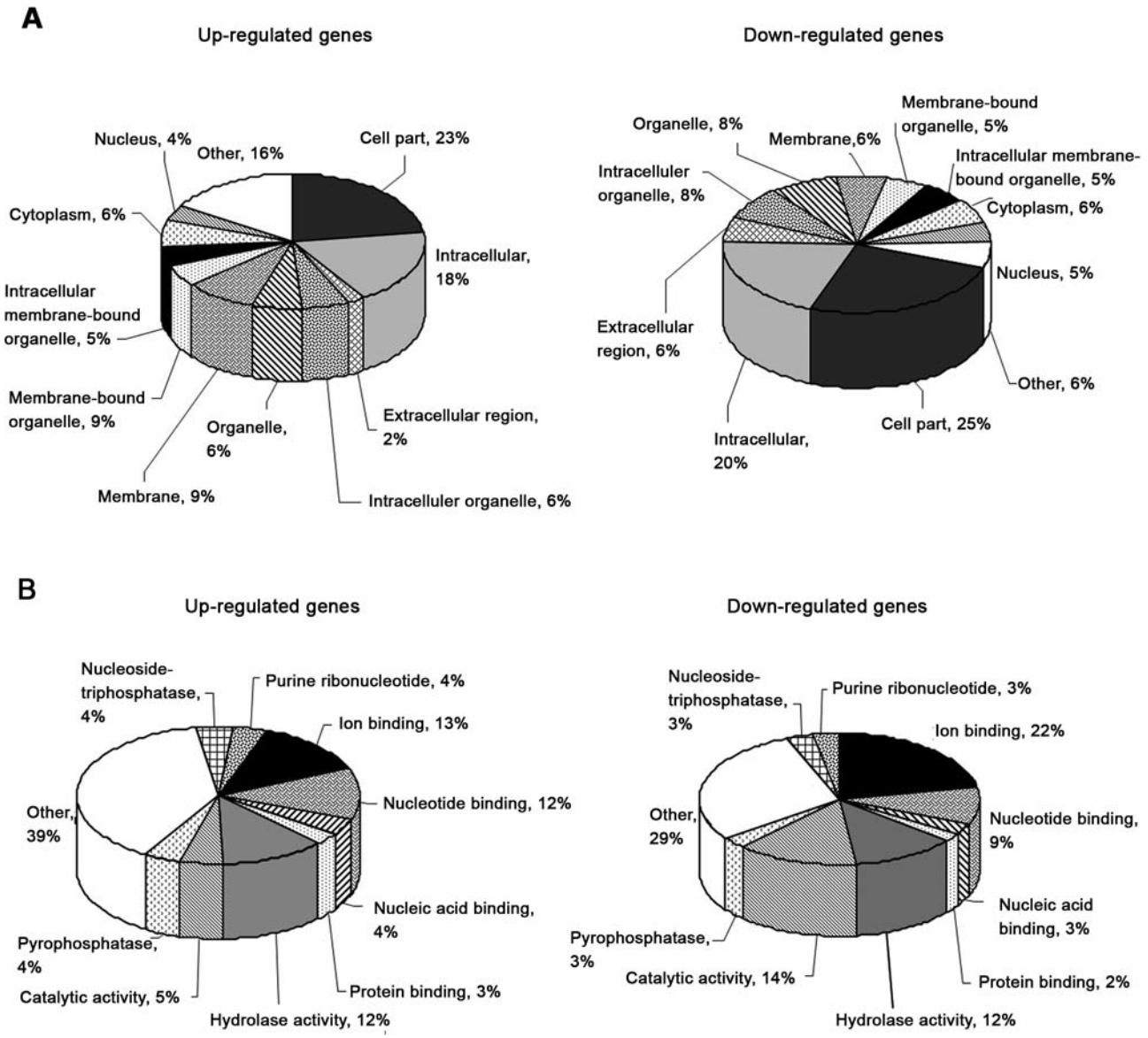

Figure 2. *Pie chart showing classification based on the cellular component (A) and the molecular function (B) of genes up- and down-regulated by melphalan in the lung tissues of E7-Tg mice.*

modulating effects of melphalan on expression of *E7*-specific biomarkers in lung tissues of Tg mice. H&E staining revealed that there were no significant alterations in the lung tissues between melphalan-treated and non-treated mice (Figure 1A). In addition, there were no adenomas developed even in lung tissues of melphalan treated *E7*-Tg mice. To investigate the effect of melphalan on expression of the *E7* oncogene in *E7*-Tg mice, *E7* expression level was detected by real-time qPCR analysis. *E7* expression was dramatically enhanced in *E7*-Tg mice compared to non-Tg mice (1.0-fold difference) and fully inhibited by treatment with melphalan (Figure 1B).

Omics analysis of the lung tissues of melphalan-treated E7-Tg mice. Genes and proteins modulated by melphalan in *E7*-Tg mice were analysed by Affymetrix GeneChip3.1 software and LC-ESI-MS/MS, respectively. Up- and down-regulated genes were categorised according to their cellular components and molecular functions. The expression levels of genes related to the nucleus, intracellular, extracellular, and membrane parts based on cellular components (Figure 2A), as well as binding, catalytic, and phosphatase activity based on molecular function (Figure 2B) were mostly modulated by melphalan. As shown in Table II, genes involved in cell cycle, proliferation, apoptosis, and

Table II. *Genes regulated in melphalan treated E7-Tg mice.*

A. Genes up-regulated in lung tissue

Gene	Log2 ratio
Chloride channel calcium activated 3	2.085877619
Schlafen 4	1.84621846
Interferon, alpha-inducible protein 27	1.493787361
LAG1 homolog, ceramide synthase 6	1.473648851
Immunoglobulin heavy chain complex	1.30929642
Interferon-induced protein 44	1.305403677
2'-5' Oligoadenylate synthetase-like 2	1.245273818
Interferon-induced protein with tetratricopeptide repeats 3	1.21273764
Macrophage activation 2 like	1.156806564
DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	1.113111292
Receptor transporter protein 4	1.074123527
Guanylate nucleotide binding protein 3	1.04813446
XIAP-associated factor 1	1.015173125

B. Genes down-regulated in lung tissue

Gene	Log2 ratio
Eukaryotic translation initiation factor 2, subunit 3, Structural gene Y-linked	-4.482510341
Jumonji, AT rich interactive domain 1D (Rbp2-like)	-3.677986081
Proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	-2.083898486
Arachidonate 15-lipoxygenase	-2.059860009
Inactive X-specific transcripts	-1.958953403
Serine (or cysteine) peptidase inhibitor, clade B, Member 2	-1.936284619
CD5 antigen-like	-1.48984543
Neurogranin	-1.265451499
C-type lectin domain family 1, member b	-1.138132478
Pro-platelet basic protein	-1.099598022
Peptidyl arginine deiminase, type IV	-1.094776885
Erythroid differentiation regulator 1	-1.064538821
Filamin, beta	-1.030835754
Coagulation factor V	-1.021510861

metabolic process such as interferon alpha inducible protein 27, DEAD (Asp-Glu-Ala-Asp) box polypeptide 60, XIAP-associated factor 1, and guanylate nucleotide-binding protein 3 were up-regulated, whereas several genes related to cellular processes and structure including proteoglycan 4, and filamin beta tended to decrease. The proteins expressed in *E7*-Tg mice (213 proteins) and melphalan-treated *E7*-Tg mice (130 proteins) were classified (Figure 1C). A total of 448 proteins were up-regulated and 303 down-regulated by melphalan treatment, and were classified by fold change, as shown in Table III. Proteins regulated by melphalan in the *E7*-Tg mice were classified according to the Gene Ontology database (http://www.geneontology.org) based on cellular composition and the biological process using the in-house

Table III. *Proteomics analysis of lung proteins modulated by melphalan in E7-Tg mice.*

Fold change	*Mus musculus*	
	Up-regulated	Down-regulated
<3	194	79
3~5	44	7
5~9	51	4
>10	159	213
Total	448	303

Data files were extracted using Spectrum Mill Data Extractor with the parameters of (MH+) 600 to 40000 and minimum signal-to-noise (S/N) 25. Searches were carried out against the human NCBInr database in both forward and reverse directions using the Spectrum Mill program (Agilent Technologies) with the following parameters: specific to trypsin with two missed cleavage; ±2.5 Da precursor-ion tolerance; and ±0.7 Da fragment-ion tolerance. The initial results were validated as described in the Materials and Methods section.

FindGo program as shown in Figure 3. Analysis based on the cellular composition (Figure 3A) showed that the nucleus (1->2%) and extracellular region proteins (6->5%) were slightly modulated by melphalan. Proteins were also classified based on their involvement in biological processes, as shown in Figure 3B. The proteins were classified into the following groups: proteolysis; immume system; response; cell adhesion; metabolism; transport and localisation; organisation and biogenesis; cell cycle and cytokinesis; translation and transcription and nucleotide; and protein modification. It was found that proteins involved in metabolism (24->25%), response (2->3%), proteolysis (3->4%), and translation/transcription/nucleotide (5->6%) were increased, while those involved in protein modification (5->4%), cell cycle/cytokinesis (6->5%), and organisation/biogenesis (12->9%) were decreased in lung tissue from melphalan-treated *E7*-Tg mice. Significantly modulated proteins found by comparing the expression profiles in the lungs of *E7*-Tg and melphalan-treated *E7*-Tg mice are listed in Table IV.

Identification of E7 specific markers modulated by melphalan in lung tissue of E7-Tg mice. To investigate the effect of mephalan on the expressions of *E7* modulators in the lung tissues of *E7*-Tg mice previously selected as putative *E7* biomarkers (13), quantitative real-time PCR analysis was used and the quantity of genes regulated by the melphaln in *E7*-Tg mice was measured. Non-Tg mice were used as the negative control, and *E7*-Tg mice and a melphalan-treated non-Tg mice were used as positive control *versus* melphalan-treated *E7*-Tg mice. Real-time

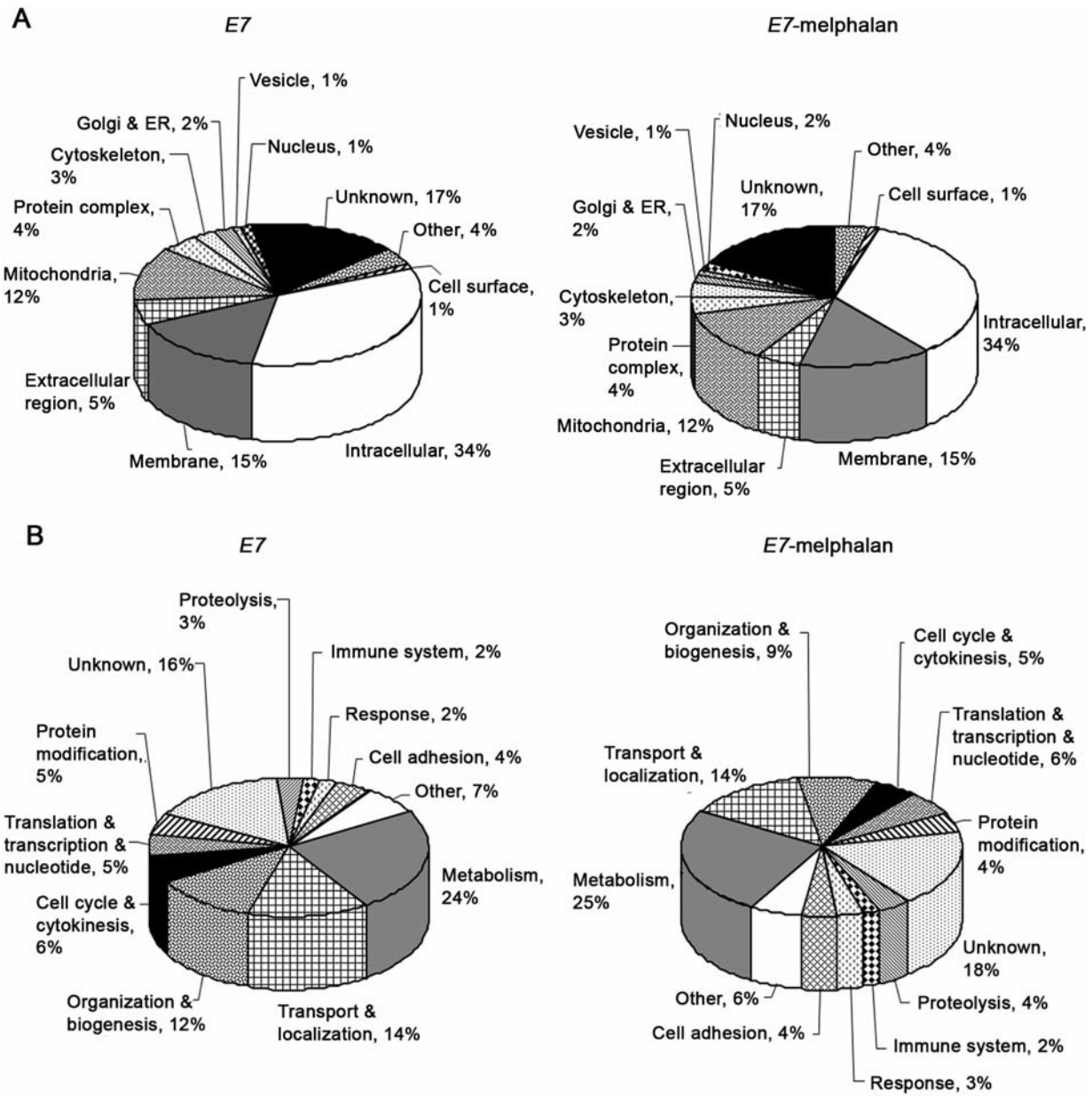

Figure 3. *Pie chart showing proteins expressed in E7-Tg control mice and melphalan treated E7-Tg mice based on the cellular composition (A) and the biological process (B).*

PCR was performed for selected genes related to cell adhesion, cell cycle and migration, proliferation and apoptosis listed in Table III and IV. In a previous study, it was reported that the *E7* oncogene significantly up-regulated *Calnexin* and *Gelsolin* (>10-fold) and weakly increased Cyclin B1, Cyclin E2, cell division cycle 46 (*CDC46*), activated leukocyte cell adhesion molecule CD166 (*CD166*), actin alpha 1 (*Acta1*), which may lead to oncogenesis. The expression of *Cofilin, Vimentin, Destrin* and protein disulfide isomerase associated 3 (*Pdia3*) were down-regulated by *E7* oncogene in lung tissue (11). These expression patterns of *E7* down-regulated genes such as *Vimentin* and *Cofilin* were recovered by melphalan treatment except *Destrin* and *Pdia3* (Figures 4 and 5). The proteins considered to be related to cancer cell adhesion, signal transduction, migration, and cancer development were selected by proteomics analysis. According to the gene and protein profiling data analysis, new target genes such as *Filamin*, serin proteinase inhibitor, clade B, member 1 (*Serpin b1*), X-linked inhibitor of apoptosis

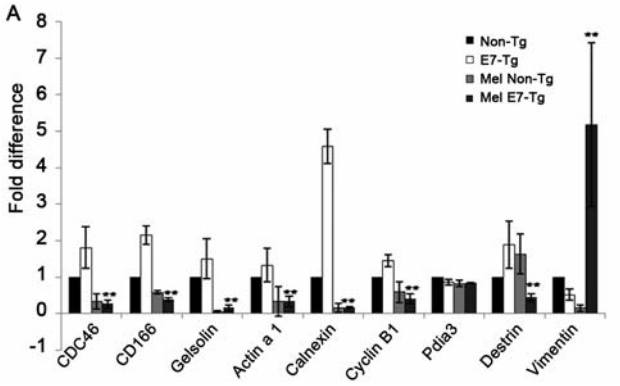

Figure 4. *The effect of melphalan on the expression of E7-modulated genes in E7-Tg mice. The gene expression level obtained from real-time qPCR was normalized by β-actin. The respective fold changes were expressed by comparing with the values of non-Tg mice which were set as 1. Data are the mean±SD of three independent experiments, each performed in triplicates. * and ** indicate statistical significance at p<0.05 and p<0.01, respectively.*

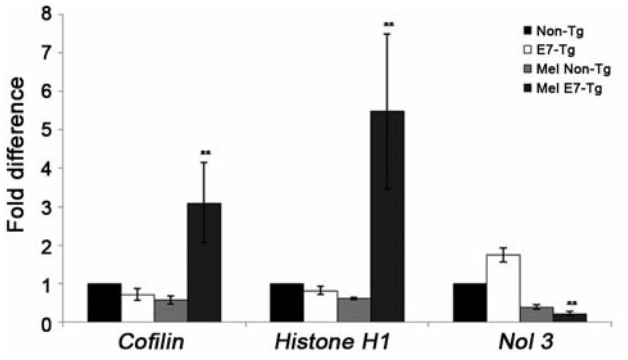

Figure 5. *The effect of melphalan on common biomarkers in the lung tissues of E7-Tg mice. **Statistically significant at p<0.01.*

protein associated factor 1 (*Xiap-1*), 2'-5' oligoadenylate synthetase-like 2 (*2'-5' Oas*), checkpoint kinase 1 (*Chk1*), and sarcolipin (*Sln*) were selected and evaluated by real-time qPCR. The gene and protein expression data were consistent with real-time qPCR analysis (Figure 4 and Table III, IV).

The effect of melphalan on common cancer biomarkers in the lung tissue of E7-Tg mice. Additional experiments were performed for gene profiling of several markers such as *Cofilin, Histone H1* and nucleolar protein 3 (*Nol-3*) known as predictive markers in tumour (20, 21). In order to investigate whether these predictive markers would be modulated by melphalan in *E7*-Tg mice, expression levels were assessed by RT-qPCR analysis. *Cofilin* and *Histone H1* were down-regulated while *Nol-3* was up-regulated by *E7* oncogene (Figure 5). Melphalan treat-ment reversed their expression levels. In particular, *Cofilin* and *Histone H1* were significantly increased in melphalan-treated *E7*-Tg mice (Figure 5).

Discussion

It has been reported that approximately 15-20% of all kinds of human malignancies might be related to oncogenic HPV (1-4, 22, 23). HPV DNA has been identified in cervical tumours, with types 16, 18, 31, 33, 45, and 51 most frequently associated with malignancies, and of these HPVs, HPV-16 is the most common found in cervical cancer (11). Depending on the risk of malignancy, HPVs are grouped as high- and low-risk types. HPV-16 E6/E7 cause malignant progression of lesions, while the low-risk HPVs do not induce malignancy (24, 25). The HPV E7 oncoprotein has been shown to bind to pRb and inactivate its function by preventing binding of pRb to E2F then transcription factor. E2F activates expression of genes required for the transition of G_1/S phase and the cells continually disrupt the cell cycle, leading to uncontrolled cellular proliferation (26). Lung cancer is a major cause of cancer-related death and a possible role of the *E7* oncogene in lung carcinogenesis is suggested by the presence of HPV DNA in lung tumours (27, 28). Many previous studies have therefore examined *E7* oncogene and lung tumours in lung cancer cell lines, as well as in transgenic mice (1, 11, 29). In this study, the effect of melphalan, known as an anticancer drug and a genotoxic carcinogen, on the expression of genes related to cancer development in lung tissue of *E7*-Tg mice was investigated. In order to elucidate whether melphalan would cause progression of malignant tumours, like a genotoxic carcinogen, or inhibit *E7*-modulated genes, like an anticancer drug, several non-Tg and *E7*-Tg were injected intraperitoneally with 0.6 mg/kg of melphalan for 8 weeks at two-day intervals. Histological analysis revealed no malignancy in lung tissue of treated compared to non-Tg and *E7*-Tg mice. A relatively small difference of lesion was detected in melphalan treated *E7*-Tg mice (Figure 1A). However, in the Omics approaches, transcripts and proteins related to cancer development were modulated by *E7* oncogene (11) or melphalan in lung tissue of *E7*-Tg mice. Taken together, melphalan did not cause progression of malignant tumours as a genotoxic carcinogen but modulated *E7*-specific biomarkers related to cell migration and cancer development. In a previous study, specific biomarkers in the lung tissues modulated by *E7* oncogene in E7-Tg mice were elucidated (11). *E7* oncogene up-regulated expression levels of genes related to cell cycle (*Cyclin B1, Cyclin E2*), cell adhesion, and cell migration (*Actin alpha 1, CD166*) factors (11). It was suggested that the *E7* oncogene may accelerate

Table IV. *Protein regulated in melphalan-treated E7-Tg mice*.

A. Proteins up-regulated in lung tissue

Protein name	Accession No.	Peptide unique no.	Mel-E7-Tg/ E7-Tg control
GAG12	29420429	2	
Caldesmon	21704156	1	
Coronin, actin binding protein 1A	31418362	1	
Creatine kinase, muscle	6671762	5	
Histone 1, H1d	34328365	1	
Lamin-B receptor	90101398	2	>>50
Muscle glycogen phosphorylase	6755256	2	
Myosin, light polypeptide 1	29789016	6	
Procollagen, type IV, alpha 2	36031080	1	
Troponin I, cardiac	6678393	1	
Vacuolar ATP synthase subunit E	1718091	1	
Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 isoform 1	46559389	11	<<50 >>10
Integrin alpha 3	7305189	3	
Laminin, gamma 1	31791057	3	<<10
Cofilin 1, non-muscle	6680924	5	>>6
AHNAK nucleoprotein isoform 1	61743961	36	
Related RAS viral (r-ras) oncogene homolog 2	13399308	5	
Annexin A2	6996913	16	
Calponin 1	31560611	3	
Integrin alpha 1	84370023	3	<<5
Aldolase 1, A isoform	6671539	6	>>*2*
Transferrin	20330802	12	
Cathepsin H	7106279	1	
Tropomyosin 4	47894398	5	
Rho GDP dissociation inhibitor (GDI) alpha	31982030	5	
Ubiquitin C	21070950	4	
Annexin A4	7304889	7	
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	10946952	1	
Moesin	70778915	25	<<2
Galectin-3	126679	1	>>1
Transgelin	6755714	6	
Fibronectin 1	46849812	6	
Vimentin	55408	20	
Tubulin, beta 2c	13542680	16	

List of the proteins, identified by their accession number and peptide unique number, up-regulated more than 1- to 50-fold in melphalan-treated *E7*-Tg *vs*. untreated *E7*-Tg mice. Because several intensities of *E7* are '0', fold changes of data up-regulated in melphalan-treated *E7*-Tg, represent the ratio of melphlan-*E7 vs*. *E7* as >> 50.

Table IV. *continued*

more progression of malignant tumours in a transgenic mouse model. The expression pattern of these genes was changed in melphalan-treated *E7*-Tg mice (Figure 4A). Cell cycle-, cancer cell adhesion-, and migration-related genes such as *CDC46, CD166, Actin alpha 1* and *Cyclin B1*, which may play important roles in cellular transformation in cancer, were significantly inhibited by melphalan treatment. Calnexin inhibits tumour suppressor genes (30) and destrin promote cytoskeletal dynamics by depolymerising actin filaments. Genes of both these proteins were increased in A549 human malignant lung epithelial cancer cell (31). Expression levels of calnexin and destrin were down-regulated in melphalan treated E7-Tg mice. In addition, melphalan down- regulated gelsolin, a key regulation factor

Table IV. *continued*

B. Proteins down-regulated in lung tissue

Protein name	Accession No.	Peptide unique no.	Mel-E7-Tg/ E7-Tg control
Alpha-tropomyosin striated-2	386003	1	
Proline arginine-rich end leucine-rich repeat	31543506	2	
Cxc14 protein	38174332	1	
Destrin	9790219	5	
Endothelial cell-selective adhesion molecule	22094995	5	<<1/100
Programmed cell death 8	6755004	1	
Serine (or cysteine) proteinase inhibitor, clade B, member 6b	15826844	3	
T-complex protein 1 subunit beta	22654291	3	
Tropomodulin-1 (E-Tmod)	1729983	1	
Profilin 1	6755040	6	<<0.1
Actin related 2/3 complex, subunit 5	13385866	1	>>0.05
Dynein light chain 2	18087731	1	
Capping protein (actin filament) muscle Z-line, beta isoform a	83649737	2	
Latexin	31980632	1	
Calnexin	6671664	3	<<1
Protein disulfide-isomerase precursor	129729	10	>>0.2
Calreticulin	6680836	6	
Diaphorase 1	19745150	7	
Gelsolin	28916693	7	

List of the proteins, identified by their accession number and peptide unique number, down-regulated more than 1~100 fold in melphalan-treated E7-Tg *vs*. untreated E7-Tg mice. Because several intensities of melphalan-E7 are '0', fold changes of data down-regulated in melphalan-treated E7-Tg, represent the ratio of mel-E7 *vs*. E7 as << 1/100.

of actin filament assembly and disassembly which inhibits apoptosis by stabilising the mitochondrial membrane potential loss and cytochrome c release (32), while melphalan up-regulated vimentin which plays a significant role in regulation of cell attachment and subcellular organisations (33). These results suggest that *E7* oncogene induces genes related to cancer development, and *E7* modulated genes are modulated by melphalan in lung tissues of E7-Tg mice. Expressions of new genes such as filamin, serine peptidase inhibitor, clade B, member 1 (*Serpinb1*), XIAP associated factor 1 (*XIAP*), 2'-5' oligoadenylate synthetase like 2 (*2'-5' Ods*), check point kinase 1 (*Chk1*), and sarcolipin (Table III, IV), selected on the basis of genomics and proteomics data, were analysed by real time RT-qPCR in E7-Tg mice treated with melphalan for 8 weeks. Filamin, an actin-binding protein that cross-links actin filaments in cortical cytoplasm and participates in the anchoring of membrane proteins for the actin cytoskeleton, and serpin b1, involved in cell shape and migration (34, 35), were up-regulated by the *E7* oncogene and their expressions were suppressed by melphalan (Figure 4B). In particular, serpin b1 is used as a possible biomarker for oral cancer metastasis and associated with the protease release, resulting in airway inflammation and damage of lung tissue (35). In other hand, *XIAP, Chk1, 2'-5' Ods* and *Sarcolipin* were up-regulated by melphalan (Figure 4B). Xiap, a member of the inhibitor of apoptosis family of proteins, and Chk1 prevent progression into mitosis in the presence of DNA damage, and regulate cell cycle progression and apoptosis by the interaction during the mitosis (36). 2'-5' Ods is a well-known mediator of BRCA1/IFN-γ-induced apoptosis, and it has been reported as a negative regulator of proliferation in several cancer cell lines (37, 38). Sarcolipin is highly expressed in skeletal muscles and involved in regulation of sarcoplasmic reticulum calcium ATPase pump in muscle cell (39). In the present study, it was investigated whether the expression of common biomarkers in tumour is regulated by melphalan in *E7*-Tg mice. Cofilin-1, histone H1, and nucleolar protein-3 (Nol-3) are used as common biomarkers for tumours, and *Histone H1* and *Cofilin-1* were confirmed to be up regulated while nol-3 was down regulated by melphalan. Cofilin is an actin binding protein which is important for the regulation of actin polymerisation and depolymersation in many cell types. It is arguably a key factor in cell invasion and cell migration in tumour cells. It has been reported that cofilin overexpression inhibits the

invasiveness of human lung cancer H1299 cells (40). Increasing levels of phosphorylated histone H1, a family of nucleosomal proteins, have been correlated with cell cycle progression and anti-tumour effects (41). Nol-3 is an endogenous inhibitor of apoptosis that can antagonise both the extrinsic and the intrinsic apoptosis pathways, and it has been observed that Nol-3 is expressed in the epithelium of human breast, colon, and cervical cancer (42). In conclusion, oncogenic *E7* triggers the progression of cancer, inducing markers related to cancer development, inflammation, cell adhesion, migration, and cell cycle progression. Genes and proteins related to carcinogenesis or *E7*-mediated lung cancer are considered to be candidates to respond to anti-cancer and genotoxic signals for enhanced carcinogenesis in the *E7*-Tg mice. The expression patterns of *E7*-specific markers in *E7*-Tg mice were modulated by melphalan treatment for 8 weeks. Most of the genes, selected by Omics data based on the association with cancer development in the lung tissue of *E7*-Tg mice, were down-regulated by melphalan treatment. In addition, common tumour markers were modulated by melphalan. Taken together, it is suggested that melphalan suppresses *E7*-mediated oncogenesis and modulates expression of *E7*-specific biomarkers, inhibiting *E7*-expression in lung tissues of *E7*-Tg mice, and resulting in tumour suppression.

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