Human Molecular Genetics, 2006, Vol. 15, No. 7 ***1181–1186***
doi:10.1093/hmg/ddl033
Advance Access published on February 23, 2006

Genetic polymorphisms of ataxia telangiectasia mutated affect lung cancer risk

Jin Hee Kim[1], Heon Kim[3], Kye Young Lee[5], Kang-Hyeon Choe[4], Jeong-Seon Ryu[6], Ho Il Yoon[1,7], Sook Whan Sung[2,8], Keun-Young Yoo[1] and Yun-Chul Hong[1,9,*]

[1]Department of Preventive Medicine and [2]Department of Thoracic and Cardiovascular Surgery, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Republic of Korea, [3]Department of Preventive Medicine and [4]Department of Internal Medicine, College of Medicine, Chungbuk National University, 12 Kaeshin-dong, Hungdok-gu, Cheongju-si, Chungbuk 361-763, Republic of Korea, [5]Department of Internal Medicine, School of Medicine, Konkuk University, No. 4-12 Hwayang-dong, Gwangjin-gu, Seoul 143-729, Republic of Korea, [6]Department of Internal Medicine, College of Medicine, Inha University, 7-206 Shinhung-dong 3ga, Jung-gu, Incheon 400-103, Republic of Korea, [7]Department of Internal Medicine and [8]Department of Thoracic and Cardiovascular Surgery, National University Bundang Hospital, 300 Gumi-dong, Bundang-gu, Seongnam, Geongi-do 463-707, Republic of Korea and [9]Institute of Environmental Medicine, SNUMRC, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Republic of Korea

Received January 6, 2006; Revised and Accepted February 15, 2006

The ataxia telangiectasia mutated (*ATM*) gene is known to be activated by DNA damage and involved in cell cycle arrest, apoptosis and DNA repair. Therefore, *ATM* gene polymorphisms may act as important factors predicting individual susceptibility to lung cancer. To evaluate the role of *ATM* gene polymorphisms in lung cancer development, genotypes of the *ATM* polymorphisms, −4518A>G, IVS21 − 77C>T, IVS61 − 55T>C, and IVS62 + 60G>A, were determined in 616 lung cancer patients and 616 cancer-free controls. When the effects of selected *ATM* genotypes were evaluated separately, only one *ATM* genotype (IVS62 + 60G>A) showed an association with lung cancer risk. Subjects with the A allele at the site (IVS62 + 60G>A) have significantly higher risk of lung cancer than those with the G allele [odds ratio (OR) = 1.6, 95% confidence interval (CI) 1.1–2.1]. When the haplotypes of four *ATM* single nucleotide polymorphism sites (−4518A>G, IVS21 − 77C>T, IVS61 − 55T>C and IVS62 + 60G>A) were studied, the ATTA haplotype showed significantly increased risk of lung cancer compared with the GCCA haplotype, the most common haplotype (OR = 7.6, 95% CI 1.7–33.5). Furthermore, subjects with the (NN)TA haplotype showed highly significant and increased risk of lung cancer when compared with those without the (NN)TA haplotype (OR = 13.2, 95% CI 3.1–56.1). Therefore, our results suggest that polymorphisms or haplotypes of the *ATM* gene play an important role in the development of lung cancer.

INTRODUCTION

Ataxia telangiectasia mutated (ATM) is the product of the gene mutated in patients with the autosomal recessive disorder ataxia telangiectasia (AT) and a member of the phosphatidylinositol 3/phosphatidylinositol 4-kinase family (1,2). This protein is known to be activated by DNA damage caused by ionizing radiation or reactive oxygen intermediates to induce the transactivation of various proteins that function in cell cycle arrest, apoptosis, DNA repair and centrosome duplication (3–5). In particular, ATM plays an important regulatory role in phosphorylation of p53, a tumor suppressor, thereby abolishing p53–Mdm2 interaction and allowing p53 to accumulate (6). ATM also phosphorylates Mdm2 which interferes with the nucleo-cytoplasmic shuttling of the Mdm2–p53 complex and contributes to p53 stabilization (7). In addition, ATM functions as a regulator of a wide variety of downstream proteins, including tumor suppressor BRCA1, checkpoint kinase

*To whom correspondence should be addressed. Tel: +82 27408394; Fax: +82 27474830; Email: ychong1@snu.ac.kr

CHK2, checkpoint protein RAD50 and DNA repair protein NBS1 (1,2).

Lung cancer is a leading cause of cancer-related death worldwide. Extensive epidemiological data clearly establish cigarette smoking as the major cause of this malignancy. Although up to 90% of lung cancer is attributable to smoking, only 5–10% of smokers develop lung cancer (8). This observation suggests that individual variation may determine susceptibility to lung cancer. Individual susceptibility to lung cancer varies with the presence of single nucleotide polymorphisms (SNPs) in genes related to cellular cycling such as cell cycle arrest and apoptosis (9). Therefore, *ATM* gene polymorphisms may act as important factors indicating individual susceptibility to lung cancer. A number of studies have evaluated the association between *ATM* gene polymorphisms and cancer development. However, *ATM* gene polymorphisms have not been studied about association with lung cancer, although several *ATM* polymorphisms have been suggested to pre-dispose those with specific genotypes or haplotypes to increased risk of breast, ovarian cancer or hereditary non-polyposis colorectal cancer (10–15).

In this study, we explored genetic polymorphisms of *ATM* to evaluate association with lung cancer risk in a case–control study of a Korean population.

RESULTS

Characteristics of lung cancer cases and control subjects were shown in Table 1. Age, sex and family history of cancer were not significantly different between cases and controls. However, body mass index (BMI) and smoking were shown to affect the odds ratio (OR) of lung cancer [BMI: OR = 1.1, 95% confidence interval (CI) 1.1–1.1; smoking: OR = 2.4, 95% CI 1.9–3.1, respectively].

The allele frequencies of four loci (−4518A, 0.43; IVS21 − 77C, 0.63; IVS61 − 55T, 0.46 and IVS62 + 60G, 0.44) of *ATM* gene in this Korean population were shown to be different from those of the SNP500Cancer database (0.55, 0.29, 0.57 and 0.67, respectively).

When we tested Hardy–Weinberg equilibrium (HWE) of *ATM* genotypes, both cases and controls were found to be on the HWE (all of four SNPs, $P > 0.05$). Moreover, in the analysis of the relative disequilibrium (D') between two from four sites, all sites were found to be strongly linked to each other ($D' \geq 0.84$, $P < 0.0001$) (Fig. 1).

When the effects of the selected *ATM* genotypes on lung cancer risk were evaluated separately (Table 2), only one *ATM* genotype (rs664143) was shown to affect lung cancer risk. Subjects with the A allele at the site (IVS62 + 60G>A) have significantly higher risk of lung cancer than those with only the G allele (adjusted OR = 1.6, 95% CI 1.1–2.1).

When haplotypes were composed of four *ATM* SNP sites (rs189037, rs664677, rs664982 and rs664143), the distributions of haplotypes were shown to be significantly different between cases and controls ($P < 0.001$) (Table 3). Moreover, subjects with the ATTA haplotype showed significantly increased risk of lung cancer compared with those with GCCA, the most common haplotype (adjusted OR = 7.6, 95% CI 1.7–33.5). In particular, the ACTA haplotype showed large difference between cases and controls with the absence of control subject, although its risk did not be expressed. The GCTA haplotype also showed the absence of control subject like ACTA. Because all three haplotypes (ATTA, ACTA and GCTA) carry the sequence (NN)TA, we estimated the effect of the (NN)TA sequence on lung cancer development. Subjects with the (NN)TA haplotype showed highly significant and increased risk of lung cancer compared with those without the (NN)TA haplotype (adjusted OR = 13.2, 95% CI 3.1–56.1) (Table 4). In addition, subjects with a (NN)TA/(NN)TA diplotype were not found in either cases or controls.

Lung cancers are composed of heterogenous cell types, and the baseline characteristics are different according to the cell type. For this reason, the effect of the (NN)TA haplotype on lung cancer risk was estimated according to the cell type of lung cancer. Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) showed very different effect of the (NN)TA haplotype on each cancer development (NSCLC: adjusted OR = 15.3, 95% CI 3.6–65.0 and SCLC: adjusted OR = 2.1, 95% CI 0.2–23.3, respectively).

Table 1. Comparison of baseline characteristics for lung cancer cases and control subjects

Variables	Case ($N = 616$)	Control ($N = 616$)	OR (95% CI)
Age (mean ± SD) (years)	65.3 ± 10.2	65.3 ± 10.2	1.0 (1.0, 1.0)
Sex (number of male) (%)	483 (78.4)	483 (78.4)	1.0 (0.8, 1.3)
BMI (mean ± SD) (kg/m^2)	22.0 ± 3.3	22.9 ± 3.1	1.1 (1.1, 1.1)[a]
Cigarette smoking (number of smoker) (%)	490 (79.6)	381 (61.9)	2.4 (1.9, 3.1)[a]
Family history of cancer (% present)	118 (22.7)	105 (20.9)	1.1 (0.8, 1.5)
Cell type of lung cancer (number of each type) (%)			
Adenocarcinoma	168 (27.3)		
Squamous cell carcinoma	240 (39.0)		
Other NSCLCs	112 (18.1)		
SCLC	96 (15.6)		

[a]Significant.

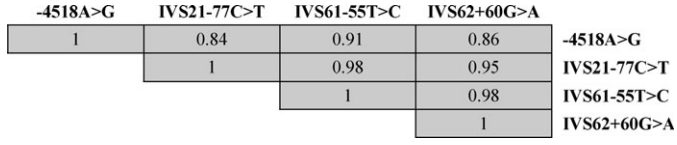

Figure 1. The relative disequilibrium (D') among four alleles of the *ATM* gene. Statistical significances of D' values were evaluated by Fisher's exact test. Gray boxes indicate statistical significance ($P < 0.0001$).

DISCUSSION

In a study on the cancer risks in heterozygous *ATM* mutation carriers, the relative risk for cancers except breast cancer was 2.05 (95% CI 1.09–3.84) in female carriers (16). Moreover, another study reported a 100-fold increased risk of developing cancer in AT patients compared with the general population (17). Our results that the IVS62 + 60G>A polymorphism or

Table 2. The distribution of selected *ATM* genotypes and lung cancer risk

Genotypes (rs number)	Case, *N* (%)	Control, *N* (%)	OR (95% CI)[a]
−4518A>G (189037)			
AA	105 (17.2)	113 (18.4)	1.0 (ref.)
AG	316 (51.7)	306 (49.8)	1.2 (0.9, 1.7)
GG	190 (31.1)	195 (31.8)	1.1 (0.8, 1.6)
AG + GG	506 (82.8)	501 (81.6)	1.2 (0.9, 1.6)
IVS21−77C>T (664677)			
CC	233 (38.3)	230 (38.1)	1.0 (ref.)
CT	294 (48.3)	303 (50.2)	0.9 (0.7, 1.1)
TT	82 (13.4)	71 (11.7)	1.0 (0.7, 1.5)
CT + TT	376 (61.7)	374 (61.9)	0.9 (0.7, 1.2)
IVS61−55T>C (664982)			
TT	119 (20.8)	118 (20.1)	1.0 (ref.)
TC	301 (52.7)	298 (50.7)	1.1 (0.8, 1.5)
CC	151 (26.5)	172 (29.2)	1.0 (0.7, 1.4)
TC + CC	452 (79.2)	470 (79.9)	1.1 (0.8, 1.4)
IVS62 + 60G>A (664143)			
GG	87 (14.2)	119 (19.3)	1.0 (ref.)
GA	346 (56.4)	312 (50.7)	1.6 (1.2, 2.3)[b]
AA	180 (29.4)	185 (30.0)	1.4 (1.0, 2.1)[b]
GA + AA	526 (85.8)	497 (80.7)	1.6 (1.1, 2.1)[b]

[a]OR adjusted with BMI and smoking status.
[b]Significant.

Table 3. The effect of *ATM* haplotypes on lung cancer risk

Haplotype[a]	Case, *N* (%)	Control, *N* (%)	OR (95% CI)[b]
GCCA	569 (50.8)	594 (51.4)	1.0 (ref.)
ATTG	383 (34.2)	396 (34.2)	1.0 (0.8, 1.2)
ACTG	69 (6.1)	88 (7.6)	0.9 (0.6, 1.2)
GTTG	35 (3.1)	33 (2.8)	0.9 (0.5, 1.5)
ACCA	19 (1.7)	30 (2.6)	0.6 (0.3, 1.2)
ATTA	18 (1.6)	2 (0.2)	7.6 (1.7, 33.5)[c]
GCTG	6 (0.5)	5 (0.4)	1.6 (0.4, 6.1)
ACTA	11 (1.0)	0 (0.0)	—
GTCA	3 (0.3)	2 (0.2)	1.7 (0.3, 10.5)
GTCG	2 (0.2)	3 (0.3)	0.6 (0.1, 3.9)
GCCG	2 (0.2)	3 (0.3)	1.9 (0.2, 21.8)
GCTA	3 (0.3)	0 (0.0)	—
Total	1120 (100)	1156 (100)	

[a]Composed of four polymorphic sites: −4518A>G, IVS21 − 77C>T, IVS61 − 55T>C and IVS62 + 60G>A.
[b]OR adjusted with BMI and smoking status.
[c]Significant.

the (NN)TA haplotype of the *ATM* gene was associated with increased risk of lung cancer support other study findings, suggesting that the *ATM* gene may play an important role in the development of cancer.

The most common *ATM* mutations in a large multiethnic cohort were reported to be associated with specific SNP haplotypes (18). In addition, the typical phenotypes in AT patients such as pre-disposition to cancer and chromosomal instability were found to be caused by *ATM* alleles that affect mRNA splicing or gene expression to either truncate or severely destabilize the ATM protein (19–21). These study results suggest the possibility that specific haplotypes related to the activity of the *ATM* gene may induce low expression or destabilization of the ATM protein and thereby increase the risk of cancer. In this study, the associations between one SNP (−4518A > G) in the promoter

Table 4. Combined diplotypes of *ATM* and lung cancer risk

Haplotype pairs[a]		Case, *N* (%)	Control, *N* (%)	OR (95% CI)[b]
Others[c]	Others[c]	528 (94.3)	576 (99.7)	1.0 (ref.)
(NN)TA	Others[c]	32 (5.7)	2 (0.3)	13.2 (3.1, 56.1)[d]
(NN)TA	(NN)TA	0 (0)	0 (0)	—

[a]Composed of four polymorphic sites: −4518A>G, IVS21 − 77C>T, IVS61 − 55T>C and IVS62 + 60G>A.
[b]OR adjusted with BMI and smoking status.
[c]Other than (NN)TA combination.
[d]Significant.

and three intronic SNPs (IVS21 − 77C > T, IVS61 + 55T>C and IVS62 + 60G>A) and lung cancer risk were evaluated and all four SNPs have a possibility that may regulate ATM protein activity due to regulation function of promoter and intron as shown in most genes. Our finding that the specific haplotype (NN)TA which consisted of these SNPs increases the risk of lung cancer strongly supports previous study results that specific genotypes or haplotypes of *ATM* may play an important role in carcinogenesis through expression regulation or alternative splicing of the *ATM* gene (22). In fact, the last two sites of (NN)TA are very close loci (178 bp distance) existing in two introns interrupted by exon 61. Moreover, when we examined the effects of two sites on splicing using the web-based tool (Improbizer, http://www.cse.ucsc.edu/%7Ekent/improbizer/improbizer.html NumMotifs and MEME version 3.5.0, http://meme.sdsc.edu/meme/website/meme.html), data obtained from these tools showed that both sites exist in protein-binding motifs having a potential as binding sites of intronic splicing enhancer or repressor, indicating a possibility that both sites may be related to splicing process of exon 61 to lead to inaccurate splicing. However, this hypothesis should be confirmed through a variety of functional studies including RT–PCR of mRNA using biosamples obtained from subjects with the haplotype.

Traditional genetic analysis methods have not shown sensitivity for a genetic contribution when it is subtle or when several genes are working together in multifactorial diseases such as cancer (23). In fact, the attempts to correlate single-locus alleles with cancer development have produced mixed results. To solve this problem and increase statistical sensitivity, the study of haplotypes has been suggested as a powerful methodology for the detection of the relationship of genes with diseases (23,24). In addition, haplotypes consisting of SNP sites in a large gene such as *ATM* were found to be useful (23). In particular, linkage disequilibrium (LD)-based strategies for detection of the genetic contributions to common diseases may be a very powerful approach. In this study, pairwise LD among four polymorphisms in the *ATM* gene was very high ($D' \geq 0.84$, $P < 0.0001$). Moreover, the closely linked (NN)TA haplotype showed highly increased risk of lung cancer (adjusted OR = 13.2, 95% CI 3.1–56.1). In particular, subjects with an (NN)TA/(NN)TA diplotype were not observed either in cases or in controls. Our data also showed considerable difference between NSCLC and SCLC in regard to the effect of the (NN)TA haplotype on lung cancer risk. Because NSCLC and SCLC are different diseases in every aspect from cellular origin, genetic/molecular

Table 5. Primers used in polymerase chain reaction

rs number	Primer		
	Forward	Reverse	Primer for SNP determination
189037[a]	5′-AAGCGGGAGTAGGTAGCTG-3′	5′-TCAAAGTAGTATCAACCGCG-3′	5′-AACGGAGAAAAGAAGCCGTGGCC-3′
664677[b]	5′-ATGTTGGCATATTCCACATAATG-3′	5′-TGAAATCAAGTAAGTTTTCCAAAGT-3′	5′-AGAAAGACATATTGGAAGTAACTTA-3′
664982[b]	5′-AAGTATTATGCTATTTTGAGATACA GATATGT-3′	5′-TTTTCTCACAGCATCTAGAGTCAA-3′	5′-ACATGAGAGTATACAGATAAAGATA-3′
664143[b]	5′-TGTAGAGGTAAAGTATTTTATAAG GAAGACT-3′	5′-ATGTTAAAATGAAGCAGTGCTCTT-3′	5′-TCTTACCAGGTAGACTGTGTATCTC-3′

[a]SNaPshot.
[b]SNP-IT™.

changes to clinical features, it might be possible to show different impact of genetic polymorphisms on lung cancer risk according to the cell type (25–28). These results indicate a possibility that the (NN)TA haplotype may function as a very risky factor in the development of lung cancer, particularly NSCLC. This finding supports our hypothesis that specific haplotype of the *ATM* gene may act as a risk factor in regard to cancer development and survival (29–31). However, our study showed remarkable difference in the allele frequencies in comparisons of Korean (−4518A, 0.43; IVS21 − 77C, 0.63; IVS61 − 55T, 0.46 and IVS62 + 60G, 0.44) and Caucasians (0.66, 0.34, 0.66 and 0.66), African or African-American (0.27, 0.21, 0.31 and 0.63), Hispanic (0.70, 0.21, 0.22 and 0.20) or Pacific Rim (0.54, 0.40, 0.56 and 0.60) obtained from the SNP500Cancer database. For this reason, whether distribution of *ATM* haplotypes in Korean population has similar trend with those in other ethnic group regardless of this difference in genetic composition should be confirmed in the future.

In conclusion, the results of this study suggest that genetic polymorphisms of *ATM* play an important role in lung cancer development. However, we need to clearly understand the function of SNPs or haplotypes in *ATM* in the development of lung cancer through laboratory research.

MATERIALS AND METHODS

Study population

A total of 616 patients with newly diagnosed lung cancer at Chungbuk National University Hospital, Dankook University Hospital and Inha University Hospital between 2001 and 2003 were recruited. There were no age, histological or stage restrictions, and all lung cancer cases were histologically confirmed. The control subjects consisted of non-cancer patients admitted to the same hospitals or participants in the health checkup program of each hospital. They were individually matched to the cases by age (± 2 years) and sex. Detailed information on diet, smoking habits, lifestyle, weight, height, medical treatment and other socio-economic characteristics were collected by trained interviewers using a structured questionnaire. The study protocol was approved by the institutional review board at each hospital, and written informed consent was provided by all study participants. Venous blood samples from all subjects were collected into heparinized tubes and stored at −70°C until used for DNA extraction.

Genotyping

In a study on SNP using limited number of subjects, low minor allele frequency of the SNP may lead to null result although the SNP is meaningful for the risk of target disease. Therefore, we first selected five ATM SNPs (rs189037, rs664677, rs600329, rs664982 and rs664143) which have >10% minor allele frequencies based on the SNP500Cancer database (http://snp500cancer.nci.nih.gov/home.cfm) and then Dan Stram's haplotype-tagging SNP program (*tagSNPsv2.exe*) was used to determine the best set of haplotype-tagging SNPs. Of these five SNPs, four (rs189037, rs664677, rs664982 and rs664143; min RSQ = 0.9936) were selected and successfully genotyped.

DNA was extracted from whole blood sample using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). SNP genotyping was performed by SNP-IT™ assays (for rs664677, rs664982 and rs664143) using SNPstream 25K® System (Orchid Biosciences, Princeton, NJ, USA) and single base primer extension assay (for rs189037) using SNaPShot assay kit (ABI, Foster City, CA, USA) according to manufacturers' recommendation.

In SNP-IT assays, the genomic DNA region spanning the polymorphic site was PCR-amplified using one phosphothiolated primer and one regular PCR primer. The amplified PCR products were then digested with exonuclease (Amersham Biosciences, Uppsala, Sweden). The 5′ phosphothiolates were used in this study to protect one strand of the PCR product from exonuclease digestion. The single-stranded PCR template generated from exonuclease digestion was overlaid onto a 384-well plate that was pre-coated covalently with the primer extension primers, SNP-IT primers (Table 5). These SNP-IT primers were designed to hybridize immediately adjacent to the polymorphic site. After hybridization of template strands, SNP-IT primers were then extended by a single base with DNA polymerase at the polymorphic site of interest. The extension mixtures contained two labeled terminating nucleotides (one FITC, one biotin) and two unlabeled terminating nucleotides. The final single base incorporated was identified with serial colorimetric reactions with anti-Fluorescein-AP (Roche, Basel, Switzerland) and streptavidin-HRP (Pierce, Rockford, IL, USA), respectively. The results of blue and/or yellow color developments were analyzed with an ELISA reader and the final genotyping (allele) calls were made with the QCReview program.

In the single base primer extension assay, the genomic DNA region containing both of the SNP was amplified with a PCR reaction. Each PCR reaction contained: 10.0 ng of DNA, 1 × PCR buffer, 0.125 U of AmpliTaq Gold DNA polymerase (ABI), 3.0 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5 pmol of each primer in 10 μl reaction volume. Reactions were incubated at 95°C for 10 min, then cycled 30 times at (95°C for 30 s, 60°C for 1 min, 72°C for 1 min) followed by 72°C for 5 min. After amplification, the PCR products were treated with 1 U of shrimp alkaline phosphatase (SAP) (Roche) and exonuclease I (USB Corporation) at 37°C for 60 min and 72°C for 15 min to purify the amplified products. One microliter of the purified amplification products were added to a SNaPshot Multiplex Ready reaction mixture containing 0.15 pmol of genotyping primer (Table 5). The primer extension reaction was carried out for 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 30 s. The reaction products were treated with 1 U of SAP at 37°C for 1 h and 72°C for 15 min to remove excess fluorescent dye terminators. One microliter of the final reaction samples containing the extension products were added to 9 μl of Hi-Di formamide (ABI). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by electrophoresis in ABI Prism 3730 DNA analyzer. Results were analyzed using Gene Mapper software (ABI).

All genotyping was done blindly to the case–control status and the repeatability test was conducted for 5% of total subjects, resulting in a 98.9% concordance rate.

Statistical analysis

Unconditional logistic regression was used to estimate ORs and 95% CI according to the *ATM* gene polymorphisms in lung cancer patients and control subjects. Incomplete data missing at least one of four SNPs were excluded, and then individual haplotypes were estimated from genotype data using the PHASE program (ver. 2.0.2). The χ^2 test was used to compare the distribution of haplotypes in the cases and the controls. To find out whether each SNP site are on the HWE, the distributions of observed genotype frequency and expected genotype frequency, which was calculated from observed allele frequency, were compared using the χ^2 test. Pairwise LD among four polymorphic sites was estimated as relative disequilibrium (D') (13). A probability level of 0.05 was used as criterion for statistical significance. SAS, version 8.1 (SAS Institute Inc., Cary, NC, USA), was used for statistical analysis.

ACKNOWLEDGEMENTS

This study was supported by the Eco-technopia 21 project of Ministry of Environment, South Korea.

Conflict of Interest statement. None declared.

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