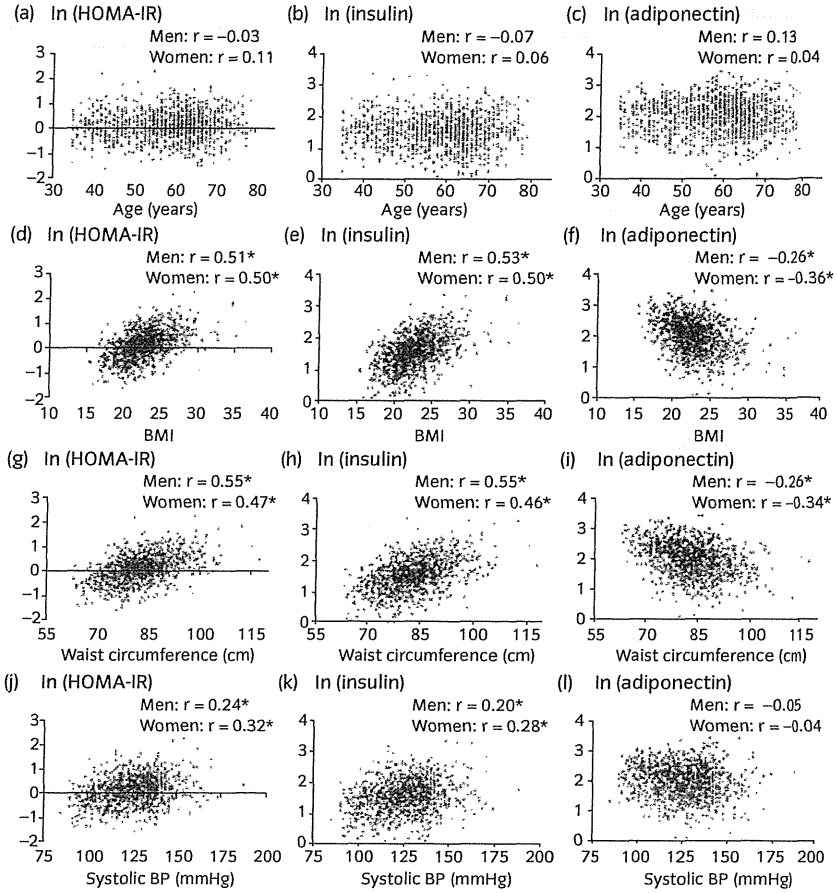

Fig. 1 Scatter plots with the *r*-values for correlation coefficients between (a–c) natural logarithm (ln) homeostasis model assessment of insulin resistance (HOMA-IR), ln (insulin), ln (adiponectin) and age, (d–f) body mass index (BMI), (g–i) waist circumference, and (j–l) systolic blood pressure (BP) by sex. *$P < 0.0001$. (∘), Men; (•), women.

Table 3 The prevalence of subjects with self-reported kidney stone and age-adjusted OR (95% CI) across homeostasis model assessment of insulin resistance tertiles

	Tertile			*P* for trend
	Lower	Middle	Upper	
Men ($n = 529$)	$n = 176$	$n = 177$	$n = 176$	
Median HOMA-IR value (range)	0.62 (0.20–0.89)	1.13 (0.90–1.42)	2.03 (1.43–9.91)	
Self-reported kidney stone, *n* (%)	31 (17.6)	28 (15.8)	25 (14.2)	0.38*
Age-adjusted OR (95% CI)	1.00	0.89 (0.51–1.57)	0.78 (0.44–1.39)	0.41**
Women ($n = 507$)	$n = 168$	$n = 170$	$n = 169$	
Median HOMA-IR value (range)	0.64 (0.25–0.91)	1.10 (0.91–1.32)	1.75 (1.33–9.13)	
Self-reported kidney stone, *n* (%)	8 (4.8)	11 (6.5)	16 (9.5)	0.09*
Age-adjusted OR (95% CI)	1.00	1.31 (0.51–3.37)	1.89 (0.78–4.60)	0.14**

*P-values for trend in proportions of subjects with self-reported kidney stone using Mantel–Haenszel chi-square test. **P-values calculated using median of tertiles as a continuous variable in logistic regression models.

stone history, due in part to the small number of subjects. With an increased number of study subjects, the differences might reach significance in men. However, impacts of Mets components on kidney stone formation in men would not be greater than those in women.

Primarily, the basis for sex differences in kidney stone formation is unclear, though kidney stones are more common in men than women. Some hormonal factors might be involved. In the present study, women with a history of kidney stone formation were older than women without a

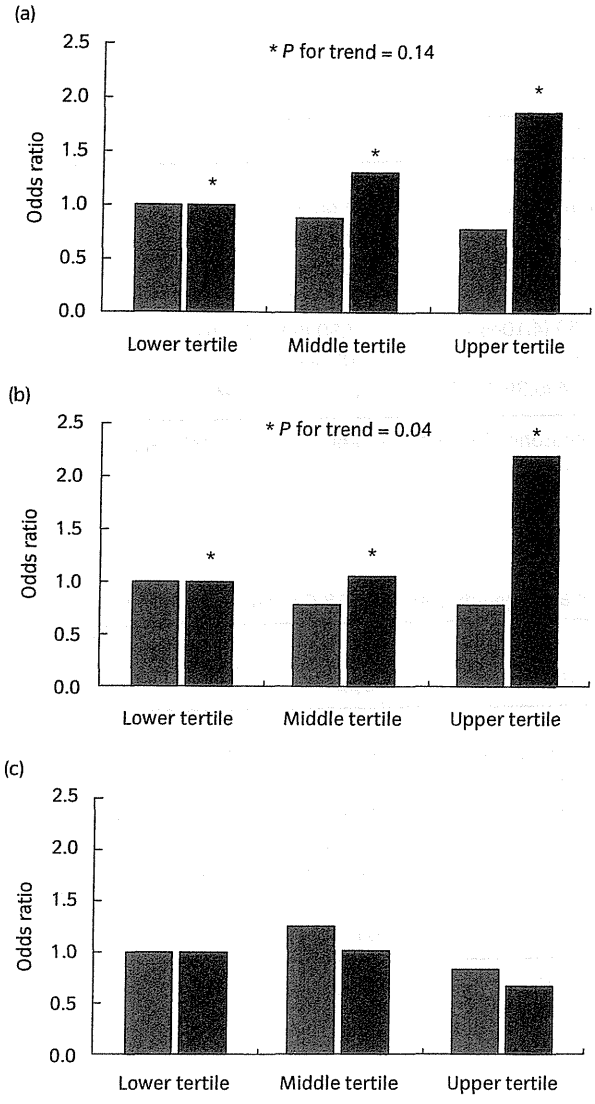

Fig. 2 Age-adjusted odds ratio for a self-reported history of kidney stones across tertiles of (a) homeostasis model assessment of insulin resistance (HOMA-IR), (b) insulin and (c) adiponectin. (▩), Men; (▩), women.

history of kidney stone formation, and postmenopausal status was significantly more frequent in women with a history of kidney stone formation. Estrogen, which reduces urinary excretion of calcium, is considered a protective factor for kidney stone formation.[19] Therefore, menopause could be a risk for kidney stone formation by increasing urinary calcium excretion in women.[20,21] However, no independent association was observed between menopause or postmenopausal hormone use and the risk of kidney stone formation.[22] In the present study, data regarding postmenopausal hormone use were lacking; however, only approximately 5% of postmenopausal Japanese women have been reported to take hormone replacement therapy.[23] Because age at the first onset of kidney stone was not surveyed in the present study, we could not examine these issues accurately.

Relationships of insulin resistance as assessed by HOMA-IR and insulin with history of kidney stone formation were also identified in the present study; HOMA-IR and insulin were significantly higher in women with a history of kidney stone formation, but not in men. Furthermore, the age-adjusted OR for history of kidney stone formation showed a positive trend across insulin tertiles (*P*-value for trend = 0.04: Table 4 & Fig. 2b) in women. Although the age-adjusted OR of women with a self-reported history of kidney stones increased across HOMA-IR tertiles, the *P*-value for the trend did not reach significance (*P*-value for trend = 0.14: Table 3 & Fig. 2a). In the present study, the "gold standard" for assessment of insulin resistance, the glucose clamp method, was not used,[24] and diabetic patients were not excluded. HOMA-IR might not be appropriate for assessing insulin resistance in advanced diabetic patients, because HOMA-IR relies on the product of fasting glucose and insulin. Therefore, HOMA-IR as a parameter of insulin resistance could have affected the results of the present study. However, many previous studies have shown that HOMA-IR has a close correlation with the insulin resistance measured using the glucose clamp method in patients with or without DM.[14,25,26]

The mechanism of hyperinsulinemia and insulin resistance on kidney stone formation has been described. High insulin resistance and hyperinsulinemia could contribute to the development of calcium stones by lowering urinary citrate excretion[27] or increasing urinary calcium excretion.[28–30] In both sexes, HOMA-IR and insulin were positively correlated with BMI, waist circumference, and systolic and diastolic BP in the present study (Fig. 1) However, the prevalence of self-reported hypertension, DM and dyslipidemia was not associated with kidney stones in either sex, due in part to the small number of patients.

In contrast, adiponectin was not different between subjects with and without a history of kidney stones in either sex, though we might have expected adiponectin to show a negative relationship with kidney stones. In both sexes, adiponectin was negatively correlated with BMI and waist circumference in the present study (Fig. 1). However, *r*-values for correlation coefficients between ln (adiponectin) and obesity estimated by BMI and waist circumference were lower than those between ln (HOMA-IR), ln (insulin) and obesity in both sexes (Fig. 1). Therefore, the effect of adiponectin on kidney stones through obesity could not be detected. In the present study, the participants were apparently healthy; larger numbers of subjects with hypoadiponectinemia are needed to correctly evaluate the effect of adiponectin on kidney stone disease.

The limitations of the present study are as follows. First, a history of kidney stones by self-reported questionnaire is

Table 4 The prevalence of subjects with self-reported kidney stone and age-adjusted OR (95% CI) across insulin tertiles

	Tertile			*P* for trend
	Lower	Middle	Upper	
Men ($n = 529$)	$n = 180$	$n = 171$	$n = 178$	
Median insulin (range), μU/mL	2.70 (1.10–3.70)	4.60 (3.80–5.70)	7.90 (5.80–27.50)	
Self-reported kidney stone, *n* (%)	33 (18.3)	25 (14.6)	26 (14.6)	0.33*
Age-adjusted OR (95% CI)	1.00	0.77 (0.44–1.36)	0.77 (0.44–1.36)	0.41**
Women ($n = 507$)	$n = 167$	$n = 172$	$n = 168$	
Median insulin (range), μU/mL	3.00 (1.10–4.00)	4.80 (4.10–5.70)	7.30 (5.80–29.10)	
Self-reported kidney stone, *n* (%)	8 (4.8)	9 (5.2)	18 (10.7)	0.03*
Age-adjusted OR (95% CI)	1.00	1.04 (0.39–2.77)	2.20 (0.92–5.26)	0.04**

**P*-values for trend in proportions of subjects with self-reported kidney stone using Mantel-Haenszel χ^2-test. ***P*-values calculated using median of tertiles as a continuous variable in logistic regression models.

Table 5 The prevalence of subjects with self-reported kidney stone and age-adjusted OR (95% CI) across adiponectin tertiles

	Tertile			*P* for trend
	Lower	Middle	Upper	
Men ($n = 529$)	$n = 176$	$n = 177$	$n = 176$	
Median adiponectin (range), μg/mL	3.70 (1.07–4.68)	5.83 (4.71–7.01)	9.56 (7.05–31.80)	
Self-reported kidney stone, *n* (%)	27 (15.3)	33 (18.6)	24 (13.6)	0.66*
Age-adjusted OR (95% CI)	1.00	1.25 (0.71–2.19)	0.85 (0.47–1.55)	0.48**
Women ($n = 507$)	$n = 169$	$n = 171$	$n = 167$	
Median adiponectin (range), μg/mL	6.08 (2.09–8.01)	9.57 (8.03–11.70)	14.40 (11.80–31.90)	
Self-reported kidney stone, *n* (%)	13 (7.7)	13 (7.6)	9 (5.4)	0.41*
Age-adjusted OR (95% CI)	1.00	1.03 (0.46–2.31)	0.67 (0.28–1.62)	0.36**

**P*-values for trend in proportions of subjects with self-reported kidney stone using Mantel–Haenszel χ^2-test. ***P*-values calculated using median of tertiles as a continuous variable in logistic regression models.

susceptible to recall bias. However, a self-reported history of kidney stones was validated in a previous study.[31] Second, the present study was limited by several absent data. Kidney stones form in response to environmental and/or metabolic risk factors, such as diet and fluid intake. The traditional Japanese diet has changed remarkably to a Western-style diet rich in animal fat and dairy foods in the post-World War II era.[32] Therefore, diet is an essential variable to investigate the risk for kidney stones in a prospective study. Because the present study was cross-sectional, adjustment for dietary factors that might alter the development of kidney stones could not be carried out. Data on kidney stone composition were also lacking; however, both calcium oxalate and calcium phosphate stones accounted for over 90% of all kidney stones in Japanese patients in 2005.[4] No urinalysis data were available. Thus, whether HOMA-IR, insulin and adiponectin affect urinary pH and calcium excretion was not determined in the present study.

In Japanese women, a history of kidney stones was positively associated with obesity, high BP, HOMA-IR and insulin, and there was a significant positive trend in age-adjusted OR for a history of kidney stones across insulin tertiles. The present result suggests that MetS components could increase the risk of kidney stones through subclinical hyperinsulinemia or insulin resistance in Japanese women. Because of the cross-sectional design of the present study, the risk of hyperinsulinemia and insulin resistance for kidney stone formation needs to be confirmed in a prospective study.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research on Special Priority Areas of Cancer (No. 17015018) and for Scientific Research C (No. 19590643) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

1 Stamatelou KK, Francis ME, Jones CA, Nyberg LM, Curhan GC. Time trends in reported prevalence of kidney stones in the United States: 1976–1994. *Kidney Int.* 2003; **63**: 1817–23.

2 Hesse A, Brandle E, Wilbert D, Kohrmann KU, Alken P. Study on the prevalence and incidence of urolithiasis in Germany comparing the years 1979 vs. 2000. *Eur. Urol.* 2003; **44**: 709–13.

3 Trinchieri A, Coppi F, Montanari E, Del Nero A, Zanetti G, Pisani E. Increase in the prevalence of symptomatic upper urinary tract stones during the last ten years. *Eur. Urol.* 2000; **37**: 23–5.

4 Yasui T, Iguchi M, Suzuki S, Kohri K. Prevalence and epidemiological characteristics of urolithiasis in Japan: national trends between 1965 and 2005. *Urology* 2008; **71**: 209–13.

5 World Health Organization. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. *World Health Organ. Tech. Rep. Ser.* 2000; **894**: 1–253.

6 MHLW. The National Nutrition Survey in Japan developed by the Ministry of Health, Labor and Welfare in 2006. [Cited 9 Oct 2009.] Available from URL: http://www.mhlw.go.jp

7 Curhan GC, Willett WC, Rimm EB, Speizer FE, Stampfer MJ. Body size and risk of kidney stones. *J. Am. Soc. Nephrol.* 1998; **9**: 1645–52.

8 Taylor EN, Stampfer MJ, Curhan GC. Obesity, weight gain, and the risk of kidney stones. *JAMA* 2005; **293**: 455–62.

9 Cappuccio FP, Strazzullo P, Mancini M. Kidney stones and hypertension: population based study of an independent clinical association. *BMJ* 1990; **300**: 1234–6.

10 Taylor EN, Stampfer MJ, Curhan GC. Diabetes mellitus and the risk of nephrolithiasis. *Kidney Int.* 2005; **68**: 1230–5.

11 West B, Luke A, Durazo-Arvizu RA, Cao G, Shoham D, Kramer H. Metabolic syndrome and self-reported history of kidney stones: the National Health and Nutrition Examination Survey (NHANES III) 1988–1994. *Am. J. Kidney Dis.* 2008; **51**: 741–7.

12 Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 2001; **285**: 2486–97.

13 Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr. Rev.* 2005; **26**: 439–51.

14 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–19.

15 Definition and the diagnostic standard for metabolic syndrome – Committee to Evaluate Diagnostic Standards for Metabolic Syndrome. *Nippon Naika Gakkai Zasshi* 2005; **94**: 794–809.

16 Gillen DL, Coe FL, Worcester EM. Nephrolithiasis and increased blood pressure among females with high body mass index. *Am. J. Kidney Dis.* 2005; **46**: 263–9.

17 Powell CR, Stoller ML, Schwartz BF *et al.* Impact of body weight on urinary electrolytes in urinary stone formers. *Urology* 2000; **55**: 825–30.

18 Taylor EN, Curhan GC. Body size and 24-hour urine composition. *Am. J. Kidney Dis.* 2006; **48**: 905–15.

19 Heller HJ, Sakhaee K, Moe OW, Pak CY. Etiological role of estrogen status in renal stone formation. *J. Urol.* 2002; **168**: 1923–7.

20 Nordin BE, Need AG, Morris HA, Horowitz M, Robertson WG. Evidence for a renal calcium leak in postmenopausal women. *J. Clin. Endocrinol. Metab.* 1991; **72**: 401–7.

21 Adami S, Gatti D, Bertoldo F *et al.* The effects of menopause and estrogen replacement therapy on the renal handling of calcium. *Osteoporos. Int.* 1992; **2**: 180–5.

22 Mattix Kramer HJ, Grodstein F, Stampfer MJ, Curhan GC. Menopause and postmenopausal hormone use and risk of incident kidney stones. *J. Am. Soc. Nephrol.* 2003; **14**: 1272–7.

23 Otsuka R, Tamakoshi K, Yatsuya H *et al.* Eating fast leads to insulin resistance: findings in middle-aged Japanese men and women. *Prev. Med.* 2008; **46**: 154–9.

24 DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am. J. Physiol.* 1979; **237**: E214–23.

25 Bonora E, Targher G, Alberiche M *et al.* Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* 2000; **23**: 57–63.

26 Emoto M, Nishizawa Y, Maekawa K *et al.* Homeostasis model assessment as a clinical index of insulin resistance in type 2 diabetic patients treated with sulfonylureas. *Diabetes Care* 1999; **22**: 818–22.

27 Cupisti A, Meola M, D'Alessandro C *et al.* Insulin resistance and low urinary citrate excretion in calcium stone formers. *Biomed. Pharmacother.* 2007; **61**: 86–90.

28 Kerstetter J, Caballero B, O'Brien K, Wurtman R, Allen L. Mineral homeostasis in obesity: effects of euglycemic hyperinsulinemia. *Metabolism* 1991; **40**: 707–13.

29 Shimamoto K, Higashiura K, Nakagawa M *et al.* Effects of hyperinsulinemia under the euglycemic condition on calcium and phosphate metabolism in non-obese normotensive subjects. *Tohoku J. Exp. Med.* 1995; **177**: 271–8.

30 Nowicki M, Kokot F, Surdacki A. The influence of hyperinsulinaemia on calcium-phosphate metabolism in renal failure. *Nephrol. Dial. Transplant.* 1998; **13**: 2566–71.

31 Curhan GC, Willett WC, Rimm EB, Stampfer MJ. A prospective study of dietary calcium and other nutrients and the risk of symptomatic kidney stones. *N. Engl. J. Med.* 1993; **328**: 833–8.

32 Kuriki K, Tokudome S, Tajima K. Association between type II diabetes and colon cancer among Japanese with reference to changes in food intake. *Asian Pac. J. Cancer Prev.* 2004; **5**: 28–35.

Cancer Epidemiology 35 (2011) 66–72

Contents lists available at ScienceDirect

Cancer Epidemiology

The International Journal of Cancer Epidemiology, Detection, and Prevention

journal homepage: www.cancerepidemiology.net

Genetic polymorphisms of glutathione S-transferase genes and susceptibility to colorectal cancer: A case–control study in an Indian population

Jingwen Wang [a,*], Jing Jiang [b], Yang Zhao [a], Vendhan Gajalakshmi [c], Kiyonori Kuriki [d], Sadao Suzuki [a], Teruo Nagaya [a], Seiichi Nakamura [e], Susumu Akasaka [f], Hideki Ishikawa [g], Shinkan Tokudome [a,h]

[a] Department of Public Health, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan
[b] Department of Hematology and Oncology, The First Hospital, Jilin University, Changchun 130021, China
[c] Epidemiological Research Center, Chennai 600-010, India
[d] Hygiene and Preventive Medicine, School of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka 422-8526, Japan
[e] Health Research Foundation, Kyoto 606-8225, Japan
[f] Osaka Prefectural Institute of Public Health, Osaka 537-0025, Japan
[g] Kyoto Prefectural University of Medicine, Graduate School of Medical Sciences, Kyoto, 602-8566, Japan
[h] National Institute of Health and Nutrition, Tokyo 162-8636, Japan

ARTICLE INFO

Article history:
Accepted 2 July 2010
Available online 4 August 2010

Keywords:
Genetic polymorphisms
Glutathione S-transferases (GSTs)
Colorectal cancer
Susceptibility

ABSTRACT

Background: Susceptibility to sporadic colorectal cancer is multifactorial and arises from interactive combinations of allelic variants in low-penetrance genes and relevant environmental risk factors. Genetic polymorphisms in metabolic enzymes as gene susceptibility factors may modify colorectal cancer risk. We evaluated the risk of colorectal cancer associated with respective or combined *glutathione S-transferase (GST)* polymorphisms and assessed the interactions between genes and environmental factors in a case–control study in an Indian population. *Methods*: The study included 59 colon and 243 rectal cancer cases, and 291 cancer-free healthy controls. *GST* genotypes were detected by multiplex PCR-based and PCR-RFLP methods. The risk of cancer associated with *GST* polymorphisms was estimated by calculation of odds ratios (ORs) and confidence intervals (95% CIs) using unconditional logistic regression. *Results*: The *GSTM1* null genotype was found to be associated with a significantly increased rectal cancer risk (OR = 1.55; 95% CI, 1.05–2.30), while the *GSTT1* null genotype with a greater risk of colon cancer (OR = 2.15; 95% CI, 1.04–4.32). A substantial increase of both colon (OR = 10.81; 95% CI, 1.11–107.22) and rectal (OR = 4.80; 95% CI, 0.94–35.91) cancer risk was shown for the combination of *GSTM1* null, *GSTT1* null and *GSTP1* 105Val allele. The combined *GSTM1* null and *GSTP1* 114Val allele also revealed an increased risk for either colon cancer (OR = 4.69; 95% CI, 0.84–23.87) or rectal cancer (OR = 5.68; 95% CI, 1.79–22.16). Furthermore, the combination of *GSTM1* null, *GSTT1* null and *GSTP1* 114Val allele was found in 2 rectal cancer cases. *Conclusion*: Our results suggest that co-exist of *GSTM1* null, *GSTT1* null and the variant *GSTP1* 105Val or 114Val allele may be predisposing risk factors for colorectal cancer in Indian population.

1. Introduction

Colorectal cancer is the second most common cancer in developed countries [1], while the incidence of colorectal cancer has also apparently been increasing in many developing countries with Westernized lifestyles. Susceptibility to sporadic colorectal cancer is multifactorial and arises from interactive combinations of allelic variants in low-penetrance genes and relevant environmental factors such as dietary and lifestyle habits [2,3]. In particular, each low-penetrance allele may contribute a subtle effect on the risk of colorectal cancer, but its interactions with other susceptibility alleles and environmental risk factors can result in a substantial increase in colorectal cancer risk [3–5]. Susceptibility genes can be involved in many different biological pathways such as the metabolic process, while metabolic enzymes (including activating and detoxifying enzymes) play a leading role in the metabolism of endogenous and exogenous chemicals such as polycyclic aromatic hydrocarbons (PAHs) that are ubiquitous environmental, dietary, and tobacco carcinogens. Therefore, polymorphisms in genes that encode metabolic enzymes may

* Corresponding author at: Department of Public Health, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku, Nagoya 467-8601, Japan. Tel.: +81 52 853 8176; fax: +81 52 842 3830.
E-mail addresses: wjw@med.nagoya-cu.ac.jp, wjw66@hotmail.co.jp (J. Wang).

1877-7821/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.canep.2010.07.003

result in varying activity levels of these enzymes, and then may modify colorectal cancer risk [6].

The glutathione S-transferases (GSTs), a superfamily of phase II metabolic enzymes, catalyze the conjugation between glutathione and chemotherapeutic drugs, carcinogens, environmental pollutants, and a broad spectrum of xenobiotics [7]. GSTs detoxify potentially mutagenic and cytotoxic DNA-reactive metabolites produced by phase I reactions, and serve to protect cellular macromolecules from damage [8]. In humans, the GST enzymes can be divided into five main classes: Alpha (GSTA), Mu (GSTM), Pi (GSTP), Theta (GSTT), and Zeta (GSTZ). Each class consists of one or more isoenzymes (i.e., A1-A4, M1-M5, P1, T1-T2 and Z1), each with a different, but sometimes overlapping substrate specificity [9]. Several polymorphisms occurring in the genes encoding GSTs such as *GSTM1*, *GSTT1*, *GSTP1* and *GSTZ1* have been identified [10–14] and widely discussed in connection with susceptibility to various diseases. The polymorphisms of the *GSTM1* and *GSTT1* loci arise from the complete deletion (null genotype) of each gene [11,15], which causes a lack of enzyme activity [16]. The polymorphisms at the *GSTP1* and *GSTZ1* loci result in amino acid substitutions that lead to reduced activity [17–21].

The situation of colorectal cancer in the Indian population has been described in detail elsewhere [22]. Briefly, although the incidence of colorectal cancer in India is low, and rectal cancer is more common than colon cancer, a significant increase has been reported among both men and women over the last 2 decades. There are geographical and ethnic variations in the genotype frequencies of *GST* genes [23], and the association of *GST* genetic polymorphisms with colorectal cancer has been widely investigated in various ethnic populations, but with inconsistent results [24]. However, since little is known about the impact of *GST* genetic polymorphisms on susceptibility to colorectal cancer in Indian populations, we therefore conducted the present case–control study to estimate the risk of colorectal cancer associated with *GST* genetic polymorphisms both individually or in combinations, and to assess the interactions between genes and environmental factors in terms of tobacco consumption and alcohol intake.

2. Patients and methods

2.1. Participant selection and data collection

Our participant selection and data collection methods have been described previously in detail [22]. In brief, this present case–control study encompassed 302 cases (including 59 colon and 243 rectal cancer patients) and 291 controls. All subjects were recruited at the Cancer Institute at Chennai in South-Eastern India. Cases were first diagnosed as primary colorectal carcinoma, and were histologically confirmed between 1999 and 2001. Colon cancer cases aged from 22 to 72 years old (mean ± SD 48.5 ± 12.0) included 67.8% men, and rectal cancer cases aged from 17 to 75 years old (mean ± SD 49.1 ± 14.1) included 64.6% men. Controls were comprised of cancer-free individuals selected from relatives/visitors to patients with cancers other than gastrointestinal cancers during the same period of our case collection, aged from 20 to 75 years old (mean ± SD 47.3 ± 12.6) included 62.5% men, and frequency matched to cases for sex and age (within 5 years). Informed consent was obtained from all study subjects. Using a standard questionnaire and trained interviewers, information was gathered on demographic variables, education, religion, mother tongue, marital status, socioeconomic conditions, and family history of cancer. Data on smoking status, alcohol consumption and chewing habits were also obtained.

2.2. Genotyping

Genomic DNA was extracted from leukocytes of blood samples. The multiplex PCR-based method was used to detect deletions of *GSTM1* and *GSTT1*, using primers 5′-GAACTCCCTGAAAAGC-TAAAGC-3′ and 5′-GTTGGGCTCAAATATACGTGG-3′ for *GSTM1*, and 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGAT-CATGGCCAGCA-3′ for *GSTT1*. A 273-bp fragment of the β-globin gene was coamplified using primers 5′-CAACTTCATCCACGTT-CACC-3′ and 5′-GAAGAGCCAAGGACAGGTAC-3′ as an internal standard [25].

Genotyping for *GSTP1* and *GSTZ1* was carried out by the PCR-RFLP method. The *GSTP1* gene variants are caused by base-pair transitions at nucleotide +313 (Ile105Val, A–G) in exon 5 and +341 (Ala114Val, C–T) in exon 6 [17]. The *GSTP1* Ile105Val polymorphism was analyzed using the primers 5′-CAGTGACTGTGTGTT-GATCA-3′ and 5′-TGCTCACATAGTTGGTGTAGATGAGGGATA-3′, followed by digestion of the PCR products with *SnaB* I [26]. The *GSTP1* Ala114Val polymorphism was detected with the primers 5′-GTTGTGGGGAGCAAGCAGAGG-3′ and 5′-CACAATGAAGGTCTTGCC-TCCC-3′, with the PCR products being digested by *Aci* I [17]. The polymorphic sites of *GSTZ1* are located at nucleotides 23 (Leu8Pro, T–C), 94 (Lys32Glu, A–G), 124 (Arg42Gly, A–G) and 245 (Thr82Met, C–T) [21]. The *GSTZ1* Lys32Glu polymorphism was detected using primers 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGAT-CATGGCCAGCA-3′, and then *BsmA* I digestion of the PCR products was conducted [13].

2.3. Statistical analysis

Differences in general characteristics between cases and controls were assessed with the Chi-square test and *t*-test, and the disparity in genotypes as well as the Hardy–Weinberg equilibrium was also examined with the Chi-square test. The association between *GST* polymorphisms and colorectal cancer was modeled by unconditional logistic regression analysis using the software package SAS (version 8.2), controlling for potential confounding factors such as age, sex, household income, education, religion, mother tongue, tobacco, alcohol, chewing habits and vegetarianism. Odds ratios (ORs) and confidence intervals (95% CIs) were used to analyze the frequencies of *GST* genotypes occurring in patients with colorectal cancer compared to control groups. The reference group consisted of individuals with putative low-risk genotypes, i.e., the presence of *GSTM1*, *GSTT1*, and homozygous *GSTP1* Ile-105 or Ala-114, and *GSTZ1* Lys-32 functional alleles, the combined effects of *GST* genotypes were calculated at two or three loci. We also assessed the joint effects between genotypes and tobacco consumption or alcohol intake using non-smokers or non-drinkers with low-risk genotypes as the reference. A likelihood ratio test was used to examine the interaction of variables with respect to the risk of colorectal cancer. All statistical tests were two-sided, and statistical significance was determined as $p < 0.05$.

3. Results

Since the general characteristics of the study participants were previously presented in detail [22], they were omitted here. The frequencies of *GST* genotypes by case–control status and the association of *GST* polymorphisms with cancers are shown in Table 1. The frequencies of *GSTM1* and *GSTT1* null genotypes, *GSTP1* 105Val, 114Val and *GSTZ1* 32Glu alleles were 0.31, 0.25, 0.36, 0.05 and 0.83 among colon cancer cases, while 0.34, 0.17, 0.30 0.08 and 0.82 among rectal cancer cases, compared with 0.26, 0.15, 0.27, 0.05 and 0.79 among controls. In the control group, genotype distributions of *GSTP1* Ile105Val (Ile/Ile, 55.0%; Ile/Val, 36.8%; Val/

Table 1
Genotype frequencies and adjusted ORs[a] for colon, rectal and colorectal cancer with polymorphisms of *GSTM1*, *GSTT1*, *GSTP1* and *GSTZ1*.

Genotype	Controls (n=291)	Colon cancer (n=59)	ORs (95% CI)	Rectal cancer (n=243)	ORs (95% CI)	Colorectal cancer (n=302)	ORs (95% CI)
	n (%)	n (%)		n (%)		n (%)	
GSTM1							
Present	215 (73.9)	41 (69.5)	1 (Ref)	161 (66.3)	1 (Ref)	202 (66.9)	1 (Ref)
Null	76 (26.1)	18 (30.5)	1.20 (0.62–2.26)	82 (33.7)	1.55 (1.05–2.30)	100 (33.1)	1.47 (1.02–2.14)
GSTT1							
Present	247 (84.9)	44 (74.6)	1 (Ref)	201 (82.7)	1 (Ref)	245 (81.1)	1 (Ref)
Null	44 (15.1)	15 (25.4)	2.15 (1.04–4.32)	42 (17.3)	1.17 (0.72–1.97)	57 (18.9)	1.33 (0.85–2.09)
GSTP1 Ile105Val							
Ile/Ile	160 (55.0)	27 (45.8)	1 (Ref)	114 (46.9)	1 (Ref)	141 (46.7)	1 (Ref)
Ile/Val	107 (36.8)	22 (37.3)	1.15 (0.60–2.16)	110 (45.3)	1.44 (0.99–2.09)	132 (43.7)	1.37 (0.96–1.95)
Val/Val	24 (8.2)	10 (16.9)	2.31 (0.92–5.57)	19 (7.8)	1.12 (0.56–2.21)	29 (9.6)	1.29 (0.70–2.40)
Val[b]	131 (45.0)	32 (54.2)	1.35 (0.75–2.44)	129 (53.1)	1.37 (0.96–1.97)	161 (53.3)	1.35 (0.97–1.90)
GSTP1 Ala114Val							
Ala/Ala	263 (90.4)	53 (89.8)	1 (Ref)	208 (85.6)	1 (Ref)	261 (86.4)	1 (Ref)
Ala/Val	27 (9.3)	6 (10.2)	1.24 (0.42–3.20)	32 (13.2)	1.65 (0.88–3.16)	38 (12.6)	1.40 (0.78–2.56)
Val/Val	1 (0.3)	0 (0.0)	NA	3 (1.2)	2.33 (0.25–51.38)	3 (1.0)	1.98 (0.22–43.32)
Val[c]	28 (9.6)	6 (10.2)	1.15 (0.39–2.94)	35 (14.4)	1.69 (0.91–3.17)	41 (13.6)	1.43 (0.80–2.55)
GSTZ1 Lys32Glu							
Lys/Lys	15 (15.1)	2 (3.4)	1 (Ref)	10 (4.1)	1 (Ref)	12 (4.0)	1 (Ref)
Lys/Glu	93 (32.0)	16 (27.1)	1.08 (0.26–7.43)	66 (27.2)	0.78 (0.32–1.98)	82 (27.1)	0.89 (0.38–2.11)
Glu/Glu	183 (62.9)	41 (69.5)	1.46 (0.37–9.77)	167 (68.7)	1.05 (0.44–2.56)	208 (68.9)	1.17 (0.52–2.71)
Glu[d]	276 (94.9)	57 (96.6)	1.31 (0.34–8.64)	238 (95.9)	0.96 (0.41–2.34)	290 (96.0)	1.07 (0.48–2.45)

[a] Adjusted for gender, age, household income, education, religion, mother tongue, smoking, drinking, chewing and vegetarianism.
[b] Ile/Val or Val/Val.
[c] Ala/Val or Val/Val.
[d] Lys/Glu or Glu/Glu.

Val, 8.2%), *GSTP1* Ala114Val (Ala/Ala, 90.4%; Ala/Val, 9.3%; Val/Val, 0.3%) and *GSTZ1* Lys32Glu (Lys/Lys, 15.1%; Lys/Glu, 32.0%; Glu/Glu, 62.9%) were all in agreement with the Hardy–Weinberg equilibrium (p = 0.31; 0.73; 0.48, respectively). A significant association was found between *GSTM1* null genotype and rectal cancer (OR = 1.55; 95% CI, 1.05–2.30), as well as between *GSTT1* null genotype and colon cancer (OR = 2.15; 95% CI, 1.04–4.32). A non-statistically significant increase in rectal cancer risk was found in both variant *GSTP1* 105Val (OR = 1.37; 95% CI, 0.96–1.97) and 114Val (OR = 1.69; 95% CI, 0.91–3.17) alleles. No significant association was found between *GSTZ1* Lys32Glu polymorphism and colorectal cancer.

The combined effects of two putative risk genotypes of *GST* polymorphisms are summarized in Table 2. The combination of *GSTM1* null with *GSTT1* null showed that the risk was increased 6.2-fold for colon cancer (95% CI, 1.62–22.61) and 2.6-fold for rectal cancer (95% CI, 0.94–7.56). The combined *GSTM1* null genotype and *GSTP1* 114Val allele also revealed a 4.7-fold increase in colon cancer risk (95% CI, 0.84–23.87) and a 5.7-fold rise in rectal cancer risk (95% CI, 1.79–22.16). Those individuals who carried the combined *GSTM1*/*GSTT1* null genotype and *GSTP1* 105Val allele also suffered somewhat increased colon and rectal cancer risks. With respect to colorectal cancer (overall colon and rectal cancers), a significantly increased risk was found in the combination of *GSTM1* null genotype with *GSTT1* null genotype (OR = 2.98; 95% CI, 1.19–8.18); with *GSTP1* 105Val allele (OR = 2.14; 95% CI, 1.25–3.69) and *GSTP1* 114Val allele (OR = 4.71; 95% CI, 1.60–17.34), as well as in the combination of *GSTT1* null genotype with *GSTP1* 105Val allele (OR = 1.89; 95% CI, 1.01–3.59), and *GSTP1* 105Val allele with *GSTZ1* 32Glu allele (OR = 2.84; 95% CI, 1.03–9.13).

We further investigated the combined effects of three putative risk genotypes (see Table 3). An increased risk for colon (OR = 10.81; 95% CI, 1.11–107.22), rectal (OR = 4.80; 95% CI, 0.94–35.91) and colorectal (OR = 4.63; 95% CI, 1.03–32.87) cancers was found in individuals with combined *GSTM1* null, *GSTT1* null genotype and *GSTP1* 105Val allele compared to combined *GSTM1* present, *GSTT1* present and *GSTP1* 105Ile/Ile genotypes. The combined *GSTM1* null, *GSTT1* present genotype and *GSTP1* 114Val allele also suggested a significantly increased risk for both colon (OR = 6.31; 95% CI, 1.03–35.42) and rectal (OR = 4.67; 95% CI, 1.28–20.53) cancers using the combined low-risk genotypes (*GSTM1* present, *GSTT1* present and *GSTP1* 114Ala/Ala genotypes) as the reference. For the combination of *GSTM1* null, *GSTT1* null genotype and *GSTP1* 114Val allele, only 2 rectal cancer cases were found among all study participants. Since both *GSTP1* 114Val allele and *GSTZ1* 32Lys/Lys genotype were rare among our study subjects, the other combinations of three risk genotypes were unable to be conducted.

The interactions of gene-tobacco are presented in Table 4. For colon cancer, no significant tobacco effect modification was found for *GSTM1* and *GSTT1* genotypes. Though both *GSTP1* 105Val and 114Val alleles showed an increased risk among smokers, it did not reach statistical significance. For rectal cancer, except for *GSTM1* null genotype, *GSTT1* null genotype (OR = 2.32; 95% CI, 0.91–6.27), *GSTP1* 105Val allele (OR = 2.05; 95% CI, 1.05–4.08), and 114Val allele (OR = 3.30; 95% CI, 0.89–15.87) were shown to have a positive association among smokers, though only *GSTP1* 105Val allele reached statistical significance. The interaction of the *GSTZ1* Lys32Glu polymorphism with smoking was also analyzed, but no significant relationship was found (data not shown).

As to the interactions of gene–alcohol, no significant effect modification was observed to a risk of either colon or rectal cancer (data not shown).

4. Discussion

We investigated the role of *GST* polymorphisms in the development of colorectal cancer in an Indian population. The *GSTM1* null genotype was found to be associated with a significantly increased rectal cancer risk (OR = 1.55; 95% CI, 1.05–2.30), while the *GSTT1* null genotype was related to a greater risk of colon cancer (OR = 2.15; 95% CI, 1.04–4.32). Both variant *GSTP1* 105Val (OR = 1.37; 95% CI, 0.96–1.97) and 114Val (OR = 1.69; 95% CI, 0.91–3.17) alleles were found to be at a somewhat increased rectal cancer risk. No significant association was found between the *GSTZ1* polymorphism and the colorectal

Table 2
ORs[a] for colon, rectal and colorectal cancer by combined *GSTM1*, *GSTT1*, *GSTP1* and *GSTZ1* genotypes.

Combined genotypes		Controls *n* (%)	Colon cancer *n* (%)	ORs (95% CI)	Rectal cancer *n* (%)	ORs (95% CI)	Colorectal cancer *n* (%)	ORs (95% CI)
GSTM1	GSTT1							
Present	Present	178 (61.2)	31 (52.5)	1 (Ref)	129 (53.1)	1 (Ref)	160 (53.0)	1 (Ref)
Present	Null	37 (12.7)	10 (17.0)	1.56 (0.65–3.53)	32 (13.2)	1.16 (0.66–2.01)	42 (13.9)	1.21 (0.73–2.04)
Null	Present	69 (23.7)	13 (22.0)	0.97 (0.45–2.00)	72 (29.6)	1.51 (0.99–2.30)	85 (28.1)	1.40 (0.93–2.08)
Null	Null	7 (2.4)	5 (8.5)	6.19 (1.62–22.61)	10 (4.1)	2.59 (0.94–7.56)	15 (5.0)	2.98 (1.19–8.18)
GSTM1	GSTP1 Ile105Val							
Present	Ile/Ile	115 (39.5)	18 (30.5)	1 (Ref)	77 (31.7)	1 (Ref)	95 (31.5)	1 (Ref)
Present	Val[b]	100 (34.4)	23 (39.0)	1.31 (0.65–2.68)	84 (34.6)	1.24 (0.81–1.92)	107 (35.4)	1.26 (0.84–1.90)
Null	Ile/Ile	45 (15.5)	9 (15.3)	1.14 (0.43–2.82)	37 (15.2)	1.31 (0.75–2.29)	46 (15.2)	1.31 (0.78–2.21)
Null	Val[b]	31 (10.6)	9 (15.3)	1.75 (0.66–4.40)	45 (18.5)	2.30 (1.31–4.08)	54 (17.9)	2.14 (1.25 –3.69)
GSTM1	GSTP1 Ala114Val							
Present	Ala/Ala	191 (65.6)	38 (64.4)	1 (Ref)	138 (56.8)	1 (Ref)	176 (58.3)	1 (Ref)
Present	Val[c]	24 (8.3)	3 (5.1)	0.60 (0.14–1.91)	23 (9.5)	1.33 (0.68–2.56)	26 (8.6)	1.11 (0.59–2.08)
Null	Ala/Ala	72 (24.7)	15 (25.4)	0.97 (0.47–1.91)	70 (28.8)	1.44 (0.95–2.19)	85 (28.1)	1.32 (0.89–1.96)
Null	Val[c]	4 (1.4)	3 (5.1)	4.69 (0.84–23.87)	12 (4.9)	5.68 (1.79–22.16)	15 (5.0)	4.71 (1.60–17.34)
GSTM1	GSTZ1 Lys32Glu							
Present	Lys/Lys	10 (3.4)	2 (3.4)	1 (Ref)	5 (2.1)	1 (Ref)	7 (2.3)	1 (Ref)
Present	Glu[d]	205 (70.5)	39 (66.1)	0.80 (0.19–3.51)	156 (64.2)	1.00 (0.33–3.40)	195 (64.6)	1.01 (0.36–2.96)
Null	Lys/Lys	5 (1.7)	0 (0.0)	NA	5 (2.1)	1.55 (0.28–8.96)	5 (1.7)	1.20 (0.23–6.34)
Null	Glu[d]	71 (24.4)	18 (30.5)	1.03 (0.23–7.37)	77 (31.7)	1.55 (0.50–5.38)	95 (31.4)	1.51 (0.53–4.50)
GSTT1	GSTP1 Ile105Val							
Present	Ile/Ile	136 (46.7)	20 (33.9)	1 (Ref)	98 (40.3)	1 (Ref)	118 (39.1)	1 (Ref)
Present	Val[b]	111 (38.1)	24 (40.7)	1.42 (0.73–2.79)	103 (42.4)	1.28 (0.87–1.90)	127 (40.0)	1.30 (0.90–1.88)
Null	Ile/Ile	24 (8.3)	7 (11.9)	2.42 (0.83–6.49)	16 (6.6)	0.94 (0.45–1.91)	23 (7.6)	1.18 (0.61–2.27)
Null	Val[b]	20 (6.9)	8 (13.6)	2.73 (0.96–7.40)	26 (10.7)	1.79 (0.91–3.53)	34 (11.3)	1.89 (1.01–3.59)
GSTT1	GSTP1 Ala114Val							
Present	Ala/Ala	223 (76.6)	38 (64.4)	1 (Ref)	173 (71.2)	1 (Ref)	211 (69.9)	1 (Ref)
Present	Val[c]	24 (8.2)	6 (10.2)	1.54 (0.52–4.04)	28 (11.5)	1.49 (0.80–2.78)	34 (11.3)	1.44 (0.80–2.62)
Null	Ala/Ala	40 (13.8)	15 (25.4)	2.45 (1.17–5.04)	35 (14.4)	1.10 (0.65–1.85)	50 (16.5)	1.32 (0.82–2.14)
Null	Val[c]	4 (1.4)	0 (0.0)	NA	7 (2.9)	2.63 (0.73–10.69)	7 (2.3)	2.07 (0.58–8.36)
GSTT1	GSTZ1 Lys32Glu							
Present	Lys/Lys	13 (4.5)	1 (1.7)	1 (Ref)	7 (2.9)	1 (Ref)	8 (2.7)	1 (Ref)
Present	Glu[d]	234 (80.4)	43 (72.9)	1.83 (0.33–34.36)	194 (79.8)	1.08 (0.41–3.01)	237 (78.5)	1.19 (0.48–3.14)
Null	Lys/Lys	2 (0.7)	1 (1.7)	4.08 (0.11–159.75)	3 (1.2)	1.86 (0.23–18.08)	4 (1.3)	1.97 (0.29–17.82)
Null	Glu[d]	42 (14.4)	14 (23.7)	3.84 (0.62–75.06)	39 (16.1)	1.23 (0.43–3.70)	53 (17.5)	1.55 (0.58–4.36)
GSTP1 Ile105Val	GSTP1 Ala114Val							
Ile/Ile	Ala/Ala	160 (55.0)	27 (45.8)	1 (Ref)	114 (46.9)	1 (Ref)	141 (46.7)	1 (Ref)
Val[b]	Ala/Ala	103 (35.4)	26 (44.0)	1.37 (0.74–2.55)	94 (38.7)	1.27 (0.86–1.87)	120 (39.7)	1.28 (0.89–1.84)
Val[b]	Val[c]	28 (9.6)	6 (10.2)	1.27 (0.43–3.34)	35 (14.4)	1.80 (1.00–3.25)	41 (13.6)	1.63 (0.93–2.87)
GSTP1 Ile105Val	GSTZ1 Lys32Glu							
Ile/Ile	Lys/Lys	15 (5.2)	0 (0.0)	1 (Ref)	5 (2.1)	1 (Ref)	5 (1.7)	1 (Ref)
Ile/Ile	Glu[d]	145 (49.8)	27 (45.8)	NA	109 (44.9)	1.76 (0.63–5.70)	136 (45.0)	2.33 (0.85–7.51)
Val[b]	Lys/Lys	0 (0.0)	2 (3.4)	NA	5 (2.1)	NA	7 (2.3)	NA
Val[b]	Glu[d]	131 (45.0)	30 (50.8)	NA	124 (51.0)	2.21 (0.80–7.17)	154 (51.0)	2.84 (1.03–9.13)
GSTP1 Ala114Val	GSTZ1 Lys32Glu							
Ala/Ala	Lys/Lys	15 (5.2)	2 (3.4)	1 (Ref)	9 (3.7)	1 (Ref)	11 (3.6)	1 (Ref)
Ala/Ala	Glu[d]	248 (85.2)	51 (86.4)	1.29 (0.33–8.59)	199 (81.9)	1.04 (0.44–2.61)	250 (82.8)	1.16 (0.51–2.71)
Val[c]	Lys/Lys	0 (0.0)	0 (0.0)	NA	1 (0.4)	NA	1 (0.3)	NA
Val[c]	Glu[d]	28 (9.6)	6 (10.2)	1.40 (0.27–10.69)	34 (14.0)	1.60 (0.59–4.52)	40 (13.3)	1.60 (0.62–4.23)

[a] Adjusted for gender, age, household income, education, religion, mother tongue, smoking, drinking, chewing and vegetarianism.
[b] Ile/Val or Val/Val.
[c] Ala/Val or Val/Val.
[d] Lys/Glu or Glu/Glu

cancer. Although the respective *GST* polymorphisms showed a subtle effect on the colorectal cancer risk, that risk rose as putative risk genotypes increased from the combinations of two or three of *GSTM1* null, *GSTT1* null, *GSTP1* 105Val and 114Val alleles.

In our control group, the frequencies of *GSTM1* and *GSTT1* null genotypes, *GSTP1* 105Val, 114Val and *GSTZ1* 32Glu alleles were, respectively, 0.26, 0.15, 0.27, 0.05 and 0.79, which were in accordance with the low prevalence of *GSTM1* null genotype (0.22–0.27) and similar to the frequencies of *GSTT1* null genotype (0.07–0.18) and *GSTP1* 105Val allele (0.22–0.25) reported in Indian population [27–30]. We first detected the distributions of *GSTP1* Ala114Val and *GSTZ1* Lys32Glu polymorphisms, and found that the variant *GSTP1* 114Val allele was rare, while the *GSTZ1* 32Glu allele was common among Indian subjects.

GSTs, as detoxifying enzymes, play an important role in the cellular defense system. GSTM1 is known to detoxify active metabolites of PAHs [16], GSTT1 is involved in the detoxification of several environmental carcinogens such as 1,3-butadiene and ethylene oxide in tobacco smoke and ambient air [31]. Whereas GSTP1 is widely expressed in normal epithelial tissues and has been shown to be highly over-expressed in colon cancer [32,33], it metabolizes numerous carcinogenic compounds including benzo[a]pyrene, a tobacco carcinogen [26]. GST Zeta catalyzes the metabolism of a series of alpha-haloacids including the carcinogen dichloroacetate [34,35], a common contaminant of chlorinated drinking water. GSTZ1, as a maleylacetoacetate isomerase, also participates in the catabolic pathway of phenylalanine and tyrosine [36]. Due to the inactive form of the enzymes (null genotype of *GSTM1* or *GSTT1*, the variant allele of *GSTP1* 105Val, 114Val or *GSTZ1* 32Glu), their capacity to detoxify activated carcinogen is diminished, leading to a progression of cancer. Interindividual differences in cancer susceptibility may be partly

Table 3
ORs[a] for colon, rectal and colorectal cancer by combined *GSTM1*, *GSTT1* and *GSTP1*genotypes.

Combined genotypes			Controls n (%)	Colon cancer n (%)	ORs (95% CI)	Rectal cancer n (%)	ORs (95% CI)	Colorectal cancer n (%)	ORs (95% CI)
GSTM1	GSTT1	GSTP1 Ile/Val							
Present	Present	Ile/Ile	96 (33.0)	14 (23.7)	1 (Ref)	66 (27.1)	1 (Ref)	80 (26.5)	1 (Ref)
Present	Present	Val[b]	82 (28.2)	17 (28.8)	1.43 (0.63–3.27)	63 (25.9)	1.11 (0.69–1.80)	80 (26.5)	1.15 (0.74–1.81)
Present	Null	Ile/Ile	19 (6.5)	4 (6.8)	1.69 (0.42–5.67)	11 (4.5)	0.79 (0.33–1.84)	15 (5.0)	0.92 (0.42–2.00)
Present	Null	Val[b]	18 (6.2)	6 (10.2)	2.24 (0.66–7.13)	21 (8.6)	1.59 (0.76–3.38)	27 (8.9)	1.68 (0.84–3.43)
Null	Present	Ile/Ile	40 (13.7)	6 (10.2)	0.86 (0.27–2.48)	32 (13.2)	1.20 (0.66–2.18)	38 (12.6)	1.15 (0.66–2.02)
Null	Present	Val[b]	29 (10.0)	7 (11.8)	1.64 (0.54–4.61)	40 (16.5)	2.07 (1.14–3.77)	47 (15.6)	1.95 (1.11–3.47)
Null	Null	Ile/Ile	5 (1.7)	3 (5.1)	7.16 (1.19–38.13)	5 (2.1)	1.89 (0.40–7.37)	8 (2.6)	2.59 (0.80–9.10)
Null	Null	Val[b]	2 (0.7)	2 (3.4)	10.81 (1.11–107.22)	5 (2.1)	4.80 (0.94–35.91)	7 (2.3)	4.63 (1.03–32.87)
GSTM1	GSTT1	GSTP1 Ala/Val							
Present	Present	Ala/Ala	158 (54.3)	28 (47.5)	1 (Ref)	111 (45.7)	1 (Ref)	139 (46.0)	1 (Ref)
Present	Present	Val[c]	20 (6.9)	3 (5.1)	0.81 (0.17–2.79)	18 (7.4)	1.27 (0.57–2.88)	21 (7.0)	1.05 (0.49–2.24)
Present	Null	Ala/Ala	33 (11.3)	10 (16.9)	1.82 (0.73–4.29)	27 (11.1)	1.12 (0.57–2.17)	37 (12.2)	1.36 (0.75–2.48)
Present	Null	Val[c]	4 (1.4)	0 (0.0)	NA	5 (2.1)	2.10 (0.46–10.55)	5 (1.7)	1.71 (0.39–7.84)
Null	Present	Ala/Ala	65 (22.3)	10 (16.9)	0.70 (0.29–1.59)	62 (25.5)	1.52 (0.93–2.50)	72 (23.8)	1.29 (0.82–2.03)
Null	Present	Val[c]	4 (1.4)	3 (5.1)	6.31 (1.03–35.42)	10 (4.1)	4.67 (1.28–20.53)	13 (4.3)	4.35 (1.35–17.05)
Null	Null	Ala/Ala	7 (2.4)	5 (8.5)	5.57 (1.37–21.64)	8 (3.3)	2.13 (0.64–7.49)	13 (4.3)	2.43 (0.86–7.51)
Null	Null	Val[c]	0 (0.0)	0 (0.0)	NA	2 (0.8)	NA	2 (0.7)	NA

[a] Adjusted for gender, age, household income, education, religion, mother tongue, smoking, drinking, chewing and vegetarianism.
[b] Ile/Val or Val/Val.
[c] Ala/Val or Val/Val.

attributed to the polymorphic variability in the activation and detoxification of carcinogens.

Although most previous studies of different ethnic populations suggested no significant association of colorectal cancer with *GSTM1* null genotype [24], two did show an increased colorectal cancer risk among Caucasians [37,38], while two others recently conducted in the European-Asian area (Hungary and Turkey) also reported a positive association [39,40]. Moreover, the *GSTM1* null genotype showed a significantly increased risk of developing rectal cancer in our study (OR = 1.55; 95% CI, 1.05–2.30). Several studies have demonstrated a strong association of *GSTT1* null genotype with colorectal cancer [40–43]. We found a significantly increased colon cancer risk (OR = 2.15; 95% CI, 1.04–4.32) in the present study, and a weak association with colorectal cancer (OR = 1.33; 95% CI, 0.85–2.09) similar to that in total tendency (OR = 1.37; 95% CI, 1.17–1.60) [24]. In agreement with several studies [14,40,44], *GSTP1* 105Val allele showed a slightly increased colorectal cancer risk in our study (OR = 1.35; 95% CI, 0.97–1.90). Unlike previous reports [14,45], we also found a non-statistically elevated colorectal cancer risk with *GSTP1* 114Val allele (OR = 1.43; 95% CI, 0.80–2.55).

We assessed the combined effects of two or three putative risk genotypes (*GSTM1* null, *GSTT1* null, *GSTP1* 105Val, or 114Val and *GSTZ1* 32Glu alleles) compared to low-risk genotypes (*GSTM1* present, *GSTT1* present, *GSTP1* 105Ile/Ile or 114Ala/Ala and *GSTZ1* 32Lys/Lys genotypes). The combination of *GSTM1* null with *GSTT1* null showed a 6.2-fold increased colon cancer risk (95% CI, 1.62–22.61). The combined *GSTM1* null and *GSTP1* 114Val allele also

Table 4
Assessments of interaction between tobacco and *GST* genetic polymorphisms in colon, rectal and colorectal cancer.

Smoking status	Genotypes	Controls (n = 291)	Colon cancer (n = 59)	ORs (95% CI)[a]	Rectal cancer (n = 243)	ORs (95% CI)[a]	Colorectal cancer (n = 302)	ORs (95% CI)[a]
Non-smokers		225 (77.3)	44 (74.6)	1 (Ref)	188 (77.4)	1 (Ref)	232 (76.8)	1 (Ref)
Smokers		66 (22.7)	15 (25.5)	1.27 (0.58–2.71)	55 (22.6)	1.02 (0.63–1.64)	70 (23.2)	1.03 (0.66–1.62)
	GSTM1							
Non-smokers	Present	173 (59.5)	29 (49.2)	1 (Ref)	117 (48.2)	1 (Ref)	146 (48.3)	1 (Ref)
Non-smokers	Null	52 (17.9)	15 (25.4)	1.65 (0.78–3.39)	71 (29.2)	2.24 (1.43–3.52)	86 (28.5)	2.10 (1.37–2.45)
Smokers	Present	42 (14.4)	12 (20.3)	1.84 (0.77–4.30)	44 (18.1)	1.64 (0.95–2.85)	56 (18.5)	1.64 (0.98–2.75)
Smokers	Null	24 (18.2)	3 (5.1)	0.82 (0.18–2.85)	11 (4.5)	0.69 (0.30–1.53)	14 (4.6)	0.67 (0.32–1.43)
	GSTT1							
Non-smokers	Present	189 (65.0)	30 (50.9)	1 (Ref)	160 (65.8)	1 (Ref)	190 (62.9)	1 (Ref)
Non-smokers	Null	36 (12.4)	14 (23.7)	2.53 (1.16–5.37)	28 (11.5)	0.88 (0.50–1.54)	42 (13.9)	1.12 (0.68–1.86)
Smokers	Present	58 (19.9)	14 (23.7)	1.62 (0.71–3.60)	41 (16.9)	0.85 (0.51–1.43)	55 (18.2)	0.94 (0.58–1.52)
Smokers	Null	8 (2.7)	1 (1.7)	1.12 (0.06–7.09)	14 (5.8)	2.32 (0.91–6.27)	15 (5.0)	2.03 (0.82–5.42)
	GSTP1 Ile105Val							
Non-smokers	Ile/Ile	114 (39.2)	19 (32.2)	1 (Ref)	91 (37.4)	1 (Ref)	110 (36.4)	1 (Ref)
Non-smokers	Val[b]	111 (38.1)	25 (42.4)	1.34 (0.68–2.28)	97 (39.9)	1.07 (0.71–1.60)	122 (40.4)	1.09 (0.75–1.60)
Smokers	Ile/Ile	46 (15.8)	8 (13.5)	1.26 (0.44–3.43)	23 (9.5)	0.62 (0.33–1.17)	31 (10.3)	0.69 (0.38–1.23)
Smokers	Val[b]	20 (6.9)	7 (11.9)	2.01 (0.63–1.64)	32 (13.2)	2.05 (1.05–4.08)	39 (12.9)	1.97 (1.04–3.81)
	GSTP1 Ala114Val							
Non-smokers	Ala/Ala	200 (68.7)	41 (69.5)	1 (Ref)	161 (66.3)	1 (Ref)	202 (66.9)	1 (Ref)
Non-smokers	Val[c]	25 (8.6)	3 (5.1)	0.67 (0.15–2.14)	27 (11.1)	1.42 (0.77–2.64)	30 (9.9)	1.26 (0.70–2.30)
Smokers	Ala/Ala	63 (21.7)	12 (20.3)	1.07 (0.46–2.40)	47 (19.3)	0.95 (0.57–1.56)	59 (19.5)	0.95 (0.60–1.52)
Smokers	Val[c]	3 (1.0)	3 (5.1)	3.35 (0.57–19.67)	8 (3.3)	3.30 (0.89–15.87)	11 (3.6)	3.03 (0.89–13.92)

[a] Adjusted for gender, age, household income, education, religion, mother tongue, drinking, chewing and vegetarianism.
[b] Ile/Val or Val/Val.
[c] Ala/Val or Val/Val.

revealed a 4.7-fold increase in colon cancer risk (95% CI, 0.84–23.87) and a 5.7-fold rise in rectal cancer risk (95% CI, 1.79–22.16). Enlarged sample size enhanced the statistical power, a significant increase of colorectal cancer (including colon and rectal cancers) risk was revealed in the combination of *GSTM1* null genotype with *GSTT1* null genotype (OR = 2.98; 95% CI, 1.19–8.18); *GSTP1* 105Val allele (OR = 2.14; 95% CI, 1.25–3.69); and *GSTP1* 114Val allele (OR = 4.71; 95% CI, 1.60–17.34). Such an increased colorectal cancer risk was also found in the combination of *GSTT1* null with *GSTP1* 105Val allele (OR = 1.89; 95% CI, 1.01–3.59) as well as *GSTP1* 105Val allele with *GSTZ1* 32Glu allele (OR = 2.84; 95% CI, 1.03–9.13). Similar to that reported previously [40], the risk of colorectal cancer substantially increased as putative risk genotypes increased in the combination of *GSTM1* null, *GSTT1* null genotype and *GSTP1* 105Val allele (OR = 4.63; 95% CI, 1.03–32.87) in our study. Moreover, the highest colon cancer risk was markedly demonstrated in this combination (OR = 10.81; 95% CI, 1.11–107.22). A study conducted in the Tamilian population of south India [30] also demonstrated the most remarkable risk of upper aerodigestive tract cancer with this combination (OR = 7.8; 95% CI, 1.0–61.0). In addition, the combined *GSTM1* null, *GSTT1* present genotype and *GSTP1* 114Val allele suggested a significantly increased risk of colon (OR = 6.31; 95% CI, 1.03–35.42) and rectal (OR = 4.67; 95% CI, 1.28–20.53) cancers. Furthermore, 2 rectal cancer cases were found to carry a combined *GSTM1* null, *GSTT1* null and *GSTP1* 114Val allele in our study.

The interactions of gene–tobacco were evaluated in our study. No significant tobacco modification effect on the risk of both colon and rectal cancers was found for GSTM1 genotypes. With respect to smokers, *GSTT1* null genotype was found to be associated with a trend toward increased rectal cancer risk. Either *GSTP1* 105Val or 114Val allele also showed a weakly positive association with colon and rectal cancers. However, the statistical power to detect gene–tobacco interactions was limited in our study due to the small number of smokers. In addition, the joint effects of gene–alcohol were also estimated, with no significant modifying effect found.

In conclusion, we first estimated the association of *GST* genetic polymorphisms with colorectal cancer risk in an Indian population, and found that *GSTM1* null, *GSTT1* null genotype and the variant *GSTP1* 105Val or 114Val allele may be predisposing risk factors for colorectal cancer. Moreover, gene–gene interactions may contribute to a substantial increase in colorectal cancer risk, while the joint effects of gene–tobacco may weakly modify the development of colorectal cancer in our Indian population. Our findings suggest that GST polymorphisms may play an important role in the detection of early colorectal cancer and in the surveillance of a high-risk population in India.

Conflict of interest

None declared.

Acknowledgements

The authors are very grateful to Dr. V. Shanta and Dr. T. Rajkumar (Cancer Institute, Chennai, India) for their support and cooperation. Jingwen Wang was also financially supported by the Japan Society for the promotion of Science and its postdoctoral fellowship for researchers from abroad.

References

[1] Boring CC, Squires TS, Tong T, Montgomery S. Cancer statistics, 1994. CA Cancer J Clin 1994;44(1):7–26.
[2] de la Chapelle A. Genetic predisposition to colorectal cancer. Nat Rev Cancer 2004;4(10):769–80.
[3] Hunter DJ, Riboli E, Haiman CA, Albanes D, Altshuler D, Chanock SJ, et al. A candidate gene approach to searching for low-penetrance breast and prostate cancer genes. Nat Rev Cancer 2005;5(12):977–85.
[4] Goodman JE, Mechanic LE, Luke BT, Ambs S, Chanock S, Harris CC. Exploring SNP–SNP interactions and colon cancer risk using polymorphism interaction analysis. Int J Cancer 2006;118(7):1790–7.
[5] Tabor HK, Risch NJ, Myers RM. Candidate-gene approaches for studying complex genetic traits: practical considerations. Nat Rev Genet 2002;3(5):391–7.
[6] Hirvonen A. Genetic factors in individual responses to environmental exposures. J Occup Environ Med 1995;37(1):37–43.
[7] Mannervik B, Danielson UH. Glutathione transferases – structure and catalytic activity. CRC Crit Rev Biochem 1988;23(3):283–337.
[8] Mannervik B. The isoenzymes of glutathione transferase. Adv Enzymol Relat Areas Mol Biol 1985;57:357–417.
[9] Eaton DL, Bammler TK. Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol Sci 1999;49(2):156–64.
[10] Board PG, Webb GC, Coggan M. Isolation of a cDNA clone and localization of the human glutathione S-transferase 3 genes to chromosome bands 11q13 and 12q13–14. Ann Hum Genet 1989;53(Pt 3):205–13.
[11] Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt HM, et al. Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. Biochem J 1994;300(Pt 1):271–6.
[12] Chenevix-Trench G, Young J, Coggan M, Board P. Glutathione S-transferase M1 and T1 polymorphisms: susceptibility to colon cancer and age of onset. Carcinogenesis 1995;16(7):1655–7.
[13] Board PG, Baker RT, Chelvanayagam G, Jermiin LS, Zeta. a novel class of glutathione transferases in a range of species from plants to humans. Biochem J 1997;328(Pt 3):929–35.
[14] Harris MJ, Coggan M, Langton L, Wilson SR, Board PG. Polymorphism of the Pi class glutathione S-transferase in normal populations and cancer patients. Pharmacogenetics 1998;8(1):27–31.
[15] Seidegard J, Vorachek WR, Pero RW, Pearson WR. Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. Proc Natl Acad Sci USA 1988;85(19):7293–7.
[16] Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 1995;30(6):445–600.
[17] Ali-Osman F, Akande O, Antoun G, Mao JX, Buolamwini J. Molecular cloning, characterization, and expression in Escherichia coli of full-length cDNAs of three human glutathione S-transferase Pi gene variants. Evidence for differential catalytic activity of the encoded proteins. J Biol Chem 1997;272(15):10004–12.
[18] Watson MA, Stewart RK, Smith GB, Massey TE, Bell DA. Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. Carcinogenesis 1998;19(2):275–80.
[19] Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. Carcinogenesis 1997;18(4):641–4.
[20] Blackburn AC, Tzeng HF, Anders MW, Board PG. Discovery of a functional polymorphism in human glutathione transferase zeta by expressed sequence tag database analysis. Pharmacogenetics 2000;10(1):49–57.
[21] Blackburn AC, Coggan M, Tzeng HF, Lantum H, Polekhina G, Parker MW, et al. GSTZ1d: a new allele of glutathione transferase zeta and maleylacetoacetate isomerase. Pharmacogenetics 2001;11(8):671–8.
[22] Wang J, Gajalakshmi V, Jiang J, Kuriki K, Suzuki S, Nagaya T, et al. Associations between 5,10-methylenetetrahydrofolate reductase codon 677 and 1298 genetic polymorphisms and environmental factors with reference to susceptibility to colorectal cancer: a case–control study in an Indian population. Int J Cancer 2006;118(4):991–7.
[23] Garte S, Gaspari L, Alexandrie AK, Ambrosone C, Autrup H, Autrup JL, et al. Metabolic gene polymorphism frequencies in control populations. Cancer Epidemiol Biomarkers Prev 2001;10(12):1239–48.
[24] de Jong MM, Nolte IM, te Meerman GJ, van der Graaf WT, de Vries EG, Sijmons RH, et al. Low-penetrance genes and their involvement in colorectal cancer susceptibility. Cancer Epidemiol Biomarkers Prev 2002;11(11):1332–52.
[25] Hirvonen A, Saarikoski ST, Linnainmaa K, Koskinen K, Husgafvel-Pursiainen K, Mattson K, et al. Glutathione S-transferase and N-acetyltransferase genotypes and asbestos-associated pulmonary disorders. J Natl Cancer Inst 1996;88(24):1853–6.
[26] Saarikoski ST, Voho A, Reinikainen M, Anttila S, Karjalainen A, Malaveille C, et al. Combined effect of polymorphic GST genes on individual susceptibility to lung cancer. Int J Cancer 1998;77(4):516–21.
[27] Buch S, Kotekar A, Kawle D, Bhisey R. Polymorphisms at CYP and GST gene loci. Prevalence in the Indian population. Eur J Clin Pharmacol 2001;57(6-7):553–5.
[28] Buch SC, Notani PN, Bhisey RA. Polymorphism at GSTM1. GSTM3 and GSTT1 gene loci and susceptibility to oral cancer in an Indian population. Carcinogenesis 2002;23(5):803–7.
[29] Vettriselvi V, Vijayalakshmi K, Solomon FDP, Venkatachalam P. Genetic variation of GSTM1. GSTT1 and GSTP1 genes in a South Indian population. Asian Pac J Cancer Prev 2006;7(2):325–8.
[30] Soya SS, Vinod T, Reddy KS, Gopalakrishnan S, Adithan C. Genetic polymorphisms of glutathione-S-transferase genes (GSTM1, GSTT1 and GSTP1) and upper aerodigestive tract cancer risk among smokers, tobacco chewers and alcoholics in an Indian population. Eur J Cancer 2007;43(18):2698–706.

[31] Landi S. Mammalian class theta GST and differential susceptibility to carcinogens: a review. Mutat Res 2000;463(3):247–83.
[32] Terrier P, Townsend AJ, Coindre JM, Triche TJ, Cowan KH. An immunohistochemical study of pi class glutathione S-transferase expression in normal human tissue. Am J Pathol 1990;137(4):845–53.
[33] Moscow JA, Fairchild CR, Madden MJ, Ransom DT, Wieand HS, O'Brien EE, et al. Expression of anionic glutathione-S-transferase and P-glycoprotein genes in human tissues and tumors. Cancer Res 1989;49(6):1422–8.
[34] Tong Z, Board PG, Anders MW. Glutathione transferase zeta catalyses the oxygenation of the carcinogen dichloroacetic acid to glyoxylic acid. Biochem J 1998;331(Pt 2):371–4.
[35] Tong Z, Board PG, Anders MW. Glutathione transferase zeta-catalyzed biotransformation of dichloroacetic acid and other alpha-haloacids. Chem Res Toxicol 1998;11(11):1332–8.
[36] Fernandez-Canon JM, Penalva MA. Characterization of a fungal maleylacetoacetate isomerase gene and identification of its human homologue. J Biol Chem 1998;273(1):329–37.
[37] Zhong S, Wyllie AH, Barnes D, Wolf CR, Spurr NK. Relationship between the GSTM1 genetic polymorphism and susceptibility to bladder, breast and colon cancer. Carcinogenesis 1993;14(9):1821–4.
[38] Gawronska-Szklarz B, Lubinski J, Kladny J, Kurzawski G, Bielicki D, Wojcicki M, et al. Polymorphism of GSTM1 gene in patients with colorectal cancer and colonic polyps. Exp Toxicol Pathol 1999;51(4–5):321–5.
[39] Kiss I, Nemeth A, Bogner B, Pajkos G, Orsos Z, Sandor J, et al. Polymorphisms of glutathione-S-transferase and arylamine N-acetyltransferase enzymes and susceptibility to colorectal cancer. Anticancer Res 2004;24(6):3965–70.
[40] Ates NA, Tamer L, Ates C, Ercan B, Elipek T, Ocal K, et al. Glutathione S-transferase M1, T1, P1 genotypes and risk for development of colorectal cancer. Biochem Genet 2005;43(3–4):149–63.
[41] Deakin M, Elder J, Hendrickse C, Peckham D, Baldwin D, Pantin C, et al. Glutathione S-transferase GSTT1 genotypes and susceptibility to cancer: studies of interactions with GSTM1 in lung, oral, gastric and colorectal cancers. Carcinogenesis 1996;17(4):881–4.
[42] Zhang H, Ahmadi A, Arbman G, Zdolsek J, Carstensen J, Nordenskjold B, et al. Glutathione S-transferase T1 and M1 genotypes in normal mucosa, transitional mucosa and colorectal adenocarcinoma. Int J Cancer 1999;84(2):135–8.
[43] Butler WJ, Ryan P, Roberts-Thomson IC. Metabolic genotypes and risk for colorectal cancer. J Gastroenterol Hepatol 2001;16(6):631–5.
[44] Moisio AL, Sistonen P, Mecklin JP, Jarvinen H, Peltomaki P. Genetic polymorphisms in carcinogen metabolism and their association to hereditary nonpolyposis colon cancer. Gastroenterology 1998;115(6):1387–94.
[45] Welfare M, Monesola Adeokun A, Bassendine MF, Daly AK. Polymorphisms in GSTP1, GSTM1, and GSTT1 and susceptibility to colorectal cancer. Cancer Epidemiol Biomarkers Prev 1999;8(4 Pt 1):289–92.

Novel immunohistochemical marker, integrin $\alpha_V\beta_3$, for BOP-induced early lesions in hamster pancreatic ductal carcinogenesis

TSUKASA KITAHASHI[1], MITSUYOSHI YOSHIMOTO[2] and TOSHIO IMAI[1]

[1]Central Animal Laboratory and [2]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan

Received September 29, 2010; Accepted December 28, 2010

DOI: 10.3892/ol.2011.252

Abstract. *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced pancreatic ductal carcinomas and early ductal lesions in Syrian hamsters have been reported to show histopathological resemblance to those in humans. Specific protein expression profiles have been found in human carcinomas, but a detailed molecular approach regarding the dissection of BOP-induced pancreatic carcinogenesis has yet to be determined. The present immunohistochemical study of early and advanced hamster lesions focused on five proteins reported to be overexpressed in human patients, to clarify interspecies phenotype similarity. Integrin $\alpha_V\beta_3$ was found to be overexpressed in the epithelial cells of 13 of 14 atypical hyperplasias and 6 of 6 adenocarcinomas. This overexpression was more frequent than in the remaining four proteins. However, immunoreactivity for α-enolase in epithelial cells and for kallikrein 7 and galectin-1/3 in both epithelial and stromal cells was also evident at various frequencies. Thus, similarities of tumor-associated protein expression between human and hamster pancreatic ductal lesions were confirmed, and integrin $\alpha_V\beta_3$ was identified as a potentially useful immunohistochemical marker for early lesions in hamsters.

Introduction

Pancreatic cancer is among the 10 most frequently occurring type of cancer. In Japan, pancreatic cancer is ranked fifth as a cause of cancer-related mortality. The mortality rate associated with this type of cancer also ranks high in other developed countries (1,2). The detection of pancreatic cancer at early operable stages is difficult, combined with the lack of curative treatment approaches other than complete surgical removal; thus, 5-year relative survival rates are less than 6% (3,4). Therefore, it is crucial to develop new diagnostic and preventive methods to complement any improvements in therapeutic methods for the reduction of mortality and morbidity, and to explore specific proteins overexpressed in early lesions during pancreatic carcinogenesis.

Specific protein expression profiles revealed by immunohistochemical and proteomic analyses are currently employed in the application of individualized therapy of advanced human pancreatic carcinomas (5-8). Consequently, a number of candidates of prognostic and/or predictive markers have been established (9,10). Examples expressed in early lesions show potential for the development of novel diagnostic and preventive strategies.

Chemically induced and transgenic animal models for pancreatic ductal carcinogenesis have been the target of investigation of the impact of environmental factors and the role of specific gene alterations in multistage carcinogenesis (11-15). Among the models established thus far, the *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced hamster model is the first and most widely utilized based on similarities to human diseases in the morphological characteristics of, not only advanced cancers, but also early ductal lesions, as well as pivotal genetic alterations, including *K-ras* and *p16* (13,16-18). In particular, it is anticipated that molecular profiles are equivalent in the early stages. Although changes in the protein expression have been reported (5-8) in pancreatic carcinomas in humans, molecular details of BOP-induced pancreatic early lesions in hamsters have yet to be investigated. In the present study, an immunohistochemical analysis was conducted on pancreatic carcinomas and early lesions induced in BOP-treated hamsters, focusing on proteins already reported to be altered in human cases. As a result, integrin $\alpha_V\beta_3$ was found to be more frequently expressed in both pancreatic carcinomas and its precursors than the four remaining proteins investigated. The results obtained showed multiple similarities in tumor-associated protein expression between human and hamster pancreatic ductal lesions.

Materials and methods

Animals. A total of 12 female Syrian golden hamsters at 5 weeks of age were purchased from Japan SLC (Shizuoka, Japan). The animals were housed 3 to a plastic cage with soft woodchip bedding (Japan SLC) in an air-conditioned animal room maintained at 22±2˚C and 60±5% relative humidity, with

Correspondence to: Dr Toshio Imai, Central Animal Laboratory, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
E-mail: toimai@ncc.go.jp

Abbreviations: BOP, *N*-nitrosobis(2-oxopropyl)amine

Key words: integrin $\alpha_V\beta_3$, Syrian hamster, pancreatic ductal carcinoma, early stage, *N*-nitrosobis(2-oxopropyl)amine

Table I. Antibodies and antigen retrieval methods for immunochemistry.

Protein	Antibodies	Antigen retrieval
Integrin $\alpha_v\beta_3$	Anti-human integrin $\alpha_v\beta_3$ monoclonal (Clone LM609; Chemicon International, Temecula, CA, USA)	Autoclaved for 10 min at 121°C in 10 mM Tris-HCl buffer, including 1 mM EDTA (pH 9.0)
Kallikrein 7	Anti-kallikrein 7 goat polyclonal (R&D Systems, Minneapolis, MN, USA)	Autoclaved for 10 min at 121°C in 10 mM citrate buffer (pH 6.0)
Galectin 1	Anti-galectin-1 rabbit polyclonal (Protein Tech Group, Chicago, IL, USA)	Autoclaved for 15 min at 121°C in 10 mM citrate buffer (pH 6.0)
Galectin 3	Anti-galectin-3 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA)	Autoclaved for 10 min at 121°C in 10 mM citrate buffer (pH 6.0)
α-Enolase	Anti-α-enolase rabbit polyclonal (Aviva Systems Biology LLC., San Diego, CA, USA)	Autoclaved for 10 min at 121°C in distilled water

a 12:12 h light:dark cycle. A basal diet (CE-2; CLEA Japan, Tokyo, Japan) and water were available *ad libitum* throughout the experiment.

Treatment for pancreatic tumor induction. Following an acclimatization period of 1 week, 9 hamsters were subcutaneously injected with BOP (Nacalai Tesque, Kyoto, Japan) in saline at 10 mg/kg body weight every other day for a total of four times. Additionally, 3 hamsters treated with saline were maintained as control animals. The experimental protocols were approved by the Committee for Ethics of Animal Experimentation of the National Cancer Center and were carried out according to the Guidelines for Animal Experiments.

Histopathological examination. At 23 weeks of age, all 12 hamsters were sacrificed and each pancreas was removed, fixed in 10% buffered formalin, processed for embedding in paraffin, sectioned and stained with hematoxylin and eosin for histopathological evaluation. Pancreatic ductal proliferative lesions were classified as atypical hyperplasias (AHs) and adenocarcinomas (ACs) according to the criteria previously described (19). Briefly, AH consisted of ductules with increased cell proliferation but minimal nuclear atypia and no loss of polarity. The typical AC had a distinct tubular, cribriform or anaplastic pattern with severely atypical columnar or cuboidal epithelia.

Immunohistochemical staining. A total of five target proteins, known to be specifically expressed in pancreatic carcinoma tissues or cell lines established from pancreatic carcinomas, were selected, as previously reported (5-8). Antibodies and antigen retrieval methods used in this study are listed in Table I. The role played by each was: integrin $\alpha_v\beta_3$, a transmembrane glycoprotein, is involved in cell-to-cell and cell-to-matrix interactions and thus may contribute to cancer progression, invasiveness and metastasis (5); kallikrein 7, a member of the serine protease family, enhances pancreatic cancer cell invasion by shedding E-cadherin (6); galectin-1, a soluble β-galactoside-binding animal lectin, modulates cell adherence and plays a role in tumor progression (7); galectin-3, another soluble β-galactoside-binding animal lectin, modulates cell adherence (8); and α-enolase, a glycolytic enzyme, is involved in the conversion of 2-phosphoglycerate phosphoenolpyruvate (7,20). The streptavidin-biotin-peroxidase complex method (StreptABComplex/HRP; DakoCytomation, Glostrup, Denmark) was employed to determine the expression and localization of each protein, and the sections were lightly counterstained with hematoxylin for microscopic examination. Negative controls without primary antibody reactions were set for each protein using serial sections. The staining intensity of each protein was analyzed with reference to the positivity rate in all epithelial and stromal cells in AHs and ACs and represented as <10%, negative (-); 10-70%, moderately positive (+); and >70%, strongly positive (++).

Results

Pancreatic ductal lesions induced by BOP. A total of 14 AHs and 6 ACs were induced in the 9 hamsters treated with BOP, whereas no lesions were found in the 3 animals without carcinogen exposure. The incidences and multiplicities (mean ± SD) of AHs and ACs were 89%, 1.6±1.1 and 44%, 0.7±0.9, respectively. Only 1 case of AC showed poorly differentiated characteristics, while the remaining cases were moderately differentiated tubular ACs. All cases harbored abundant stroma (data not shown).

Immunohistochemical findings for the five proteins analyzed

Normal pancreas (Fig. 1). Pancreatic ductal and ductular cells as well as islet cells showed weak immunohistochemical staining for α-enolase in hamsters without BOP treatment. The reactions in ductal and islet cells were cytoplasmic and essentially homogeneous. Integrin $\alpha_v\beta_3$, kallikrein 7 and galectin 1/3 were almost negative in the normal pancreatic tissues.

Atypical hyperplasias (Fig. 2). Integrin $\alpha_v\beta_3$ and α-enolase were expressed predominantly in the epithelial components of AHs, whereas kallikrein 7 and galectin 1/3 were expressed in both the epithelial and adjacent stromal elements. The morphological characteristics of the numerous stromal cells positive for kallikrein 7 and/or galectin 1/3 characterized these cells as fibroblasts. Regarding subcellular staining, integrin $\alpha_v\beta_3$ was mostly localized in the cell cytoplasm, while appearing to aggregate with a granular pattern towards the apex from the nuclei in the epithelial cells. Concerning

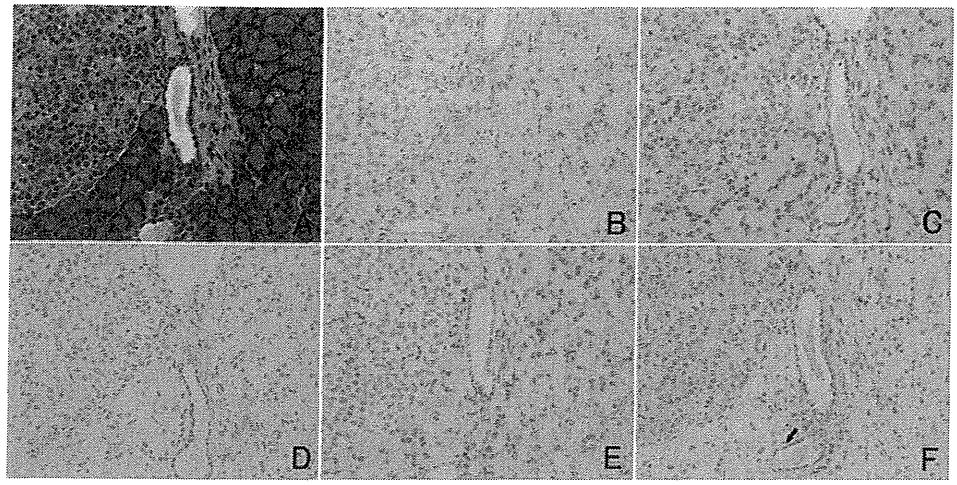

Figure 1. Normal hamster pancreatic tissue, including ductules (center panels), an islet (left panels) and acini (right panels). (A) H&E and immunohistochemistry for (B) integrin $\alpha_V\beta_3$, (C) kallikrein 7, (D) galectin 1, (E) galectin 3 and (F) α-enolase. The proteins were essentially negative, except for weak cytoplasmic positivity for (F) α-enolase in the islet and ductular cells (arrow). Original magnification, x200.

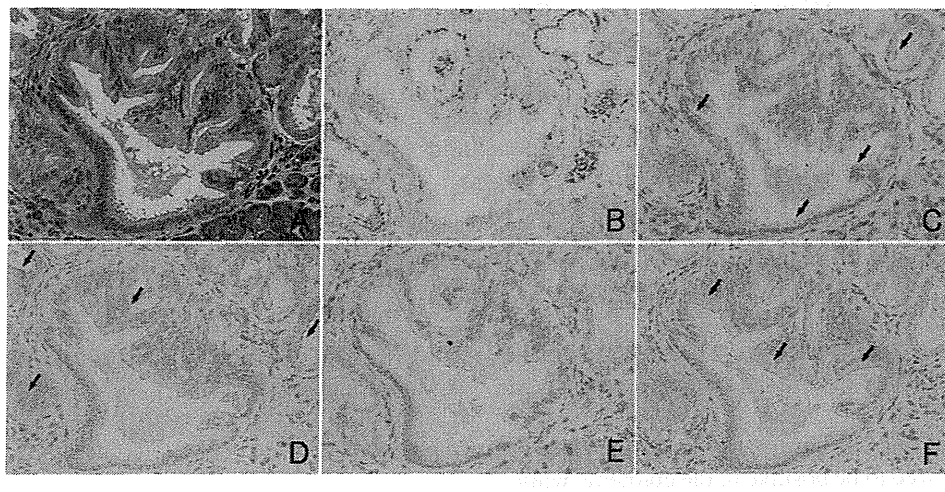

Figure 2. Atypical hyperplasia in a BOP-treated hamster. (A) H&E and immunohistochemistry for (B) integrin $\alpha_V\beta_3$, (C) kallikrein 7, (D) galectin 1, (E) galectin 3 and (F) α-enolase. (B) Integrin $\alpha_V\beta_3$ localization, particularly in the cytoplasm, with a granular pattern in the epithelial cells. In this case, (E) galectin 3 appears negative. (C, D and F) Other proteins are almost uniformly positive in the epithelial cytoplasm and/or are localized on the apical surface (arrows). Original magnification, x200.

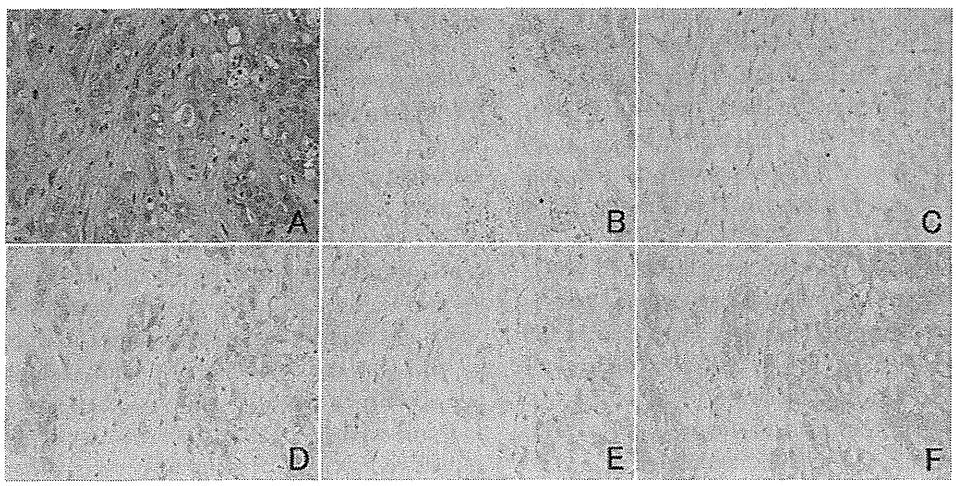

Figure 3. Adenocarcinoma in a BOP-treated hamster. (A) H&E and immunohistochemistry for (B) integrin $\alpha_V\beta_3$, (C) kallikrein 7, (D) galectin 1, (E) galectin 3 and (F) α-enolase. (B, D and F) Integrin $\alpha_V\beta_3$, galectin 1 and α-enolase are mainly positive in the epithelial tumor cells, and (C and E) kallikrein 7 and galectin 3 are mainly reactive in the stromal cells. Original magnification, x200.

Table II. Incidences and intensities of immunostaining in positive AHs and ACs.

Protein	Lesion	Location	Incidence (%)	
			Intensity (+)	Intensity (++)
Integrin $\alpha_V\beta_3$	AH (n=14)	Epithelium	8 (57)	5 (36)
		Stroma	0	0
	AC (n=6)	Epithelium	4 (67)	2 (33)
		Stroma	0	0
Kallikrein 7	AH (n=14)	Epithelium	6 (43)	0
		Stroma	4 (28)	4 (28)
	AC (n=6)	Epithelium	4 (66)	0
		Stroma	0	6 (100)
Galectin 1	AH (n=14)	Epithelium	0	3 (21)
		Stroma	1 (7)	0
	AC (n=6)	Epithelium	4 (67)	0
		Stroma	1 (17)	0
Galectin 3	AH (n=14)	Epithelium	2 (14)	1 (7)
		Stroma	1 (7)	1 (7)
	AC (n=6)	Epithelium	0	0
		Stroma	2 (33)	0
α-Enolase	AH (n=14)	Epithelium	11 (79)	0
		Stroma	0	0
	AC (n=6)	Epithelium	6 (100)	0
		Stroma	0	0

AH, atypical hyperplasia; AC, adenocarcinoma; +, moderately positive; ++, strongly positive.

kallikrein 7, galectin 1/3 and α-enolase, positivity was found almost uniformly in the cell cytoplasm and/or was localized on the apical surfaces.

Adenocarcinomas (Fig. 3). Similar to the AHs, integrin $\alpha_V\beta_3$ and α-enolase proved to be positive in the epithelia, while kallikrein 7 and galectin 1 were observed in the epithelial and stromal cells. Galectin 3 was stained only in the stromal cells. Subcellular staining patterns were similar to those in AHs.

Immunostaining incidences and intensities for the five proteins. Staining incidences and intensities for each protein were evaluated based on the positivity rates for cells (Table II). A total of 13 of14 AHs (93%) and 6 of 6 ACs (100%) were positive for integrin $\alpha_V\beta_3$. Consequently, the incidence of integrin $\alpha_V\beta_3$ was higher compared to the remaining four proteins, and in 5 of the AHs (36%) and 2 of the ACs (33%), the grading was strongly positive (++). Staining was found to be negative in the stroma. By contrast, kallikrein 7 was predominantly expressed in the stroma of 8 of 14 AHs (57%) and 6 of 6 ACs (100%), and the epithelial cells of 6 of 14 AHs (43%) were also stained. In 4 AHs (28%) and 6 ACs (100%), stromal cells were graded as strongly positive (++). Galectin 1 was predominantly expressed in the epithelium of 3 of 14 AHs (21%) and 4 of 6 ACs (67%), and all the AHs were graded as strongly positive (++). Galectin 3 was also expressed in both the epithelium and stroma of AHs (3/14, 21% and 2/14, 14%, respectively), and the stroma of ACs (2/6, 33%). Among the positive cases, 1 AH case showed strongly positive in both the epithelial and stroma cells. Expression of α-enolase was observed predominantly in the epithelial cells of 11 of 14 AHs (79%) and 6 of 6 ACs (100%). Staining intensity was moderately positive (+).

Discussion

The present immunohistochemical analysis focusing on five proteins reported to be overexpressed in human pancreatic carcinomas showed that integrin $\alpha_V\beta_3$ was found to be frequently and strongly expressed in BOP-induced hamster pancreatic early ductal lesions and carcinomas. This expression is in agreement with its reported promotion of cell migration and proliferation (21,22). Subcellular localization aggregated in the cytoplasm of epithelial cells has also been reported in human pancreatic cancer cases (5). Although a slight expression of integrin $\alpha_V\beta_3$ was found in pancreatic ductal hyperplasias, which lack cellular atypia and are thought to be initial histological focal changes, the frequency was lower than that in more advanced lesions (data not shown). The significance of the overexpression of integrin $\alpha_V\beta_3$ in pancreatic ductal carcinogenesis has yet to be elucidated. However, dysregulation in protein transportation and/or degradation functions may occur in the early stages of pancreatic ductal carcinogenesis in hamsters and humans. The relationship between integrin $\alpha_V\beta_3$-positivity and parameters, such as proliferation, apoptosis and invasiveness, has yet to be investigated. However, in human cases, 58% of pancreatic carcinomas showed positive staining and the frequency was significantly higher in primary tumors with lymph node metastasis (5). Recently, integrin $\alpha_V\beta_3$ was also studied as a target molecule for imaging diagnosis

in mammary carcinoma (23), and this BOP-induced hamster model may aid in the pre-clinical screening of integrin $\alpha_V\beta_3$ and other molecular-targeted probes and/or medicines.

Kallikrein 7 is a chymotrypsin-like serine protease, originally purified from human skin, which is specifically expressed in keratinizing squamous epithelia (24), and is involved in cell invasion by cleavage of the extracellular domain of adhesion molecules, such as E-cadherin. In the present study, Kallikrein 7 was found to be predominantly expressed in stromal cells of both AHs and ACs. Moderate-to-intense staining for kallikrein 7 has been reported in the majority of human pancreatic carcinomas, but this staining is distributed among the majority of the tumor cells (6). Although the cause of such variation remains to be determined, cytoplasmic positivity and/or localization on the apical surfaces was evident in the epithelial cells of some of our AHs and ACs in hamsters.

Galectin 1/3 are members of the family of β-galactoside-binding animal lectins (25) and play a role in a variety of cell functions, including proliferation, migration and adhesion characteristics (26,27). In this study, the expression of galectin 1/3 was observed at lower frequencies than the remaining three proteins in the epithelial and stromal cells in AHs, whereas it was strongly expressed in epithelial and stromal cells, respectively, in ACs. In human carcinoma cases, however, the opposite phenomenon has been described, with galectin 1 being stronger in the stroma and galectin 3 in the epithelial elements (7,8). The causes for this phenomenon remain to be determined.

α-Enolase, a glycolytic enzyme, is involved in the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway (28). In the present analysis, α-enolase showed the second most frequent expression in the epithelium of both AHs and ACs. On the other hand, normal hamster pancreatic islets and acinar and ductal epithelium cells were weakly positive, partially consistent with a previous report (29).

In conclusion, some similarities to the human tumor-associated protein expression were confirmed in hamster pancreatic ductal lesions. The addition of molecules may also be identified by a global analysis using cDNA microarrays and/or proteomic approaches. Additional studies using hamsters may allow for the discovery of target molecules for practical diagnostic and preventive methods for human early pancreatic carcinomas.

Acknowledgements

This study was supported by Grants-in-Aid for the Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare of Japan. We thank Dr Malcolm A. Moore for the revision of the scientific English language.

References

1. Matsuda T, Marugame T, Kamo K, Katanoda K, Ajiki W, Sobue T and the Japanese Cancer Surveillance Research Group: Cancer incidence and incidence rates in Japan in 2002: based on data from 11 population-based cancer registries. Jpn J Clin Oncol 38: 641-648, 2008.
2. Michaud DS: Epidemiology of pancreatic cancer. Minerva Chir 59: 99-111, 2004.
3. Tsukuma H, Ajiki W, Ioka A and Oshima A: Survival of cancer patients diagnosed between 1993 and 1996: a collaborative study of population-based cancer registries in Japan. Jpn J Clin Oncol 36: 602-607, 2006.
4. De Braud F, Cascinu S and Gatta G: Cancer of pancreas. Crit Rev Oncol Hematol 50: 147-155, 2004.
5. Hosotani R, Kawaguchi M, Masui T, Koshiba T, Ida J, Fujimoto K, Wada M, Doi R and Imamura M: Expression of integrin $\alpha_V\beta_3$ in pancreatic carcinoma: relation to MMP-2 activation and lymph node metastasis. Pancreas 25: e30-e35, 2002.
6. Johnson SK, Ramani VC, Hennings L and Haun RS: Kallikrein 7 enhances pancreatic cancer cell invasion by shedding E-cadherin. Cancer 109: 1811-1820, 2007.
7. Shen J, Person MD, Zhu J, Abbruzzese JL and Li D: Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry. Cancer Res 64: 9018-9026, 2004.
8. Shimamura T, Sakamoto M, Ino Y, Shimada K, Kosuge T, Sato Y, Tanaka K, Sekihara H and Hirohashi S: Clinicopathological significance of galectin-3 expression in ductal adenocarcinoma of the pancreas. Clin Cancer Res 8: 2570-2575, 2002.
9. Gold DV, Modrak DE, Ying Z, Cardillo TM, Sharkey RM and Goldenberg DM: New MUC1 serum immunoassay differentiates pancreatic cancer from pancreatitis. J Clin Oncol 24: 252-258, 2006.
10. Takayama R, Nakagawa H, Sawaki A, Mizuno N, Kawai H, Tajika M, Yatabe Y, Matsuo K, Uehara R, Ono K, Nakamura Y and Yamao K: Serum tumor antigen REG4 as a diagnostic biomarker in pancreatic ductal adenocarcinoma. J Gastroenterol 45: 52-59, 2010.
11. Rivera JA, Graeme-Cook F, Werner J, Z'graggen K, Rustgi AK, Rattner DW, Warshaw AL and Fernández-del Castillo C: A rat model of pancreatic ductal adenocarcinoma: targeting chemical carcinogens. Surgery 122: 82-90, 1997.
12. Osvaldt AB, Wendt LR, Bersch VP, de Cássia A, Schumacher R, Edelweiss MI and Rohde L: Pancreatic intraepithelial neoplasia and ductal adenocarcinoma induced by DMBA in mice. Surgery 140: 803-809, 2006.
13. Pour P, Althoff J, Kruger FW and Mohr U: A potent pancreatic carcinogen in Syrian hamsters: *N*-nitrosobis(2-oxopropyl)amine. J Natl Cancer Inst 58: 1449-1453, 1977.
14. Hingorani SR, Petricoin EF, Maitra A, *et al*: Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 4: 437-450, 2003.
15. Ueda S, Fukamachi K, Matsuoka Y, Takasuka N, Takeshita F, Naito A, Iigo M, Alexander DB, Moore MA, Saito I, Ochiya T and Tsuda H: Ductal origin of pancreatic adenocarcinomas induced by conditional activation of a human Ha-*ras* oncogene in rat pancreas. Carcinogenesis 27: 2497-2510, 2006.
16. Fujii H, Egami H, Chaney W, Pour P and Pelling J: Pancreatic ductal adenocarcinomas induced in Syrian hamsters by *N*-nitrosobis(2-oxopropyl)amine contain a c-Ki-*ras* oncogene with a point-mutated codon 12. Mol Carcinog 3: 296-301, 1990.
17. Li J, Weghorst CM, Tsutsumi M, Poi MJ, Knobloch TJ, Casto BC, Melvin WS, Tsai MD and Muscarella P: Frequent $p16^{INK4A\ CDKN2A}$ alterations in chemically induced Syrian golden hamster pancreatic tumors. Carcinogenesis 25: 263-268, 2004.
18. Tsujiuchi T, Sasaki Y, Kubozoe T, Konishi Y and Tsutsumi M: Alterations in the Fhit gene in pancreatic duct adenocarcinomas induced by *N*-nitrosobis(2-oxopropyl)amine in hamsters. Mol Carcinog 36: 60-66, 2003.
19. Konishi Y, Mizumoto K, Kitazawa S, Tsujiuchi T, Tsutsumi M and Kamano T: Early ductal lesions of pancreatic carcinogenesis in animals and humans. Int J Pancreatol 7: 83-89, 1990.
20. Roda O, Chiva C, Espuna G, Gabius H, Real FX, Navarro P and Andreu D: A proteomic approach to the identification of new tPA receptors in pancreatic cancer cells. Proteomics 6: S36-S41, 2006.
21. Carreiras F, Lehmann M, Sichel F, Marvaldi J, Gauduchon P and Le Talaer JY: Implication of the $\alpha_V\beta_3$ integrin in the adhesion of the ovarian-adenocarcinoma cell line IGROV1. Int J Cancer 63: 530-536, 1995.
22. Yokosaki Y, Monis H, Chen J and Sheppard D: Differential effects of the integrins $\alpha_9\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_6$ on cell proliferative responses to tenascin. Roles of the β subunit extracellular and cytoplasmic domains. J Biol Chem 271: 24144-24150, 1996.

23. Beer AJ, Niemeyer M, Carlsen J, Sarbia M, Nährig J, Watzlowik P, Wester HJ, Harbeck N and Schwaiger M: Patterns of $\alpha_V\beta_3$ expression in primary and metastatic human breast cancer as shown by ^{18}F-Galacto-RGD PET. J Nucl Med 49: 255-259, 2008.
24. Hansson L, Strömqvist M, Bäckman A, Wallbrandt P, Carlstein A and Egelrud T: Cloning, expression, and characterization of stratum corneum chymotryptic enzyme. A skin-specific human serine proteinase. J Biol Chem 269: 19420-19426, 1994.
25. Barondes SH, Castronovo V, Cooper DN, *et al*: Galectins: a family of animal β-galactoside-binding lectins. Cell 76: 597-598 1994.
26. Perillo, NL, Marcus ME and Baum LG: Galectins: Versatile modulators of cell adhesion, cell proliferation, and cell death. J Mol Med 76: 402-412, 1998.
27. Maeda N, Kawada N, Seki S, Ikeda K, Iwao H, Okuyama H, Hirabayashi J, Kasai K and Yoshizato K: Stimulation of proliferation of rat hepatic stellate cells by galectin-1 and galectin-3 through different intracellular signaling pathways. J Biol Chem 278: 18938-18944, 2003.
28. Zhou W, Capello M, Fredolini C, Piemonti L, Liotta LA, Novelli F and Petricoin EF: Mass spectrometry analysis of the post-translational modifications of α-enolase from pancreatic ductal adenocarcinoma cells. J Proteome Res 9: 2929-2936, 2010.
29. Ahmed M and Bergsten P: Glucose-induced changes of multiple mouse islet proteins analysed by two-dimensional gel electrophoresis and mass spectrometry. Diabetologia 48: 477-485, 2005.

High susceptibility of heterozygous (+/*fa*) lean Zucker rats to 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis

TOSHIO IMAI[1,2], YOUNG-MAN CHO[1], MAMI TAKAHASHI[2], TSUKASA KITAHASHI[2], SHIGEAKI TAKAMI[1], AKIYOSHI NISHIKAWA[1] and KUMIKO OGAWA[1]

[1]Division of Pathology, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501; [2]Central Animal Division, National Cancer Center Research Institute, Chuo-ku, Tokyo 104-0045, Japan

Received December 11, 2012; Accepted January 22, 2013

DOI: 10.3892/or.2013.2326

Abstract. Susceptibility to 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis was investigated in lean Zucker *(+/fa)* rats carrying one mutated leptin receptor gene and wild-type controls (+/+). Rats with both genotypes were given a single DMBA administration and divided into two groups, one group was fed on basal diet mixed with 10% corn oil and the other was fed on basal diet alone. The minimum latency period of palpable carcinomas in +/*fa* rats of both groups was 8 weeks following DMBA treatment, in contrast to the 11-12 weeks in +/+. The incidence and multiplicity of carcinomas increased or showed a tendency for increase in the early stages in +/*fa* rats of both groups as compared to the +/+ counterparts. The volumes of carcinomas showed a tendency to increase in the corn oil diet groups of both genotypes. The major histopathological phenotype of carcinomas in all groups was well-differentiated without distinct atypia (multiplicity, 0.69-1.09/rat), but moderately/poorly differentiated carcinomas with atypia were also found, predominantly in +/*fa* rats (0.09-0.21). These latter tumors were characterized by elevated ERK activity but not estrogen receptor expression. Serum leptin concentrations in +/*fa* rats at 7 weeks of age were higher than those in +/+ and were elevated by the corn oil diet; however, no obvious change was detected in other serum parameters examined. In conclusion, +/*fa* rats proved more susceptible to DMBA-induced mammary carcinogenesis than +/+ controls, and hyperleptinemia was suggested to contribute to tumor growth as well as to susceptibility to tumorigenesis and more aggressive phenotypes in Zucker lean rats.

Correspondence to: Dr Toshio Imai, Central Animal Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
E-mail: toimai@ncc.go.jp

Key words: mammary, carcinoma, leptin, 7,12-dimethylbenz(*a*)-anthracene, Zucker rat

Introduction

A relationship between obesity and breast cancer risk has been proposed based on epidemiological data, a positive association with increasing body mass index being found particularly in postmenopausal women (1-4). Although the underlying mechanisms have yet to be fully clarified, increased concentrations of circulating sex hormones are likely to contribute at least in part (5). In addition, circulating levels of an adipokine leptin, which is secreted mainly from adipose tissue and limits food intake and increases energy expenditure (6), was recently suggested to have a role independent of obesity indices in breast tumorigenesis (7). In estrogen receptor (ER)-positive breast cancer cells, leptin has been demonstrated to stimulate aromatase expression and cell proliferation, and both in ER-positive and -negative breast cancer cells, leptin induced transactivation of ErbB tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR) and ErbB-2 (HER2/Neu), resulting in the induction of cell proliferation and increased survival (8-10).

To investigate the effects of obesity on mammary carcinogenesis, a number of animal models, featuring inherited obesity or feeding of a high fat/calorie diet, were employed. Fatty Zucker (*fa/fa*) rats, which have autosomal recessive mutation in the leptin receptor gene (11), develop hyperinsulinemia, but blood glucose remains at normal levels (12). In addition, they demonstrate significantly increased serum triglyceride, total cholesterol and leptin levels (12,13). Lean Zucker (+/*fa* and +/+) rats, by contrast, exhibit normal appearing metabolic functions and have been utilized as controls in chemically-induced mammary carcinogenesis investigations (14-17). In a previous study, the latency period and/or the incidence of mammary carcinomas were reported to be shorter and greater, respectively, in female *fa/fa* than +/*fa* and +/+ rats treated with 7,12-dimethylbenz(*a*)anthracene (DMBA) (15,17). However, in another study, female Zucker (*fa/fa*) rats treated with *N*-methyl-*N*-nitrosourea (MNU) showed a lower incidence of mammary carcinomas compared to lean Zucker controls (+/*fa* and +/+) (14). A number of factors may contribute to the discrepancy between the DMBA- and MNU-treated rats, and it remains unclear which obesity-associated internal parameters, such as hyperin-

sulinemia, hyperleptinemia or hyperlipidemia, fundamentally affect mammary carcinogenesis.

We recently compared serum biochemical parameters between lean Zucker (+/*fa*) and (+/+) rats in combination with or without an obesity-inducing 10% corn oil diet, to clarify whether lean Zucker (+/*fa*) rats might also be more sensitive to the high fat diet than the +/+ controls (18). Serum leptin concentrations were higher in the (+/*fa*) case at 7 weeks of age (~140 pg/ml as compared to ~80 pg/ml in +/+; $P<0.01$), although the difference was significantly smaller at 12 weeks of age, and serum concentrations of other parameters including insulin, triglycerides and total cholesterol were similar between the two genotypes. In addition, both +/*fa* and +/+ rats fed basal diet mixed with 10% corn oil showed higher serum leptin levels than those fed basal diet alone, but no other parameters examined were altered by the obesity-inducing diet.

In the present study, to clarify the effects of hereditary and dietary hyperleptinemia on mammary carcinogenesis, lean Zucker (+/*fa*) rats with and without 10% corn oil feeding were utilized in a DMBA-induced mammary carcinogenesis model along with control lean Zucker (+/+) rats. In the present study, latency period and growth rates of mammary carcinomas were assessed by regular palpation, and at the termination, histopathological, immunohistochemical and western blot analyses were performed to determine expression profiles of estrogen- and intracellular signaling cascade-related proteins in the mammary carcinomas, as well as serum biochemistry for obesity-associated parameters. The data demonstrated +/*fa* rats to indeed be more susceptible to DMBA-induced mammary carcinogenesis than +/+ controls, with hyperleptinemia appearing to be partly associated with tumor growth as well as with susceptibility to tumorigenesis and a more aggressive phenotype in an estrogen-independent manner.

Materials and methods

Chemicals and animals. DMBA was purchased from Sigma Chemical (St. Louis, MO, USA) and dissolved in sesame oil at 10 mg/ml prior to administration. A total of 100 female Zucker rats (lean phenotype) at 5 weeks of age were purchased from Charles River Japan (Kanagawa, Japan) and acclimated for 1 week prior to genotyping by the method of Phillips *et al* (19). Throughout the acclimatization and experimental periods, the animals were housed at a maximum of 3 or 4 per plastic cage with white wood chips (Sankyo Laboratory Service, Tokyo, Japan) for bedding and transferred to clean cages with fresh bedding twice a week in a standard air-conditioned animal room (24±1°C, 55±5% relative humidity, 12 h light and dark cycle). All animals had free access to basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and tap water until the start of the experiment.

Experimental protocol. Sixty-six +/*fa* and 32 +/+ rats at 7 weeks of age received an intragastric administration of DMBA (50 mg/kg body weight) by gavage, and the animals of each genotype were then divided into basal diet (CRF-1; 357 kcal/100 g) and 10% corn oil diet (CRF-1-based, Oriental Yeast; 414 kcal/100 g) groups. The present dose level of DMBA at 50 mg/kg body weight was selected based on our previous experiments, in which palpable mammary tumors were induced at adequate incidences for detection of endogenous and exogenous tumor promoting and/or inhibitory factors in Sprague-Dawley (20) and F344 rats (21). The dietary concentration of corn oil at 10% was selected based on the previously reported effective concentrations of linoleic acid for promotion of rat mammary tumor development (22). General conditions and mortality were checked daily and body weight was measured once a week during the experimental period. The amounts of supplied and residual diet were weighted weekly in order to calculate the average daily food intake per week. Following DMBA administration, a veterinary scientist (T.I.) palpated cervix, thorax and abdomen of awake rats to detect mammary tumors once weekly. The length, width and height of each tumor were measured using a caliper and tumor volumes were calculated as follows: Volume = (length) x (width) x (height) x $\pi/6$.

For endpoints for this study, the rats were sacrificed when demonstrating over 20% decrease in body weight excluding total tumor weight and/or when symptoms of poor physical condition, such as decrease in locomotor activity, were found. Volume of mammary tumors was not considered important in this regard, since change in tumor volume was a key item for evaluation of the effects of rat genotype and corn oil diet. All remaining rats were sacrificed at 32 weeks following DMBA administration. The present study design was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences.

Necropsy and histopathology. At the end of the experimental period, blood samples were collected from the abdominal aorta of all surviving animals under ether anesthesia. Serum was separated and maintained at -80°C until use. Following euthanasia by exsanguination under ether anesthesia, animals were subjected to necropsy. Whole skins with mammary glands and tumors were removed, and the sizes of all mammary tumors were recorded. Tumor volumes were calculated in the same manner as for palpable tumors. Sections of frozen tissue of randomly selected mammary tumors of rats in all groups were prepared with liquid nitrogen and stored at -80°C until use. The remaining tumor and mammary tissues were fixed in 10% neutral buffered formalin, processed routinely to paraffin-embedded sections at 4-5 μm, and stained with hematoxylin and eosin (H&E) for histopathological analysis. Animals that died or that were sacrificed on becoming moribund were similarly necropsied and included for the sequential palpable tumor and postmortem analyses.

Immunohistochemistry. Primary antisera for the leptin receptor (goat polyclonal; Neuromics Antibodies, Edina, MN, USA; 1:1,000 dilution), smooth muscle actin (mouse clone 1A4; Dako, Glostrup, Denmark; 1:200), leptin (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200), ER α (mouse clone 6F11; Novocastra, Newcastle, UK; 1:50 or 1:500), ER β (rabbit polyclonal; Affinity BioReagents, Rockford, IL, USA; 1:100) and aromatase (rabbit polyclonal; Abcam, Cambridge, MA, USA; 1:500), were utilized for immunohistochemistry. Analyzed mammary tumors were selected from all groups, on the basis of the genotype, diet and phenotypes. Paraffin sections 5, 5, 10 and 10 carcinomas from the +/+-basal diet, +/+-corn oil diet, +/*fa*-basal diet and

+/*fa*-corn oil diet groups, respectively, were used for leptin receptor, leptin, smooth muscle actin and ER α, and frozen sections of 3, 4, 4 and 5 each were for ER β and aromatase. Antigen retrieval for paraffin sections was carried out in an autoclave for 10 min at 121˚C in 10 mM citrate buffer (pH 6.0) for leptin receptor, smooth muscle actin and ER α. The streptavidin-biotin-peroxidase complex method (StreptABComplex/HRP; Dako) was used to determine the expression and localization of each antigen, and sections were lightly counterstained with hematoxylin for microscopic examination. Negative controls without primary antibody reactions were set for each antigen using serial sections. The positivities for ER α in over 1,000 mammary adenocarcinoma cells were assessed on each paraffin section to give percentage values.

Western blot analysis. Twelve mammary tumors and four normal mammary tissue samples of the +/+-basal diet, +/+-corn oil diet and +/*fa*-basal diet groups were homogenized in extraction buffer (50 mM Tris-HCl pH 7.4, 3 mM EDTA, 100 mM NaCl, 1% Tween-20, 10 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin) and centrifuged at 14,000 g for 20 min. Equal amounts of protein samples (50 μg) from collected supernatants were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5-20% gradient acrylamide gels (ATTO, Tokyo, Japan), and the separated proteins were transferred to polyvinylidene difluoride membranes (Whatman, Sanford, ME, USA). Immunoblotting was performed using rabbit polyclonal antibodies against ER β (Affinity BioReagents), aromatase (Abcam), signal transducer and activator of transcription (STAT)3 and phospho-STAT3 (Thy705) (Cell Signaling Technology, Danvers, MA, USA), extracellular signal-regulated kinase (ERK)1/2 and phospho-ERK1/2 (R&D Systems, Minneapolis, MN, USA) or monoclonal antibodies against β-actin (mouse clone AC-15; Sigma), followed by exposure to peroxidase-labeled anti-rabbit or mouse polyclonal goat antibodies (Dako) and development of signals with TMB 3,3',5,5' tetramethylbenzidine (ATTO). Semi-quantitative analyses were performed using Scion Image (alpha4.0.3.0; Scion, Frederick, MD, USA).

Serum biochemistry. Concentrations of serum leptin, adiponectin, insulin and insulin-like growth factor (IGF)-I were determined for randomly selected almost half and one third of samples from +/+- and +/*fa*-groups, respectively, using rat/mouse enzyme immunoassay kits from Yanaihara Institute (Shizuoka, Japan), Adipogen (Incheon, Korea), Mercodia (Uppsala, Sweden) and R&D Systems, respectively. Other serum biochemical parameters including triglyceride, total cholesterol and glucose were measured for all samples except for those lost due to sampling error at SRL (Tokyo, Japan).

Statistical analysis. The survival rates and incidence of palpable or histopathologically defined mammary tumors were analyzed for inter-group differences by the Fisher's exact probability test. Data for body weights and multiplicity, volume and latency of mammary tumors, ER α-positivity in mammary adenocarcinoma sections, serum biochemistry and western blot analysis data were examined with the Student's or the Welch's t-test following the F-test. Significance was inferred at the 5, 1 and 0.1% levels.

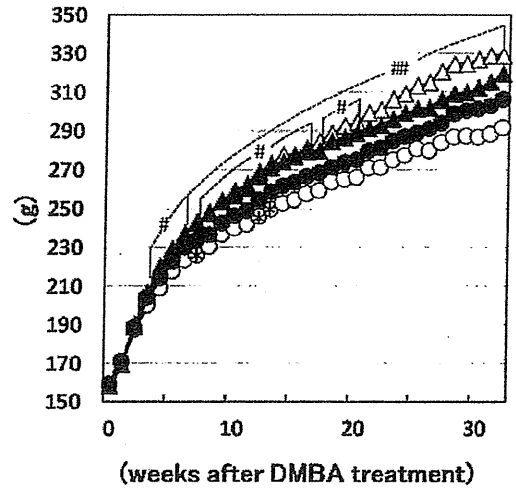

Figure 1. Body weight curves. Open circles, +/+-basal diet; open triangles, +/+-10% corn oil diet; closed circles, +/*fa*-basal diet; closed triangles, +/*fa*-10% corn oil diet. *P<0.05 vs. +/+-basal diet (difference with the genotype basis); #P<0.05, ##P<0.01 vs. +/+-basal diet or +/*fa*-basal diet (difference with the diet basis).

Results

Survival rates, body weights and food intake. At the end of the experiment, survival rates were 94% (15/16), 81% (13/16), 85% (28/33) and 85% (28/33) in the +/+-basal diet, +/+-corn oil diet, +/*fa*-basal diet and +/*fa*-corn oil diet groups, respectively, with no significant variation among the groups. Body weight curves of each group are shown in Fig. 1. Values of the +/+-corn oil diet group were higher than those of the +/+-basal diet group from week 4 to the end of the experiment. In addition, the body weights of the +/*fa*-corn oil diet group were higher than those of the +/*fa*-basal diet group from week 8 to 20. The differences between +/+ and +/*fa* of both the basal and the corn oil diet groups were markedly smaller than those between the basal diet and corn oil diet groups in each genotype. Average food intake of the +/+-basal diet, +/+-corn oil diet, +/*fa*-basal diet and +/*fa*-corn oil diet groups were 11.8-14.2, 9.2-12.8, 11.4-14.3 and 9.7-12.6 g/rat/day, respectively, and those of the corn oil groups showed a tendency for decrease as compared to those of the basal diet groups in both genotypes.

Sequential changes in palpable mammary carcinomas. The minimum latency periods of palpable mammary carcinomas, which were histopathologically defined postmortem, were 8 weeks following DMBA administration in both the +/*fa*-basal and +/*fa*-corn oil diet groups, considerably shorter than the 11-12 weeks in the +/+-basal and +/+-corn oil diet groups (Fig. 2A). Incidence and multiplicity of palpable mammary carcinomas were increased or showed a tendency for increase in the early stages in +/*fa*-basal and +/*fa*-corn oil diet groups as compared to their +/+-counterparts, whereas their volume showed a tendency for increase in the corn oil diet groups of both +/+ and +/*fa* as compared to the basal diet groups (Fig. 2B and C).