

adiponectin to those receptors, and the associated intracellular signaling pathways in the colorectum remain unclear. In this study, we investigated the effect of adiponectin on cell growth and the intracellular signaling pathway involved in CRC cell lines.

### Materials and methods

**Reagents and antibodies.** Human globular adiponectin was purchased from BioVendor Laboratory Medicine Inc. (Brno, Czech Republic). Compound C was purchased from Calbiochem (La Jolla, CA) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-total and -phosphorylated (Thr172) AMPK, anti-total and -phosphorylated (Ser2448) mTOR, anti-total and -phosphorylated (Thr421/Ser424) p70S6 kinase, anti-total and -phosphorylated (Ser240/244) S6 ribosomal protein, and anti-rabbit horseradish-peroxidase-conjugated IgG antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-AdipoR1 (C-14), anti-AdipoR2 (N-19), and anti-goat horseradish-peroxidase-conjugated IgG antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) antibody was from Trevigen, Inc. (Gaithersburg, MD).

**Cell lines.** The human colon adenocarcinoma cell lines, HT-29, Lovo, and HCT116 were used for this study. Lovo was obtained from Health Science Research Resources Bank (Osaka, Japan), while HT-29 and HCT116 were obtained from American Type Culture Collection (Manassas, VA). HCT116 and HT-29 were cultured in McCoy's 5A, and Lovo was cultured in Ham's F12, supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) (all from Invitrogen, Carlsbad, CA) at 37°C under a humidified atmosphere of 5% CO<sub>2</sub>.

**Western blot analysis.** Cultured cells treated with the test compound for indicated time periods were rinsed with phosphate-buffer saline (PBS). For obtaining the total cell extracts, cells were harvested in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1% TritonX-100) containing a cocktail of protease inhibitors (Sigma). The lysates were incubated on ice for 30 min and centrifuged at 15,000 rpm. Protein concentrations were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA). Proteins were separated by SDS/PAGE (7.5-12.5% gels) and transferred onto a Hybond-P PVDF membrane (Amersham Biosciences, Little Chalfont, UK). After the transfer, the membranes were blocked with Blocking One-P (Nacalai Tesque, Kyoto, Japan) and probed with the primary antibodies specified below. Horseradish-peroxidase-conjugated secondary antibodies and the ECL detection kit (Amersham Biosciences, Little Chalfont, UK) were used for the detection of specific proteins. Images were captured and analyzed by LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

**Cell growth assay.** Cells were seeded in 96-well, flat-bottom microtiter plates at a density of  $5 \times 10^3$  cells per well and incubated in medium containing 1% FBS. After 24 h, the

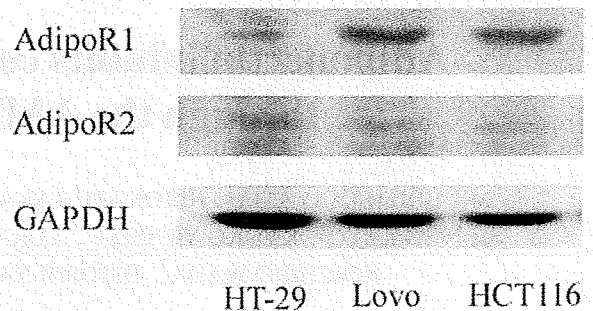


Figure 1. Expression of adiponectin receptors in colorectal cancer cells. Western blot analysis revealed that three kinds of colorectal cancer cell lines HCT116, HT-29 and Lovo cells, expressed both AdipoR1 and AdipoR2 receptors.

complete medium was replaced with test medium for 24 h at 37°C. After incubating the plates for an additional 4 h with MTT solution (0.5%), sodium dodecylsulfate was added to a final concentration of 10% and absorbance at 595 nm was determined for each well using a microplate reader (Model 550; Bio-Rad). Three independent experiments were carried out for each cell line. Annexin V-FITC and PI double staining with the Annexin V-FITC apoptosis detection kit I (Becton-Dickinson, San Jose, CA, USA) followed by FACScan flow cytometry (Becton-Dickinson) was used to identify apoptotic cells. Cell fluorescence was measured with a FACScan flow cytometer from BD Biosciences (San Jose, CA, USA). Dual parameter cytometric data were analyzed by using CellQuest software from BD Biosciences. Apoptosis measures were performed in triplicate.

**Statistical analysis.** All results are expressed as mean  $\pm$  SEM. Statistical analyses were performed using Student's t-test after analysis of variance (ANOVA). The results were considered to be statistically significant at  $p < 0.05$ .

### Results

**Expression of adiponectin receptors on colorectal cancer cells.** Western blot analysis revealed that three kinds of colorectal cancer cell lines, HCT116, HT-29 and Lovo, expressed both AdipoR1 and AdipoR2 receptors (Fig. 1). In HT-29, weak expressions of both of AdipoRs were observed. In Lovo and HCT116, strong expression of AdipoR1, and weak expression of AdipoR2 were observed.

**Globular adiponectin (g-adiponectin) inhibited human colorectal cancer cell growth.** To determine the effect of g-adiponectin on colorectal cancer cell growth, MTT assay was performed using HCT116, HT-29 and Lovo cells. To reduce the effect of adiponectin in serum, all experiments were conducted using the culture medium containing 1% FBS. G-adiponectin significantly inhibited colorectal cancer cell growth in all examined cells in a dose-dependent manner (Fig. 2).

**G-adiponectin up-regulates AMPK activity in colorectal cancer cells.** The effect of g-adiponectin on the phosphorylation of

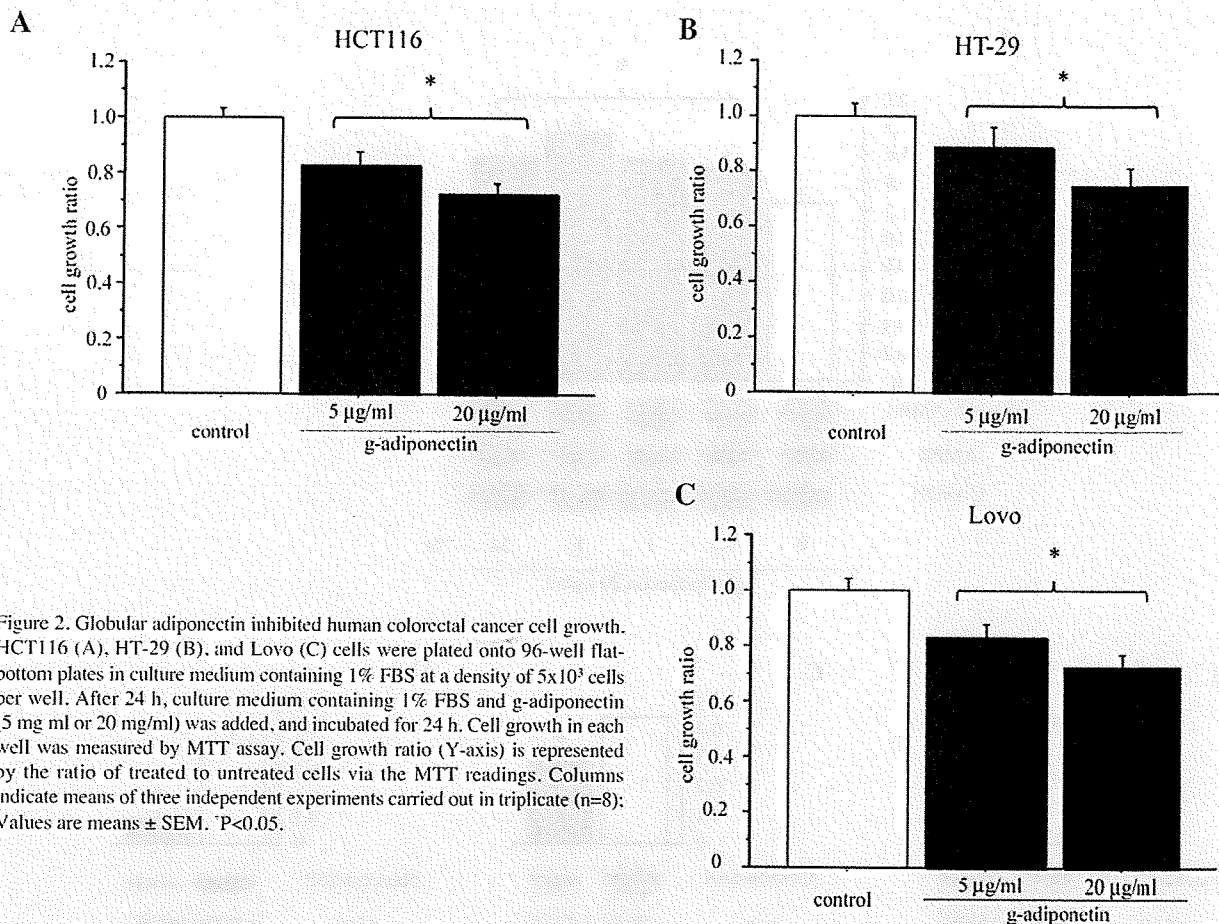


Figure 2. Globular adiponectin inhibited human colorectal cancer cell growth. HCT116 (A), HT-29 (B), and Lovo (C) cells were plated onto 96-well flat-bottom plates in culture medium containing 1% FBS at a density of  $5 \times 10^3$  cells per well. After 24 h, culture medium containing 1% FBS and g-adiponectin (5 mg/ml or 20 mg/ml) was added, and incubated for 24 h. Cell growth in each well was measured by MTT assay. Cell growth ratio (Y-axis) is represented by the ratio of treated to untreated cells via the MTT readings. Columns indicate means of three independent experiments carried out in triplicate (n=8). Values are means  $\pm$  SEM.  $P < 0.05$ .

AMPK and mTOR signaling pathway was examined. Western blot analysis revealed that g-adiponectin significantly phosphorylated AMPK and its effect on AMPK phosphorylation was maximal at 6 h after treatment (Fig. 3A). We also observed the significant phosphorylation of mTOR, p70S6K, and S6 proteins by the treatment with g-adiponectin (Fig. 3B-D). These results suggest that g-adiponectin inhibits colorectal cancer cell growth via AMPK activation and mTOR signaling pathway suppression. Selective AMPK inhibitor compound C reversed the g-adiponectin induced cell growth inhibition in HCT116 colorectal cancer cells, as detected by MTT assay (Fig. 3E). This indicates that g-adiponectin-induced cell growth inhibition is mediated by activation of AMPK.

*G-adiponectin has no effect on apoptosis.* The effect of g-adiponectin on apoptosis in HCT116 and HT-29 was evaluated using annexin V-FITC and PI double staining. There was no apoptotic effect in HCT116 and HT-29 treated with 5 µg/ml g-adiponectin or 20 µg/ml g-adiponectin in basal medium containing 1% FBS (Fig. 4).

## Discussion

The association of low plasma adiponectin level and cancer risk was previously reported (32-35). However, there are few studies regarding the association of adiponectin and colorectal cancer (15,36). In the present study, we demonstrated both of

the AdipoR expression levels and g-adiponectin activation of the AMPK, resulting in the suppression of mTOR signaling pathway in colorectal cancer cell lines. Our recent study demonstrated that AdipoRs were expressed in normal colon epithelial and colorectal cancer cells in human (37). In this study, three kinds of colorectal cancer cell lines HCT116, HT-29 and Lovo cells were shown to express AdipoRs. Furthermore, we clearly demonstrated that globular adiponectin inhibited colorectal cancer cell growth and activated AMPK, while selective AMPK inhibitor compound C reversed the effect of g-adiponectin-induced cell growth inhibition, which indicates that g-adiponectin inhibits cell growth via regulation of AMPK. mTOR is one of the enzymes downstream of AMPK. AMPK activation acts as an inhibitor of mTOR pathway and suppresses tumor development (38,39). In this study, we demonstrated that g-adiponectin suppressed the mTOR pathway following the activation of AMPK. These results suggest that g-adiponectin suppresses cancer cell growth through AMPK activation and subsequent inhibition of mTOR pathway. However, the mechanisms through which adiponectin affects cancer cells are not completely elucidated, thus there is a possibility that suppression of mTOR pathway by adiponectin is mediated through other enzymes (40,41). Further studies are needed to evaluate the molecular pathways downstream of each AdipoR.

In conclusion, this study clearly demonstrates the novel findings that g-adiponectin inhibits colorectal cancer cell

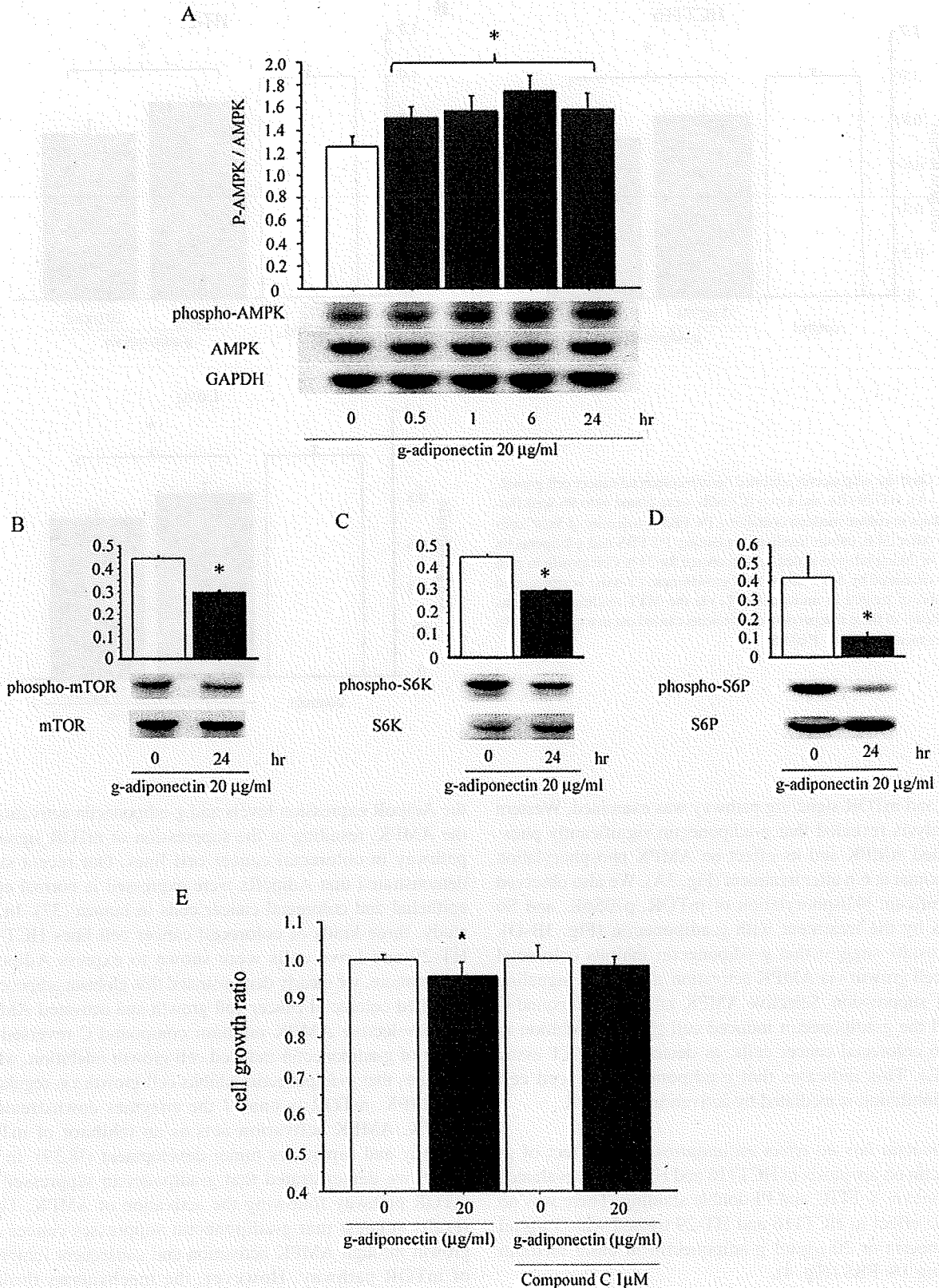


Figure 3. G-adiponectin up-regulates AMPK activity in colorectal cancer cell and selective AMPK inhibitor compound C reversed the g-adiponectin-induced cell growth. Western blot analysis was performed. HCT116 cells were treated with culture medium containing 1% FBS and g-adiponectin (20  $\mu$ g/ml) for 24 h. After harvesting, cells were lysed and prepared for immunoblot analysis. (A) G-adiponectin increases AMPK phosphorylation and its effect on AMPK phosphorylation was maximal at 6 h after treatment. (B-D) G-adiponectin reduced phosphorylation of mTOR, p70S6K and S6P in HCT116 cells. Western blot analysis was performed in triplicate. Values are means  $\pm$  SEM. \* $P$ <0.05. (E) To confirm the effect of adiponectin-induced growth inhibition via AMPK, HCT116 incubated in culture medium containing 1% FBS and adiponectin (20  $\mu$ g/ml) alone or with the addition of compound C (1  $\mu$ M) for 24 h were used for MTT assay. Columns indicate means of four independent experiments carried out in triplicate (n=8); Values are means  $\pm$  SEM. \* $P$ <0.05.

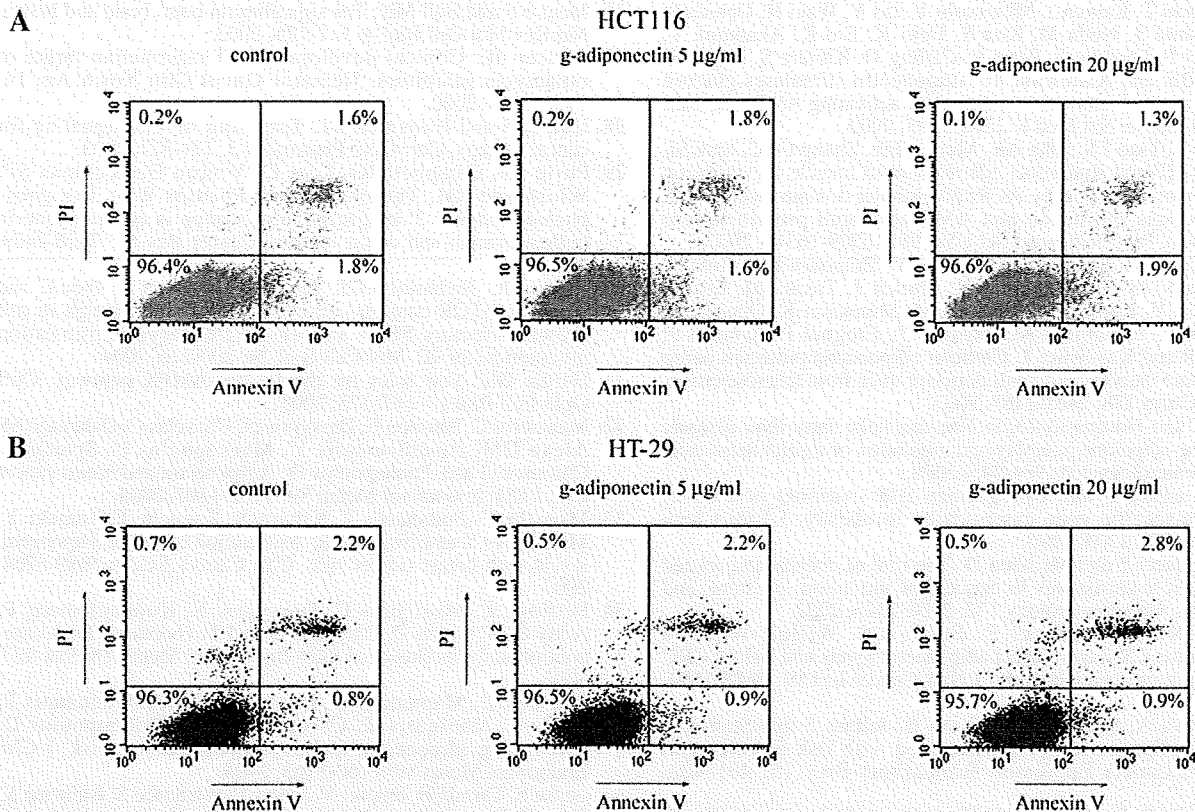


Figure 4. G-adiponectin has no effect on apoptosis. The apoptotic effect of g-adiponectin in HCT116 (A) and HT-29 (B) colorectal cancer cells incubated with culture medium containing 1% FBS and g-adiponectin (5 µg/ml or 20 µg/ml) were evaluated via annexin V-FITC and PI double staining. Apoptosis measures were performed in triplicate.

growth through the activation of AMPK and subsequent suppression of mTOR pathway. This may be a key step in the elucidation of the effect of adiponectin on colorectal cancer. Further studies are required to elucidate the function of adiponectin in colorectal cancer.

#### Acknowledgements

We thank Machiko Hiraga and Yuko Sato for their technical assistance. This work was supported in part by a Grant-in-Aid for research on the Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare, Japan to A.N., a grant from the National Institute of Biomedical Innovation (NIBIO) to A.N., a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (KIBAN-B) to A.N, and a research grant from the Princess Takamatsu Cancer Research Fund to A.N.

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# Polymorphisms of DNA damage response genes in radiation-related and sporadic papillary thyroid carcinoma

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## Abstract

Papillary thyroid carcinoma (PTC) etiologically occurs as a radiation-induced or sporadic malignancy. Genetic factors contributing to the susceptibility to either form remain unknown. In this retrospective case-control study, we evaluated possible associations between single-nucleotide polymorphisms (SNPs) in the candidate DNA damage response genes (*ATM*, *XRCC1*, *TP53*, *XRCC3*, *MTF1*) and risk of radiation-induced and sporadic PTC. A total of 255 PTC cases (123 Chernobyl radiation-induced and 132 sporadic, all in Caucasians) and 596 healthy controls (198 residents of Chernobyl areas and 398 subjects without history of radiation exposure, all Caucasians) were genotyped. The risk of PTC and SNPs interactions with radiation exposure were assessed by logistic regressions. The *ATM*G557A and *XRCC1* Arg399Gln polymorphisms, regardless of radiation exposure, associated with a decreased risk of PTC according to the multiplicative and dominant models of inheritance (odds ratio (OR)=0.69, 95% confidence interval (CI) 0.45–0.86 and OR=0.70, 95% CI 0.59–0.93 respectively). The *ATM*IVS22-77 T>C and *TP53* Arg72Pro SNPs interacted with radiation ( $P=0.04$  and  $P=0.01$  respectively). *ATM* IVS22-77 associated with the increased risk of sporadic PTC (OR=1.84, 95% CI 1.10–3.24) whereas *TP53* Arg72Pro correlated with the higher risk of radiogenic PTC (OR=1.80, 95% CI 1.06–2.36). In the analyses of *ATM/TP53* (rs1801516/rs664677/rs609429/rs1042522) combinations, the GG/TC/CG/GC genotype strongly associated with radiation-induced PTC (OR=2.10, 95% CI 1.17–3.78). The GG/CC/GG/GG genotype displayed a significantly increased risk for sporadic PTC (OR=3.32, 95% CI 1.57–6.99). The results indicate that polymorphisms of DNA damage response genes may be potential risk modifiers of ionizing radiation-induced or sporadic PTCs.

*Endocrine-Related Cancer* (2009) 16 491–503

## Introduction

Thyroid cancer accounts for more than 90% of all endocrine malignancies. The incidence of thyroid cancer in the world is increasing during the past three decades, mainly due to the papillary thyroid carcinoma (PTC) which is the predominant type of malignant thyroid tumors (Davies & Welch 2006).

Most thyroid cancer patients do not have the history of radiation exposure, yet ionizing radiation (IR) is a recognized etiological factor of the disease. An

increased risk of thyroid cancer has been documented after external irradiation (Ron *et al.* 1995) and after environmental exposure to <sup>131</sup>I, such as after the Chernobyl fallouts in Belarus, Ukraine, and Russia (Bennett *et al.* 2006).

Although radiation thyroid doses in Chernobyl PTC cases are generally greater than in controls in epidemiological studies (Cardis *et al.* 2005, Jacob *et al.* 2006, Likhtarev *et al.* 2006), thus confirming radiation to be a risk factor for thyroid cancer, those in

controls are non-zero. Furthermore, there were some 14 million residents in the contaminated territories at the time of exposure (Bennett *et al.* 2006). Conceivably, at least some of them might have accumulated thyroid doses comparable with doses in diseased individuals. However, thyroid cancer developed only in a small fraction of irradiated population.

Among the variety of DNA damage types induced by radiation, double-strand DNA breaks are considered to be the most significant for chromosomal aberrations, mutagenesis, genetic instability, and carcinogenesis (Khanna & Jackson 2001). PTC is one of the rare human cancers of epithelial origin in whose oncogenesis gene rearrangements play a noticeable role. Several variants of rearrangements are described in PTC, with RET/PTC occurring most frequently (Nikiforov *et al.* 1997, Rabes *et al.* 2000).

While in the exposed individuals DNA damage could be attributed to IR, the origination of genetic alterations in sporadic cancers remains obscure. Nevertheless, the spectrum of oncogenic changes in radiation-related and sporadic PTCs is largely common. Such similarities imply the resemblance of molecular reactions on DNA damage in exposed and non-exposed thyrocytes. These reactions involve first of all DNA damage response factors, including DNA repair and checkpoint complexes.

The vast majority of Chernobyl thyroid malignancies were PTCs which displayed wide variations in clinical course, from highly aggressive tumors developing after the shorter latency to more indolent carcinomas with the longer latent period (Williams 2006). The randomness and multiplicity of forms of genetic alterations caused by IR can only partly explain these differences in the individual reactions on exposure as well as why cancer develops only in some of the exposed individuals.

It is attractive to hypothesize that inherited variability in the genes directly or indirectly involved in the maintenance of genome stability in response to environmental carcinogens such as IR or chemicals that may play a role in susceptibility for radiation-related or sporadic PTC or may be a marker of it. In this work, we tested the relation of genetic variants of some of such genes, namely *ATM*, *TP53*, *XRCC1*, *XRCC3*, and *MTF1* to PTC of different etiology.

The ataxia-telangiectasia mutated (*ATM*) gene plays a key role in the sensing and repair of DNA double-strand breaks. Activation of the ATM protein kinase by IR results in the subsequent initiation of several molecular pathways of DNA damage repair (Shiloh 2003). One of the ATM targets is the p53 pathway.

Overexpression of *TP53* arrests the cell cycle and affects DNA repair and apoptosis.

The *ATM* and *TP53* genes play a significant role especially in the tumors that are induced by IR. A number of single-nucleotide polymorphisms (SNPs) in the *ATM* and *TP53* genes studied in populations of different ethnicities have been reported to associate with the risk of different radiogenic tumors (Hu *et al.* 2002, Angele *et al.* 2003, Thorstenson *et al.* 2003, Malmer *et al.* 2007). By contrast, studies of post-Chernobyl pediatric thyroid cancers demonstrated a low mutation and polymorphism rate in the *TP53* gene (Nikiforov *et al.* 1996, Hillebrandt *et al.* 1997). It, however, should be mentioned that after exposure to radiation p53 facilitates DNA repair in normal thyrocytes *in vitro* (Yang *et al.* 1997).

The base excision repair (BER) and homologous recombination repair (HRR) pathways are particularly important for genomic integrity restoration (Hoeijmakers 2001). The product of the X-ray repair cross complementing 1 (*XRCC1*) gene acts as a scaffold and a modulator of different enzymes involved in BER. The *XRCC1* Arg399Gln and Arg280His variants have been extensively investigated for their function and association with cancer risk; however, the results remain contradictory rather than conclusive (Hu *et al.* 2005). The *XRCC3* gene is a member of the *Rad51* DNA-repair gene family. Its product is a factor of the HRR. The *XRCC3* Thr241Met polymorphism has been controversially associated with different human malignancies (Han *et al.* 2006). Sturgis *et al.* (2005) reported 241Met allele association with the risk of differentiated thyroid cancer.

The metal-responsive transcription factor 1 (*MTF1*) gene has been implicated in tumor initiation and progression to malignant growth. MTF1 protein interacts with metallothioneins that are able to suppress cellular stresses generated by IR and other agents (Tamura *et al.* 2005). Polymorphism in murine *Mtf1* gene has been found to associate with the susceptibility to experimental  $\gamma$ -ray-induced thymic lymphomas. This observation points at possible involvement of human *MTF1* polymorphisms in the modulation of radiation-induced malignancies (Tamura *et al.* 2005).

To date no polymorphisms of the *ATM*, *XRCC1*, and *MTF1* genes have been studied neither in human sporadic or radiation-induced PTCs. Data on the *TP53* and *XRCC3* polymorphisms associations are quite limited (Hillebrandt *et al.* 1997, Boltze *et al.* 2002, Granja *et al.* 2004, Sturgis *et al.* 2005, Rogounovitch *et al.* 2006). Therefore, in this study, we addressed the relation of SNPs in aforementioned DNA damage response genes to the risk of PTCs of different etiology.

## Materials and methods

### Study population

A total of 255 histologically verified PTC cases and 596 healthy controls, all Caucasians, were included in the study. Among the patients, 123 individuals with PTC (24 males and 99 females) lived in the areas of the Russian Federation (38 patients) and Belarus (85 patients) contaminated with radionuclides from Chernobyl fallouts. At the time of the Chernobyl accident, these subjects were younger than 18 years old (mean age at exposure  $\pm$  s.d.,  $9.8 \pm 5.1$  years old; 1–18 years old, range). The mean age at diagnosis was  $24.4 \pm 4.9$  years old, range 19–37 years old (IR-induced PTCs). Information about individual radiation thyroid doses was available for PTC cases from Russia as reconstructed in previous studies (Davis *et al.* 2004, Stepanenko *et al.* 2004). The doses varied from 43 to 2640 mGy. Radiation thyroid doses for PTC patients and controls from Belarus evaluated in dosimetric investigations at the places of residence ranged 21–1500 mGy (Bouville *et al.* 2007). Among the controls, 198 individuals (65 males and 133 females, mean age at sampling  $22.2 \pm 3.2$  years old; 19–35 years old, range) were residents of the Chernobyl areas (60 from the Russian Federation and 138 from Belarus). The averaged thyroid radiation dose in the exposed control subjects from Russia is 41 mGy (Bouville *et al.* 2007). All exposed control individuals were aged < 18 years at the time of the accident (mean age at exposure  $1.8 \pm 3.2$  years old; 1–16 years old, range) (IR-exposed controls). IR-exposed controls and patients with IR-induced PTCs not were individually matched; however, they were residents of the same settlements. This, given the uncertainty with individual radiation thyroid doses, was supposed to partly reduce exposure bias. Age of IR-exposed control subjects was set to be  $\pm 3$  years of that of IR-induced PTC individuals.

One hundred and thirty-two PTC cases (21 males and 111 females, mean age at diagnosis  $47.8 \pm 11.4$  years old; 19–76 years old, range) were adults without history of radiation exposure (sporadic PTCs). The remaining 398 control participants (180 males and 218 females, mean age at sampling  $45.0 \pm 10.3$  years old; 16–65 years old, range) had no previous history of radiation exposure (non-exposed controls); their age was also set to be  $\pm 3$  years of that of patients with sporadic PTC. Both sporadic PTCs and non-exposed controls originated from the European part of Russia not contaminated by the Chernobyl fallouts.

Thyroid tissues and/or blood samples were collected from patients during surgery or further follow-up. Blood samples and information from the controls were

obtained during a routine health examination or complex screening for thyroid diseases.

Written informed consent was obtained from all participants. Protocols of the present study were approved by the Committee for Ethical Issues of Human Genome Analysis of Nagasaki University.

### SNP selection

The candidate SNPs (Table 1) were selected based on their reported functional role (if available), associations with radiosensitivity or (thyroid) cancer risk. Accordingly, we did not search for tag SNPs or account for the genetic variability in the regions of SNP location. All SNPs are listed in a public database, dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), with validated status in ethnically diverse populations. To ensure sufficient power for calculations, only SNPs with minor allele frequency (MAF) of >1% were included.

### SNP genotyping

DNA was extracted from normal thyroid tissues using proteinase K/phenol–chloroform method or from the whole blood lymphocytes with Puregene DNA Purification Kit (Gentra Systems, Inc., Minneapolis, PA, USA). All specimens were genotyped using various techniques (Table 1). Primers and probes (Table 2) were designed with Primer Express Version 1.0 (Applied Biosystems, Foster City, CA, USA) software.

Briefly, 25  $\mu$ l PCR mixtures generally contained 50 ng DNA, 1.5 mM  $MgCl_2$ , 200  $\mu$ M each dNTP, optimized concentrations of corresponding primers and 0.625 U AmpliTaq Gold (Applied Biosystems). All restriction endonucleases for PCR/RFLP were from New England BioLabs (Ipswich, MA, USA). TaqMan allelic discrimination assay for *TP53* variants was done essentially as described previously (Rogounovitch *et al.* 2006). Melting curve  $T_m$ -shift assay for *MTFI* genotyping was designed according to the described technology (Wang *et al.* 2005) and done in a Thermal Cycler Dice Real Time System TP800 (TaKaRa, Ohtsu, Japan). Technical details are available from the authors upon request.

For every SNP, some 20–30 randomly chosen DNA samples, unless otherwise specified, were also analyzed by direct sequencing with a Big Dye Terminator sequencing kit v 3.1 (Applied Biosystems) in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). A complete concordance between different techniques was observed.



Table 1 Single-nucleotide polymorphism (SNP), genotyping methods and possible functional role

SNP nucleotide/amino acid change	Database ID	Genotyping method	Chromosome/exon or intron	MAF (%) in different populations (NCBI dbSNP)	SNP effects, minor allele versus wild-type (reference)
ATM G5557A Asp1853Asn	rs1801516	PCR/RFLP <sup>a</sup> (AflII) GG (187, 30) GA (217, 187, 30) AA (217)	11/exon 39	European: 7–22 Asian: 0–2 Global: 5	Alters an exonic splicing enhancer, modulates correct splicing of exon 39 (Thorstenson et al. 2003) Decreases ATM expression level and capacity of DNA damage recognition (Heikkinen et al. 2005)
ATM IVS22 – 77 T > C T60136C	rs664677	PCR/RFLP (RsaI) TT (299) TC (299, 265, 34) CC (265, 34)	11/intron 22	European: 34–50 Asian: 44–70 Global: 35–36	No reports
ATM IVS48 + 238 C > G C113450G	rs609429	PCR/RFLP (KpnI) CC (172, 35) CG (207, 172, 35) GG (207)	11/intron 48	European: 60 Asian: 37 Global: 53	Generates a weak additional donor splice site and decreases gene expression (Angele et al. 2003)
XRCC1 G25211A Arg280His	rs25489	PCR/RFLP (RsaI) GG (155, 123) GA (278, 155, 123) AA (278)	19/exon 9	European: 3–10 Asian: 0 Global: 7	Compromises DNA repair (reviewed by Hu et al. 2005)
XRCC1 G25897A Arg399Gln	rs25487	PCR/RFLP (MspI) GG (327, 107) GA (434, 327, 107) AA (434)	19/exon 10	European: 30–46 Asian: 27 Global: 23–26	Affects IR-induced mitotic delay and hypersensitivity to IR (Hu et al. 2002) Compromises single-strand DNA breaks repair (controversially) (reviewed by Taylor et al. 2002)
TP53 G640C Arg72Pro	rs1042522	TaqMan	17/exon 4	European: 23–27 Asian: 40–51 Global: 35	Lower efficiency in apoptosis induction; higher level of G1 arrest (Pim & Banks 2004)
XRCC3 C18067T Thr241Met	rs861539	TaqMan	14/exon 7	European: 41–45 Asian: 6–14 Global: 22	Decreased DNA repair capacity (reviewed by Han et al. 2006)
MTF1 T2193A	rs11488567	Melting curve $T_m$ -shift	1/intron 1	Unknown	No reports
MTF1 G20433A	rs3912368	Melting curve $T_m$ -shift	1/intron 5	European: 25–37 Asian: 21	No reports

<sup>a</sup>Restriction enzymes, genotypes and corresponding restriction fragments sizes (bp) are indicated for the SNPs analyzed by PCR/RFLP.

Table 2 Primers and probes for genotyping

SNP	Primer/probe sequences (5'–3') <sup>a</sup>	Primer/probe concentration (μM)	Annealing temperature (°C)
ATM G5557A	F: CCATACTTGATTCATGATATTTTACcttAA R: TTCCATCTTAAATCCATCTTTCTC	0.2 0.2	57
ATM IVS22–77 T>C	F: AGTTTAGCACAGAAAGACATATTGGAAGTAACgTA R: CGGGAAAAGAAGCTGTGGTTAAATATGAAA	0.2 0.2	57
ATM IVS48+238 C>G	F: CTC AATTTCTGTTATAAAAATGAGAAGgTAC R: TTA ACTACTTGT CAGGGACTATCTTAAGGAC	0.2 0.2	57
XRCC1 G25211A	F: GTCTGAGGGAGGAGGGTCTG R: TTCTGGAAGCCACTCAGCAC	0.2 0.2	59
XRCC1 G25897A	F: CCACCAGCTGTGCCTTTG R: CCGGGACTCACTTTGAATGA	0.2 0.2	55
TP53 G640C	F: CGTCCCAAGCAATGGATGATT R: CCGGTGTAGGAGCTGCTGG w/t allele probe (FAM): CTCCCCGCGTGGCCCC Variant allele probe (VIC): CTCCCCGCGTGGCCCC	0.8 0.8 0.4 0.4	61
XRCC3 C18067T	F: AGGGCCAGGCATCTGCA R: CTTCCGCATCCTGGCTAA w/t allele probe (FAM): TCACGCAGCGTGGCCCCCAG Variant allele probe (VIC): TCACGCAGCATGGCCCCCAG	0.8 0.8 0.5 0.5	61
MTF1 T2193A	F1: <u>GCGGGCAGGGCGGCTTA</u> ACTTTAAAACCATCAAGTCATTTTTAgA F2: <u>GCGGGCTTA</u> ACTTTAAAACCATCAAGTCATTTTTAAT R: ACGCCCAGTCGGCATTGCT	0.2 0.2 0.2	58
MTF1 G20433A	F1: GCGGGCAGGGCGGCTAATTATGCTCACCTGAATATATACAGGG F2: <u>GCGGGCCTAATTATGCTCACCTGAATATATACAGGA</u> R: GAGACCTGTAGAGCTAGGTGGATATACAGAGATAT	0.075 0.2 0.2	63

<sup>a</sup>The bases shown in lowercase are mismatches introduced to generate restriction endonuclease sites (PCR/RFLP) or to optimize allelic specificity ( $T_m$ -shift). The underlined 5' portions of primer sequences correspond to GC tails in the  $T_m$ -shift method.

Raw genotyping outputs were interpreted by at least two independent investigators. Missing results due to genotyping procedure failures accounted for <1% for any SNP tested.

### Statistical analysis

Genotype frequencies in each group were determined by univariate analysis and evaluated for departure from Hardy-Weinberg equilibrium by the  $\chi^2$  test. SNP associations with PTC were assessed by multivariate logistic regression analysis for codominant, multiplicative, dominant, and recessive models to avoid assumptions regarding the mode of inheritance (see notes below Table 4). All analyses were adjusted for gender (male or female, nominal), age (years, continuous), and IR-exposure (yes or no, nominal). Besides all the parameters above, the full model included disease status (yes or no, nominal) and, depending on the mode of inheritance, genotype for each SNP (nominal variable in the codominant, dominant, and recessive models and ordinal in the multiplicative model).

Power calculations were done with the PS software (<http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize>). With given sample size, the study

had a power of 54–99% to detect an OR of 2.0 at the significance level of 5% with MAF ranging 4–45%.

Interaction between SNPs, cancer and radiation exposure were hypothesized *a priori* and evaluated by multivariate analysis with corresponding adjustments. Separate calculations of OR were done in irradiated and non-exposed case-control groups when *P* value for an interaction term did not exceed 0.05.

Statistical analysis was done using SPSS for Windows version 17.0 (SPSS, Inc., Chicago, IL, USA).

### Results

The distribution of genotypes and MAF for each SNP in the four study groups is shown in Table 3. The observed distributions in the control groups were not statistically different from those expected from Hardy-Weinberg equilibrium for all SNP except for *ATM* G5557A and *ATM* IVS22-77 T>C in the non-exposed controls. Since such deviation might point at possible genotyping error (Hosking *et al.* 2004), we reanalyzed 96 non-exposed controls for these SNPs by direct sequencing. There were no inconsistencies between PCR/RFLP and sequencing results (data not shown) ruling out technical flaw. Furthermore, allelic frequencies determined in our study are in a good agreement

**Table 3** Distribution of genotypes and minor allele frequencies by study groups

SNP, genotype	IR-induced PTC n (%)	IR-exposed controls n (%)	Sporadic PTC n (%)	Non-exposed controls n (%)
<b>ATM G5557A</b>	<i>n</i> = 122	<i>n</i> = 198	<i>n</i> = 132	<i>n</i> = 398
GG	95 (77.9)	138 (69.7)	105 (79.5)	293 (73.6)
GA	25 (20.5)	53 (26.8)	24 (18.2)	90 (22.6)
AA	2 (1.6)	7 (3.5)	3 (2.3)	15 (3.8)
<i>P</i>	0.24		0.36	
A, %	11.9	16.9	11.4	15.1
<b>ATM IVS22-77 T&gt;C</b>	<i>n</i> = 123	<i>n</i> = 195	<i>n</i> = 132	<i>n</i> = 398
TT	35 (28.4)	62 (31.8)	45 (34.1)	135 (33.9)
TC	76 (61.8)	102 (52.3)	61 (46.2)	216 (54.3)
CC	12 (9.8)	31 (15.9)	26 (19.7)	47 (11.8)
<i>P</i>	0.17		0.06	
C, %	40.6	42.0	42.8	38.9
<b>ATM IVS48+238 C&gt;G</b>	<i>n</i> = 122	<i>n</i> = 196	<i>n</i> = 132	<i>n</i> = 398
CC	37 (30.3)	68 (34.7)	41 (31.1)	131 (32.9)
CG	69 (56.6)	97 (49.5)	61 (46.2)	201 (50.5)
GG	16 (13.1)	31 (15.8)	30 (22.7)	66 (16.6)
<i>P</i>	0.47		0.28	
G, %	41.4	40.3	45.8	41.8
<b>XRCC1 Arg280His<sup>a</sup></b>	<i>n</i> = 123	<i>n</i> = 195	<i>n</i> = 132	<i>n</i> = 398
GG	113 (91.9)	176 (90.3)	117 (88.6)	366 (92.0)
GA	10 (8.1)	19 (9.7)	15 (11.4)	32 (8.0)
<i>P</i>	0.63		0.24	
A, %	4.1	4.9	5.7	4.0
<b>XRCC1 Arg399Gln</b>	<i>n</i> = 123	<i>n</i> = 197	<i>n</i> = 132	<i>n</i> = 398
GG	55 (44.7)	75 (38.1)	65 (49.2)	158 (39.7)
GA	50 (40.7)	100 (50.7)	53 (40.2)	193 (48.5)
AA	18 (14.6)	22 (11.2)	14 (10.6)	47 (11.8)
<i>P</i>	0.20		0.15	
A, %	35.1	36.5	30.7	36.1
<b>TP53 Arg72Pro</b>	<i>n</i> = 122	<i>n</i> = 197	<i>n</i> = 129	<i>n</i> = 395
GG	53 (43.4)	115 (58.4)	69 (53.5)	196 (49.6)
GC	57 (46.7)	73 (37.0)	49 (38.0)	161 (40.8)
CC	12 (9.9)	9 (4.6)	11 (8.5)	38 (9.6)
<i>P</i>	0.02		0.74	
C, %	33.2	23.1	27.5	30.0
<b>XRCC3 Thr241Met</b>	<i>n</i> = 120	<i>n</i> = 198	<i>n</i> = 132	<i>n</i> = 398
CC	53 (44.2)	82 (41.4)	55 (41.7)	161 (40.5)
CT	51 (42.5)	89 (45.0)	65 (49.2)	192 (48.2)
TT	16 (13.3)	27 (13.6)	12 (9.1)	45 (11.3)
<i>P</i>	0.89		0.78	
T, %	34.6	36.1	33.7	35.4
<b>MTF1 T2193A</b>	<i>n</i> = 122	<i>n</i> = 198	<i>n</i> = 131	<i>n</i> = 397
TT	45 (36.9)	82 (41.4)	44 (33.6)	133 (33.5)
TA	64 (52.5)	91 (46.0)	67 (51.1)	188 (47.4)
AA	13 (10.6)	25 (12.1)	20 (15.3)	76 (19.1)
<i>P</i>	0.52		0.57	
A, %	36.8	35.6	40.8	42.8
<b>MTF1 G20433A</b>	<i>n</i> = 123	<i>n</i> = 198	<i>n</i> = 132	<i>n</i> = 398
GG	62 (50.4)	100 (50.5)	66 (50.0)	192 (48.2)
GA	53 (43.1)	88 (44.4)	56 (42.4)	151 (38.0)
AA	8 (6.5)	10 (5.1)	10 (7.6)	55 (13.8)
<i>P</i>	0.85		0.16	
A, %	28.0	27.3	28.8	32.8

NOTE. Total numbers of samples in each group vary slightly due to genotyping procedures failures.

<sup>a</sup>There was no homozygous (A/A) variant of XRCC1 Arg280His among all samples tested.

with those specified for Caucasians in the dbSNP (build 129, April 2008, Table 1) thus attesting to the appropriate data quality.

As seen from Table 4, an association between *ATM* G5557A and PTC, regardless of radiation exposure, was found. The presence of the A allele significantly decreased PTC risk compared with wild-type G allele in the multiplicative model of inheritance (OR=0.69, 95% CI 0.45–0.86,  $P=0.03$ ), which is useful for risk comparison between the groups based on the analysis of allelic frequencies in them.

Main effect on PTC risk appeared also significant for the *XRCC1* gene Arg399Gln polymorphism. The presence of the minor 399Gln allele decreased PTC risk compared with the Arg/Arg genotype (OR=0.66, 95% CI 0.57–0.88,  $P=0.02$  and OR=0.70, 95% CI 0.59–0.93,  $P=0.03$ , in the co-dominant and dominant models respectively).

Analysis of combined *ATM* G5557A and *XRCC1* Arg399Gln genotypes demonstrated that increasing number of minor alleles (i.e. *ATM* 5557A and *XRCC1* 399Gln) significantly decreased PTC risk in corresponding individuals in comparison with those who do not carry minor alleles (Fig. 1).

No other SNP in any gene showed a significant main effect on PTC.

For *ATM* IVS22-77 T>C and *TP53* Arg72Pro, evidence for interaction between radiation exposure and PTC was found ( $P$  for interaction 0.04 and 0.01 respectively). As shown in Table 5, the analyses performed in IR-exposed and non-irradiated patients compared respectively, with irradiated and non-exposed controls revealed a significantly increased risk of sporadic PTC for the *ATM* IVS22-77 homozygous CC genotype carriers compared with the TC+TT genotypes (the recessive model of inheritance, OR=1.84, 95% CI 1.10–3.24,  $P=0.03$ ), whereas in the irradiated group an insignificant inverse effect of these genotypes was observed (OR=0.59, 95% CI 0.28–1.27,  $P=0.17$ ). For *TP53* codon 72 polymorphism, in all but the recessive models the increased risk of IR-induced PTC as compared with IR-exposed controls was observed. The highest risk of radiogenic PTC was in the co-dominant model (OR=2.33, 95% CI 1.15–7.21,  $P=0.03$ ). A significant risk was also found in the multiplicative model of inheritance (OR=1.70, 95% CI 1.17–2.46,  $P=0.006$ ). In addition, comparison between IR-exposed and non-exposed controls did not reveal statistically significant difference in adjusted distributions of these polymorphisms. In healthy subjects, the strongest association for the *ATM* IVS22-77 T>C was in the recessive model (OR=1.38, 95% CI 0.84–2.26,

$P=0.21$ ) and in the multiplicative model for *TP53* Arg72Pro (OR=0.70, 95% CI 0.52–1.19,  $P=0.11$ ) further emphasizing possible role of these SNPs in PTC of different etiology.

Considering multiple pathways for repairing diverse DNA damages induced by endogenous and exogenous carcinogens, genetic variants in different repair pathways may probably have a joint effect on cancer risk. In attempt to search for the stronger associations between PTC and studied SNPs, we performed the analyses of genotype combinations for the *ATM* and *TP53* polymorphisms as these genes are functionally related and three out of four SNPs included in our study showed effects on PTC. Among the possible *ATM/TP53* combinations (rs1801516/rs664677/rs609429/rs1042522) tested, two demonstrated significant differences in the subsets of both groups of PTCs (Fig. 2). Particularly, the combined *ATM/TP53* GG/TC/CG/GC genotype was strongly associated with the IR-induced PTC (OR=2.10, 95% CI 1.17–3.78,  $P=0.015$ ). Another *ATM/TP53* combination, GG/CC/GG/GG, demonstrated a significantly increased risk for sporadic PTC (OR=3.32, 95% CI 1.57–6.99,  $P=0.002$ ).

## Discussion

Our study addressed possible associations between SNPs in the genes involved in DNA damage response and the risk of PTC of different etiology. The results demonstrated that the presence of the variant 5557A allele in exon 39 of *ATM* and *XRCC1* 399Gln allele, particularly in the heterozygous state, significantly associated with the decreased risk of PTC. The *ATM* IVS22-77 CC genotype in the non-exposed group and the *TP53* 72Pro allele in the radiation-related one associated with the increased risk of PTC. Moreover, two particular *ATM/TP53* combined genotypes were found with higher frequencies in the IR-induced or sporadic PTC when compared with the controls. Altogether, these data indicate that SNPs in the studied genes may likely modify PTC risk.

A significant association between the *ATM* G5557A and bilateral breast cancer in Caucasian patients has been shown before (Heikkinen *et al.* 2005). Also, this SNP has been reported as a possible modulator of clinical radiosensitivity in cancer. The *ATM* 5557A allele was associated with severe adverse effects of radiation therapy in prostate (Hall *et al.* 1998) and breast cancer patients (Angele *et al.* 2003). Later, an enhanced radiosensitivity of human fibroblasts in the presence of the *ATM* 5557A allele was demonstrated in an experimental work (Alsbeih *et al.* 2007). In contrast

**Table 4** OR (95% CI) for papillary thyroid carcinoma (PTC) by gene polymorphism according to different models of inheritance (adjusted for age, gender and radiation exposure). *P* < 0.05 in bold

SNP	Genotype	OR (95% CI)	<i>P</i>
ATM G5557A	GG	1.00 <sup>a</sup>	
	GA	0.75 (0.49–1.15)	0.31
	AA	0.61 (0.21–1.77)	0.45
	Risk per A allele <sup>b</sup>	0.69 (0.45–0.86)	<b>0.03</b>
	GA + AA versus GG <sup>c</sup>	0.73 (0.48–1.10)	0.13
	AA versus GA + GG <sup>d</sup>	0.65 (0.23–1.87)	0.41
ATM IVS22–77 T>C	TT	1.00	
	TC	1.03 (0.70–1.50)	0.74
	CC	1.19 (0.70–2.04)	0.47
	Risk per C allele	1.08 (0.83–1.40)	0.57
	TC + CC versus TT	1.06 (0.74–1.53)	0.75
	CC versus TC + TT	1.17 (0.72–1.90)	0.52
ATM IVS48+238 C>G	CC	1.00	
	CG	1.10 (0.75–1.62)	0.55
	GG	1.14 (0.69–1.89)	0.84
	Risk per G allele	1.07 (0.84–1.37)	0.57
	CG + GG versus CC	1.11 (0.77–1.60)	0.57
	GG versus CG + CC	1.08 (0.69–1.69)	0.74
XRCC1 Arg280His <sup>e</sup>	GG	1.00	
	GA	1.12 (0.62–2.01)	0.71
	Risk per A allele	1.15 (0.70–1.87)	0.61
XRCC1 Arg399Gln	GG	1.00	
	GA	0.66 (0.57–0.88)	<b>0.02</b>
	AA	0.88 (0.50–1.57)	0.56
	Risk per A allele	0.90 (0.69–1.17)	0.41
	GA + AA versus GG	0.70 (0.59–0.93)	<b>0.03</b>
	AA versus GA + GG	0.98 (0.57–1.69)	0.94
TP53 Arg72Pro	GG	1.00	
	GC	1.02 (0.70–1.47)	0.89
	CC	1.16 (0.63–2.14)	0.38
	Risk per C allele	1.05 (0.81–1.38)	0.70
	GC + CC versus GG	1.04 (0.74–1.48)	0.82
	CC versus GC + GG	1.15 (0.64–2.08)	0.64
XRCC3 Thr241Met	CC	1.00	
	CT	0.99 (0.69–1.44)	0.99
	TT	0.96 (0.54–1.70)	0.92
	Risk per T allele	0.99 (0.76–1.28)	0.92
	CT + TT versus CC	0.99 (0.70–1.41)	0.97
	TT versus CT + CC	0.96 (0.56–1.64)	0.88
MTF1 T2193A	TT	1.00	
	TA	1.07 (0.73–1.56)	0.61
	AA	0.83 (0.49–1.41)	0.46
	Risk per A allele	0.94 (0.73–1.21)	0.63
	TA + AA versus TT	1.00 (0.70–1.44)	0.99
	AA versus TA + TT	0.80 (0.49–1.29)	0.35
MTF1 G20433A	GG	1.00	
	GA	1.14 (0.79–1.63)	0.43
	AA	0.76 (0.40–1.43)	0.21
	Risk per A allele	0.97 (0.74–1.25)	0.80
	GA + AA versus GG	1.05 (0.76–1.49)	0.76
	AA versus GA + GG	0.71 (0.39–1.32)	0.27

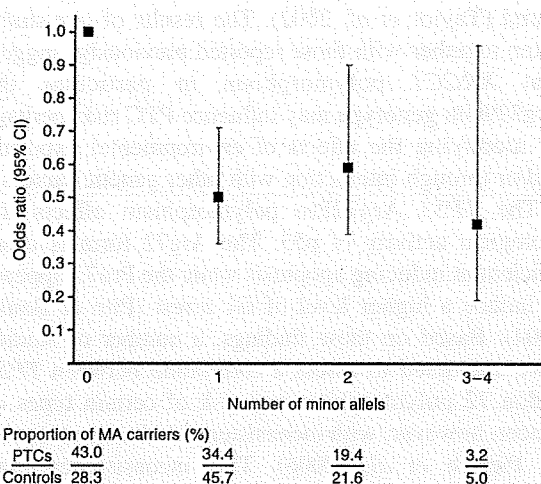
<sup>a</sup>Codominant model of inheritance (wild-type homozygous genotype serves as the reference).

<sup>b</sup>Multiplicative model of inheritance (uses allele frequencies).

<sup>c</sup>Dominant inheritance model (combined heterozygous and homozygous for the minor allele versus wild-type homozygous).

<sup>d</sup>Recessive inheritance model (minor allele homozygous versus combined heterozygous and homozygous for the wild-type allele).

<sup>e</sup>The dominant and recessive models are not shown for XRCC1 Arg280His because of the absence of homozygous (A/A) genotype among 848 samples tested.



**Figure 1** Effect of increasing number of minor alleles (MA) for *ATM* G5557A and *XRCC1* Arg399Gln (minor alleles, *ATM* 5557A, and *XRCC1* 399Gln) on PTC risk. The combined genotype with 0 MA was used as a reference. *P* values for genotypes with different MA number:  $P_{1MA} < 0.0001$ ;  $P_{2MA} < 0.01$ ;  $P_{3-4MA} < 0.05$ . Carriers of three and four minor alleles were combined because of the exceedingly low number of 4 MA carriers in both PTC and control groups.

to these reports, Edvardsen *et al.* (2007) revealed an increasing rate of side effects of radiotherapy with decreasing frequency of this variant allele. Our data are rather in agreement with the latter report and favor the protective role of the *ATM* 5557A allele in PTC development.

The intronic *ATM* polymorphisms IVS22–77 T>C and IVS48 + 238 C>G in the homozygous state have

been associated with increased breast cancer risk and in the heterozygous state with clinical radioprotection (Angele *et al.* 2003). These findings were confirmed in the *in vitro* experiments using lymphoblastoid cell lines established from corresponding patients. Our investigation demonstrated the association between the IVS22–77 CC genotype and increased risk of sporadic PTC in adult patients. By contrast, in the IR-induced PTC group, there was an inverse non-significant correlation for this genotype. At the same time, in the IR-induced PTCs, the number of patients heterozygous for IVS22–77 was somewhat, but insignificantly, higher as compared with sporadic PTCs (Table 3). The results for the IVS48 + 238 C>G tended to parallel those for the IVS22–77 T>C remaining below the threshold of significance. At present, the mechanistic and functional basis for the intronic *ATM* SNPs implications in cancer revealed in the previous studies and in ours as well is not fully understood. In a broader sense, however, they may be indicative of a role for the *ATM* gene (or its product) in the development of PTC.

As reviewed by Hu *et al.* (2005), the results of the *XRCC1* gene Arg399Glu investigations vary in different cancers for populations with different ethnicities. In relation to cancer and radiation, the 399Gln allele in combination with 280His was associated with breast cancer risk, and in pair with 194Trp with clinical radiosensitivity in Caucasian women with breast cancer. Also, the 399Gln allele was found to decrease

**Table 5** OR (95% CI) for papillary thyroid carcinoma (PTC) of different etiology by *ATM* and *TP53* polymorphisms (adjusted for gender and age). *P* < 0.05 in bold

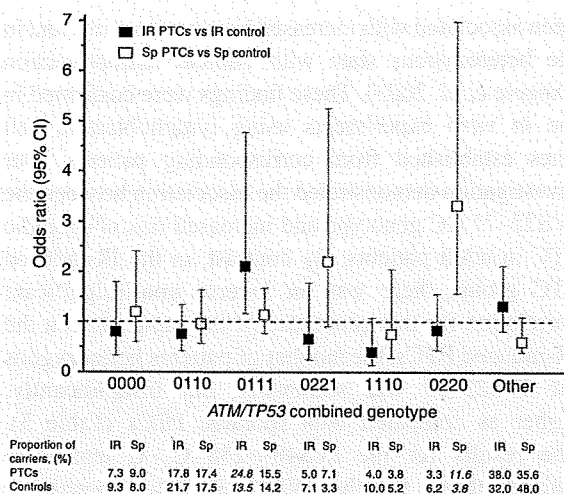
SNP	Genotype	IR-induced PTC versus IR-exposed controls		Sporadic PTC versus non-exposed controls	
		OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
<i>ATM</i> IVS22–77 T>C	TT	1.00 <sup>a</sup>		1.00	
	TC	1.38 (0.80–2.39)	0.19	0.82 (0.51–1.32)	0.50
	CC	0.73 (0.31–1.70)	0.44	1.63 (0.87–3.08)	0.09
	Risk per C allele <sup>b</sup>	0.97 (0.66–1.41)	0.86	1.18 (0.86–1.62)	0.32
	TC + CC versus TT <sup>c</sup>	1.23 (0.72–2.10)	0.44	0.97 (0.62–1.52)	0.88
	CC versus TC + TT <sup>d</sup>	0.59 (0.28–1.27)	0.17	1.84 (1.10–3.24)	<b>0.03</b>
<i>TP53</i> Arg72Pro	GG	1.00		1.00	
	GC	1.68 (1.11–2.75)	<b>0.03</b>	0.84 (0.53–1.33)	0.52
	CC	2.33 (1.15–7.21)	<b>0.03</b>	0.84 (0.39–1.79)	0.73
	Risk per C allele	1.70 (1.17–2.46)	<b>0.006</b>	0.89 (0.64–1.23)	0.47
	GC + CC versus GG	1.80 (1.06–2.36)	<b>0.01</b>	0.84 (0.54–1.29)	0.43
	CC versus GC + GG	2.06 (0.79–5.41)	0.14	0.90 (0.44–1.88)	0.79

<sup>a</sup>Codominant model of inheritance (wild-type homozygous genotype serves as the reference).

<sup>b</sup>Multiplicative model of inheritance (uses allele frequencies).

<sup>c</sup>Dominant inheritance model (combined heterozygous and homozygous for the minor allele versus wild-type homozygous).

<sup>d</sup>Recessive inheritance model (minor allele homozygous versus combined heterozygous and homozygous for the wild-type allele).



**Figure 2** The combined *ATM/TP53* genotypes and risk of PTC of different etiology. The combined genotypes were analyzed separately in the IR-exposed and sporadic PTCs versus corresponding control. Six combinations of 3 *ATM* and 1 *TP53* SNPs (rs1801516/rs664677/rs609429/rs1042522) whose frequencies were higher than 5% at least in two of four subgroups are shown. In the numerical codes for any SNP, 0 – the genotype with no MA (i.e. homozygous wild-type); 1 – 1 MA presents (heterozygous genotype); 2 – 2 MA present (homozygous variant genotype); first three numbers correspond to 3 *ATM* SNPs and the last one to *TP53* polymorphism. In the figure, the *GG/TT/CC/GG* genotype is represented by the '0000' numerical code as it does not contain minor alleles; the *GG/TC/CG/GG* corresponds to 0110, *GG/TC/CG/GC* to 0111; *GG/CC/GG/GC* to 0221; *GA/TC/CG/GG* to 1110, and *GG/CC/GG/GG* to 0220. All combinations with frequencies <5% in three or more subgroups are pooled and indicated as 'other'.

the risk of bladder cancer and squamous cell carcinoma of the head and neck.

Interestingly, not only variant but also wild-type allele (i.e. *XRCC1* 399Arg) demonstrated possible role in cancer. High-dose radiation to the chest was more strongly associated with breast cancer among white American women with *XRCC1* Arg399Arg genotype (Duell et al. 2001). Looking for potential biological explanations for these findings, the authors found a higher prevalence of *TP53* deletions in the Arg399Arg cases exposed to occupational radiation compared with exposed patients with the Gln399Gln genotypes or unexposed cases of either genotype. Figueiredo et al. (2004) observed an increased risk of disease among wild-type homozygous (Arg/Arg) and heterozygous Canadian Caucasian women with a family history of breast cancer compared with the individuals without such.

The described above data may be explained, at least in part, by the results of functional study of this polymorphism in which an equal ability for both alleles to suffice single strand break repair by *XRCC1* has been

found (Taylor et al. 2002). The results of our study, taken together with those reported previously, suggest that *XRCC1* polymorphism in particular the Arg399Gln genotype may influence PTC risk, perhaps by modifying the effects of environmental exposure and/or through interaction with other genetic factors.

The *TP53* Arg72Pro polymorphism affects the biological activity of p53. The Arg72 form is more efficient at inducing apoptosis while the Pro72 appears to induce a higher level of G1 arrest (Pim & Banks 2004). Based on these findings, a number of studies have attempted to assess a correlation between *TP53* codon 72 polymorphism and risk of certain types of cancer, however, with inconsistent results, as reviewed by Pietsch et al. (2006). This inconsistency may possibly be explained in part by the coexistence of the codon 72 polymorphism and gain of function mutations in *TP53* in some tumors (Pietsch et al. 2006, Soussi & Wiman 2007).

Several groups have investigated the *TP53* Arg72-Pro polymorphism in PTC. Boltze et al. (2002) found a small number of heterozygotes and no Pro/Pro genotype in differentiated thyroid carcinomas from Germany. By contrast, in ethnically heterogeneous Brazilian population, the Pro/Pro genotype was associated with the higher risk of differentiated thyroid cancer (Granja et al. 2004). The study of codon 72 polymorphism in thyroid tumors from Russian and Ukrainian patients demonstrated a significantly lower frequency of wild-type homozygotes (i.e. Arg/Arg) among adults with IR-induced PTC when compared with sporadic PTC cases and general population (Rogounovitch et al. 2006). Data obtained in the present work, using an independent set of samples, confirm these findings suggesting the modifying role (or as of a marker) of the *TP53* Arg72Pro polymorphism in PTC developed after exposure to IR which is further supported by the absence of significant difference in genotype distributions among our two control groups.

As shown in a genetic study, frequencies of the C allele (encoding 72Pro) do not generally differ in populations of Belarus and Russia (Khrunin et al. 2005). However, East Slavs do not form a single genetic cluster on multidimensional analysis. The 72Pro allele frequency in Belarus is about 0.3; in the two different subpopulations from the Central and Northern regions of the European part of Russia it is 0.24 and 0.32 respectively. The study of healthy population from Poland (bordering with Belarus, linguistically and culturally similar), reported the frequency of 0.28 for the 72Pro allele (Siddique et al. 2005). The 72Pro frequency reported by

Rogounovitch *et al.* (2006) in Russian healthy controls is also 0.28. Thus, the effect of population admixtures in the controls in our investigation could not be completely ruled out. Yet on the other hand, the ratio of Belarusian and Russian subjects in the IR-exposed PTCs and controls was similar (2.24 and 2.30 respectively) suggestive of an unbiased estimate and being an argument in support of *TP53* Arg72Pro polymorphism association with radiation-related PTC.

While many studies established the effect of individual SNPs on cancer, the role of SNP combinations has been less addressed. Several *ATM* and *TP53* haplotypes were associated with clinical radiosensitivity in breast cancer (Angele *et al.* 2003) and brain tumor risk (Malmer *et al.* 2007). Recently, the interactions of SNPs located on different chromosomes were investigated in various malignancies (Yen *et al.* 2008, Yoon *et al.* 2008). One experimental study, in which *ATM* Asp1853Asn, *TP53* Arg72Pro, *XRCC1* Arg399Gln, and *XRCC3* Thr241Met were genotyped, demonstrated that the increasing number of risk alleles enhanced radiosensitivity of human fibroblast cell lines and, potentially, susceptibility to radiation-induced cancers (Alsbeih *et al.* 2007). So far no studies have investigated the joint effect of gene polymorphisms on thyroid cancer. Our observations demonstrated that frequencies of particular combined *ATM/TP53* genotypes were higher in patients with radiogenic or sporadic PTC compared with corresponding control populations.

To some extent these results support the idea that genetic factors may possibly modify predisposition to thyroid cancer. A recent study by Detours *et al.* (2007) reported difference in the expression levels of some genes between Chernobyl PTCs from Ukraine and French sporadic PTCs. Although the mentioned work and the present one are different in molecular approaches, the results of both are suggestive of a possible genetic 'susceptibility signature' that may contribute to the individual predisposition to IR and other carcinogens' effects. These findings are in favor of a 'susceptibility model' that may partly explain why only a minority of the large population exposed to the IR after the Chernobyl disaster developed thyroid cancer (Yamashita & Saenko 2006, Detours *et al.* 2007, 2008).

It is necessary to note that even though nine SNPs were analyzed in our study, no correction for multiple comparisons was applied because of study design and techniques employed. The associations were tested in a one-at-a-time fashion in a limited sample size in the difficult to access groups. The need for correction in such circumstances is still debated (Rothman &

Greenland 1998). Furthermore, since data obtained in this work may be referred to as an initial screening result, non-adjusted presentation enables their inclusion in future meta-analysis. Effects of candidate SNPs that we report need validation in other studies.

In conclusion, the results presented here show that SNPs in *ATM* exon 39 and *XRCC1* exon 10 may be the markers of a decreased PTC risk in adults, whereas the *ATM* IVS22-77 and *TP53* codon 72 SNPs genes may associate with the risk of PTC development in non-irradiated and irradiated individuals. To the best of our knowledge, presented here is the first study of this kind reporting the results of genotyping of candidate DNA damage response genes in irradiated and non-irradiated PTC patients and in corresponding healthy populations. Our data support the paradigm of genetic modifiers of radiation-associated carcinogenesis and perhaps may contribute to genetic determination of PTC-prone subjects. We believe such identification will allow future personalized cancer risk prediction which is of a significant importance in view of the growing thyroid cancer incidence and also because of the relevance to occupational and radiation emergency medicine issues.

#### Declaration of interest

The authors declare no potential conflict of interest.

#### Funding

This work was supported in part by Grant-in-Aid for Scientific Research 19256003, 19510058, and 19790651 from Japan Society for the Promotion of Science.

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Research article

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## Mouse gastric tumor models with prostaglandin E<sub>2</sub> pathway activation show similar gene expression profiles to intestinal-type human gastric cancer

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Published: 17 December 2009

Received: 13 April 2009

BMC Genomics 2009, 10:615 doi:10.1186/1471-2164-10-615

Accepted: 17 December 2009

This article is available from: <http://www.biomedcentral.com/1471-2164/10/615>

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### Abstract

**Background:** Gastric cancers are generally classified into better differentiated intestinal-type tumor and poorly differentiated diffuse-type one according to Lauren's histological categorization. Although induction of prostaglandin E<sub>2</sub> pathway promotes gastric tumors in mice in cooperation with deregulated Wnt or BMP signalings, it has remained unresolved whether the gastric tumor mouse models recapitulate either of human gastric cancer type. This study assessed the similarity in expression profiling between gastric tumors of transgenic mice and various tissues of human cancers to find best-fit human tumors for the transgenic mice models.

**Results:** Global expression profiling initially found gastric tumors from *COX-2/mPGES-1* (C2mE)-related transgenic mice (*K19-C2mE*, *K19-Wnt1/C2mE*, and *K19-Nog/C2mE*) resembled gastric cancers among the several tissues of human cancers including colon, breast, lung and gastric tumors. Next, classification of the C2mE-related transgenic mice by a gene signature to distinguish human intestinal- and diffuse-type tumors showed C2mE-related transgenic mice were more similar to intestinal-type compared with diffuse one. We finally revealed that induction of Wnt pathway cooperating with the prostaglandin E<sub>2</sub> pathway in mice (*K19-Wnt1/C2mE* mice) further reproduce features of human gastric intestinal-type tumors.

**Conclusion:** We demonstrated that C2mE-related transgenic mice show significant similarity to intestinal-type gastric cancer when analyzed by global expression profiling. These results suggest that the C2mE-related transgenic mice, especially *K19-Wnt1/C2mE* mice, serve as a best-fit model to study molecular mechanism underlying the tumorigenesis of human gastric intestinal-type cancers.

### Background

Gastric cancers are classically categorized into intestinal type and diffuse type based on Lauren's histological clas-

sification [1]. Intestinal-type gastric cancers are characterized by better differentiated, cohesive and glandular-like cell groups. The intestinal type is progressed through mul-

multiple steps beginning with atrophic gastritis that is followed by intestinal metaplasia, dysplasia and carcinoma [2,3]. Diffuse type corresponds to poorly differentiated, infiltrating and non-cohesive tumor cells. Although diffuse type is not characterized by the multiple proceeding steps, this shows more metastatic phenotype with poorer prognosis.

Several genetic alterations are more frequently observed in either subtype of gastric cancer. Overexpression of *ErbB2* is selectively found in intestinal-type tumors and may serve as prognostic marker for tumor invasion [4,5]. *ErbB2* expression level was reported to correlate with lymph node or liver metastasis [6,7]. Significant decrease in the expression of E-cadherin (*CDH1*) has also been described preferentially in diffuse-type gastric cancer ranging from 20% to 90% of frequency [8-10]. The decreased expression of *CDH1* is caused by LOH or hypermethylation. Interestingly, hereditary diffuse gastric cancer is caused by germline mutations of *CDH1* gene [11,12]. In addition, mutation in adenomatous polyposis coli (*APC*) which activates Wnt/ $\beta$ -catenin pathway is predominantly found in intestinal-type gastric cancer [13]. Cyclooxygenase-2 (*COX-2*) that is one of the crucial enzymes to synthesize prostaglandin  $E_2$  is highly up-regulated in intestinal-type cancers compared with diffuse-type ones [14]. These genetic alterations could be used as a hallmark of each type of gastric cancer as well as the histological features.

Genome-wide mRNA expression profiles have identified gene signatures to distinguish intestinal- and diffuse-type gastric cancers. Boussioutas *et al.* [15] reported that the gene signature distinctive for intestinal type exhibits the up-regulation of proliferation markers related to DNA replication, spindle assembly and chromosome segregation. Down-regulated genes in the signature are associated with epithelial differentiation. Jinawath *et al.* [16] also developed another gene signature that is differentially expressed between intestinal-type and diffuse-type cancers with Japanese gastric tumor samples. The intestinal-type signature represented enhancement of cell cycle progression, while the genes associate with extracellular-matrix (ECM) are deregulated in the diffuse type signature. These signatures could provide opportunities of developing biomarkers to diagnose/distinguish the two types in both clinical and preclinical researches.

Transgenic mice that develop gastric tumors present suitable models to decipher gastric tumorigenesis, and identify novel therapeutic targets. We have previously developed several transgenic mice in which prostaglandin  $E_2$  production pathway is highly activated specifically in gastric mucosa. *K19-C2mE* mice expressing *COX-2* and microsomal prostaglandin synthase-1 (*mPGES-1*) develop inflammation-associated hyperplasia [17]. This was medi-

ated through the recruitment of mucosal macrophages. By crossing the *K19-C2mE* mice with *K19-Wnt1* mice, cooperative effect of Wnt1 and  $PGE_2$  on gastric tumorigenesis was investigated. The *K19-Wnt1/C2mE* mice led to the development of dysplastic gastric adenocarcinoma signifying the importance of the Wnt pathway activation to keep the progenitor cells undifferentiated [18]. To examine the additional effect of the suppression of BMP pathway on the prostaglandin  $E_2$  activation, the compound mice of *K19-Nog/C2mE* were established. The *K19-Nog/C2mE* mice cause the development of gastric hamartomas that are morphologically similar to juvenile polyposis (JP) [19]. Although the detailed histological and hypothesis-based molecular analysis implicated the pivotal role of prostaglandin  $E_2$ , Wnt and Nog pathway respectively in gastric tumorigenesis, it remains elusive whether the *K19-C2mE* and its compound transgenic mice show similarity to intestinal type or diffuse type of human gastric cancers when analyzed by non-biased global expression profile.

In order to identify which types of human gastric tumors (intestinal or diffuse type) the *C2mE*-related mice are more similar to, we compared expression profile of the two types of human gastric cancer with those of *K19-C2mE*, *K19-Wnt1/C2mE*, and *K19-Nog/C2mE* transgenic mice.

## Results

### Overall gene expression profiles of transgenic animals

We have previously developed several types of transgenic mice in which prostaglandin  $E_2$  pathway is activated. *K19-C2mE* mice expressing *COX-2* and *mPGES-1* induce hyperplastic gastric tumors. *K19-Wnt1/C2mE* mice in which both Wnt and prostaglandin  $E_2$  pathways are activated cause dysplastic gastric tumors. *K19-Nog/C2mE* mice expressing noggin as well as *C2mE* develop gastric hamartomas. To provide insight into the molecular mechanism of gastric tumorigenesis, gastric tissues from the transgenic mice and wild-type mice were subject to microarray analysis. Using the Affymetrix GeneChip system, mRNA expression levels were measured for 45,037 probe sets, which represent 21,066 Entrez genes and 5,324 other sequences. Increased expression of introduced gene in each transgenic mouse was observed as reported previously [17-19].

Genome-scale overview of the microarray data revealed that expression changes in the three tumor models of *K19-C2mE*, *K19-Wnt1/C2mE* and *K19-Nog/C2mE* were quite similar, whereas overexpression of Wnt1 only or Nog only led to the expression changes in a small portion of genes (Figure 1). This suggests most of expressional changes in the three transgenic mice were caused by the activation of  $PGE_2$  pathway. Hypergeometric test for gene enrichment showed that the genes involved in wound healing and