Microsomal Epoxide Hydrolase Polymorphisms, Cigarette Smoking, and Risk of Colorectal Cancer: The Fukuoka Colorectal Cancer Study

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Microsomal epoxide hydrolase (EPHX1) plays an important role in the activation and detoxification of polycyclic aromatic hydrocarbons, carcinogens found in cigarette smoke. Polymorphisms in exon 3 (Y113H) and exon 4 (H139R) of the *EPHX1* have been associated with enzyme activity. We investigated the risk of colorectal cancer in relation to the *EPHX1* Y113H and H139R polymorphisms and assessed effect modifications of cigarette smoking and the other covariates. The interaction between the *EPHX1* polymorphisms and selected genetic polymorphisms was also examined. We used data from Fukuoka Colorectal Cancer Study, a community-based case–control study, including 685 cases and 778 controls. In-person interviews were conducted to assess lifestyle factors. The *EPHX1* Y113H and H139R polymorphisms were determined by the TaqMan assay and the polymerase chain reaction-restriction fragment length polymorphism, respectively. Neither of the two polymorphisms nor the imputed EPHX1 phenotype was associated with colorectal cancer risk. Cigarette smoking and alcohol intake showed no effect modification on the association with the *EPHX1* polymorphisms or the imputed EPHX1 phenotype. Increased risks of colorectal cancer associated with the 113Y allele and imputed EPHX1 phenotype were observed among individuals with high body mass index (BMI; $\geq$25.0 kg/m^2), but not among those with low BMI (<25.0 kg/m^2). The risk decreased with an increasing number of the 139R allele in the null genotypes of *GSTM1/GSTT1*. It is unlikely that the *EPHX1* polymorphisms play an important role in colorectal carcinogenesis. The observed interactions of the *EPHX1* polymorphisms with BMI and the *GSTM1/GSTT1* genotypes warrant further investigation.

Key words: microsomal epoxide hydrolase; polymorphism; cigarette smoking; colorectal cancer

INTRODUCTION

Colorectal cancer accounts for 10% of all cancers and is the third most common cancer in the world [1]. In Japan, the temporal trend showed a marked increase in the incidence of and mortality from colorectal cancer until 1990s [2], and the rates are currently among the highest in the world [1]. Risk for colorectal cancer is influenced by both environmental and genetic factors [3]. Several lifestyle factors such as physical inactivity, alcohol use, and high intake of red meat have been implicated in increased risk of colorectal cancer [4]. It has been a matter of controversy whether smoking is related to increased risk of colorectal cancer [5]. Smoking is consistently related to increased risk of colorectal adenomas [6], and a recent meta-analysis reported a small increase in the risk of colorectal cancer associated with long-term smoking although the findings are rather

Abbreviations: EPHX1, microsomal epoxide hydrolase 1; BMI, body mass index; GST, gluthathione *S*-transferase; OR, odds ratio; CI, confidence interval; CYP, cytochrome P-450; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

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disparate [7]. While descriptive features of lung and colorectal cancers are not supportive of a causal role for smoking in colorectal carcinogenesis [8], it is possible that smoking may confer increased risk of colorectal cancer in genetically susceptible individuals in terms of the metabolism of carcinogens in tobacco smoke [9].

Microsomal epoxide hydrolase (EPHX1) is an enzyme involved in the metabolism of reactive epoxides including polycyclic aromatic hydrocarbons, carcinogens found in cigarette smoke [10]. The EPHX1 converts benzo(a)pyrene 7,8 epoxide to the less reactive and more water-soluble dihydrodiol, benzo[a]pyrene 7,8 diol [10]. Although this reaction is generally considered as a detoxification reaction, the less reactive dihydrodiol can be further activated into a highly reactive benzo(a)pyrene 7,8 dihydrodiol 9,10 epoxide [11]. Two functional polymorphisms are known in the *EPHX1* gene; one is the Y113H in exon 3 (rs 1051740), and the other is the H139R in exon 4 (rs 2234922) [12]. In vitro, the *EPHX1* 113H allele is associated with a 40% decrease in enzyme activity, and the 139R allele has an approximately 25% higher activity [12]. Individuals homogyzous or heterozygous for the 113H were shown to have decreased risks of lung cancer [13–15] and upper aerodigestive cancer [16]. Furthermore, high-activity phenotype imputed from the combined genotypes of the Y113H and H139R was associated with increased risks for cancers of the lung [13] and upper aerodigestive tract [16] among those with a high exposure to cigarette smoking. These findings suggest that the *EPHX1* polymorphisms may play a role in the development of tobacco-related cancers. The 113H allele was associated with an increased risk of bladder cancer [17], however.

Several studies have addressed the association of the *EPHX1* polymorphisms with colorectal cancer [18–23] and adenomas [23–28], reporting inconsistent findings. Individuals with the 113HH genotype had an increased risk of colorectal cancer in the earliest study [18] but a decreased risk in the subsequent study [19]. The other studies showed no measurable association of Y113H, H139R, or the imputed phenotype activity with colorectal cancer risk [20–23]. On the other hand, high-activity phenotype was associated with an increased risk of colorectal adenomas among smokers [24,25], whereas individuals homozygous for the 113H allele and those with the composite genotype representing very slow activity showed an increased risk of colorectal adenomas when they had a high exposure to smoking [28]. In the present study, we examined the risk of colorectal cancer in relation to the *EPHX1* Y113H and H139R polymorphisms and assessed the interaction between these polymorphisms and cigarette smoking in the Fukuoka Colorectal Cancer Study, a community-based case–control study in Japan. We also explored the effect modifications of alcohol intake and body mass index (BMI) and the interactions between the *EPHX1* polymorphisms and other genetic polymorphisms of the enzymes involved in tobacco carcinogens.

MATERIALS AND METHODS

Methodological issues of the survey in the Fukuoka Colorectal Cancer Study have been described previously [29]. The study was approved by the ethics committee of the Kyushu University Faculty of Medical Sciences and the collaborating hospitals except two; in the two hospitals, ethics committee was not available at the time of the survey, and the survey was done with an approval of each hospital director.

Subjects

Both cases and controls were residents of Fukuoka city or three adjacent areas. Cases consisted of consecutive patients with histologically confirmed incident colorectal cancer who were admitted to the two university hospitals and six affiliated hospitals for surgical treatment during the period of September 2000 to December 2003. Eligible cases were those aged 20–74 yr at the time of diagnosis and lived in the study area. They also had to be mentally competent to complete the interview. Exclusion criteria were patients who had history of partial or total removal of the colorectum, familial adenomatous polyposis, or inflammatory bowel disease. Of the 1,053 eligible cases, a total of 840 (80%) participated in the interview and 685 gave an informed consent for genotyping.

Controls were frequency matched with cases on sex and 10-yr age class using the same inclusion criteria as for the cases except they did not have a prior diagnosis of colorectal cancer. Exclusion criteria were the same as those for the cases. A total of 1,500 subjects were selected by a two-stage random sampling using residential registry and were invited to participate in the study by mail. Among them, 1,382 were found to be eligible; 833 (60%) participated in the survey, and 778 gave an informed consent for genotyping.

Data Collection

Lifestyle factors were ascertained by in-person interview using a uniform questionnaire. Cases were interviewed in the respective hospitals while controls were interviewed in the public community centers or collaborating clinics. The index date was defined as the date of the onset of symptoms or screening leading to the diagnosis for the cases and the date of interview for controls. BMI (kg/m^2) 10 yr earlier, which was estimated by reported height and weight, was used because the current body mass index was unrelated to colorectal cancer risk [30].

Body weight 10 yr earlier was not available for 2 cases and 10 controls and was substituted with the current body weight. Years of smoking and numbers of cigarettes smoked per day were ascertained for each decade of age if the subjects had ever smoked cigarettes daily for 1 yr or longer. Cigarette-yr until the beginning of the previous decade of age was determined by multiplying the number of cigarettes smoked per day by the years of smoking, and classified into 0, 1–399, 400–799, and ≥800 cigarette-yr. Information on alcohol consumption, type of job and non-occupational physical activity at the time of 5 yr prior to the index date was ascertained. Non-occupational physical activity was expressed as a sum of metabolic equivalents (MET) multiplied by hours of weekly participation in each activity [30].

Genotyping

DNA was extracted from the buffy coat by using a commercial kit (Qiagen GmbH, Hilden, Germany). The following genotyping procedures used 1 μl template DNA with a concentration of 10 ng/μl. Genotyping of the *EPHX1* Y113H polymorphism was carried out by the TaqMan assay (assay ID C_14938_30; Applied Biosystems, Inc., Foster City, CA), using the Stratagene Mx3000P Real-Time QPCR system (Agilent Technologies, Inc., Santa Clara, CA). The *EPHX1* H139R polymorphism was determined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described elsewhere [31], using primers 5′-GGTGCCAGAGCCTGACCGTGC-3′ (sense) and 5′-ATGGAACCTCTAGCAGCCCCGTACC-3′ (anti-sense). The PCR product of 319 bp was digested with *Rsa*I, resulting in fragments of 297 and 22 bp for the 139H allele and fragments of 177, 122, and 22 bp for the 139R allele. The digestion products were separated on a 3% agarose gel (NuSieve, Lonza, Rockland, ME).

Statistical Methods

The EPHX1 activity phenotype was imputed on the basis of the number of putative high-activity alleles (113Y and 139R) in the combined genotype [13]. Associations of the *EPHX1* genotypes with colorectal cancer risk were examined in terms of odds ratio (OR) and 95% confidence interval (CI), which were obtained from logistic regression analysis. Statistical adjustment was made for 5-yr age class (starting with the lowest class of <50 yr), sex, residence area (Fukuoka City or the adjacent areas), and smoking (0, 1–399, 400–799, or ≥800 cigarettes-yr). The results did not change with additional adjustment for BMI 10 yr ago (<22.5, 22.5–24.9, 25.0–27.4, or ≥27.5 kg/m^2), alcohol intake (0, 0.1–0.9, 1.0–1.9, or ≥2.0 units/day), type of job (sedentary, moderate, or hard), non-occupational physical activity (0, 1–15.9, or ≥16 MET-h/wk), and parental history of colorectal cancer. Thus, we presented the ORs with adjustment for age, sex, residence area, and smoking.

Trend of the association was assessed with scores 0, 1, and 2 assigned to the three genotype categories. Effect modifications of smoking and the other covariates were tested by the Wald statistic for a product term of the ordinal variable for genotype and a dichotomous variable for smoking (<400 and ≥400 cigarette-yr) [32], alcohol intake (<2.0 and ≥2.0 unit) [33], and BMI (<25.0 and ≥25.0 kg/m^2) [30] with reference to the previous results. Previously, we reported the associations with *Cytochrome P450 (CYP) 1A1*, *Gluthathione S-transferase (GST) M1*, and *GSTT1* polymorphisms in relation to colorectal cancer risk in the same study subjects [34]. Since the EPHX1 is in the interplay with the CYP1A1 and GST in the metabolism of tobacco-related carcinogens [19], interactions between the *EPHX1* polymorphisms and these other polymorphisms (*CYP1A1*2A*, *CYP1A1*2C*, and the combination of the *GSTM1* and *GSTT1* genotypes) were also explored. The Hardy–Weinberg equilibrium was tested using Pearson's χ^2-test with 1 degree of freedom. A two-sided *P*-value <0.05 was considered as statistically significant. Statistical analyses were calculated using SAS version 9.2 (SAS Institute, Cary, NC).

RESULTS

Characteristics of cases and controls have been previously reported [33]. In brief, the mean age (SD) of the cases and controls were 60.2 (8.7) and 58.6 (10.7) yr, respectively ($P = 0.003$). Males numbered 426 (62%) in the case group and 490 (63%) in the control group. As compared with controls, cases were more likely to be heavy drinkers, had greater BMI 10 yr earlier, and had a higher frequency of family history of colorectal cancer. Cases and controls were not different with respect to residence area, smoking, type of job and non-occupational physical activity.

Genotype distribution of the controls was in Hardy–Weinberg equilibrium for both the *EPHX1* Y113H ($P = 0.35$) and H139R ($P = 0.41$). Frequencies of the *EPHX1* 113H allele were 0.42 in cases and 0.44 in controls, and frequencies of the *EPHX1* 139R allele were 0.16 in cases and 0.18 in controls. As compared with the *EPHX1* 113YY genotype, the *EPHX1* 113HH genotype was associated with a slightly decreased risk of colorectal cancer. The *EPHX1* 139R allele tended to be related to a decreased risk. These decreases in risk were far from the statistical significance, however. The imputed EPHX1 phenotype activity was unrelated to colorectal cancer (Table 1). Sex-specific analyses showed no difference in the association with the *EPHX1* Y113H, H139R polymorphisms, and the imputed EPHX1 phenotype activity between men and women ($P = 0.94$, 0.82, and 0.93, respectively). The associations did not differ in two age groups of <50 and ≥50 yr ($P = 0.29$ for Y113H, 0.70 for H139R, and 0.37 for the imputed phenotype). Furthermore, we

Table 1. The *EPHX1* Polymorphisms and Colorectal Cancer Risk

Genotype	*N* (%)		OR (95% CI)[a]	*P*-value
	Cases, $n = 685$	Controls, $n = 778$		
Y113H (exon 3)				
YY	228 (33.3)	239 (30.7)	1.00 (referent)	
YH	342 (49.9)	396 (50.9)	0.89 (0.70–1.12)	0.32
HH	115 (16.8)	143 (18.4)	0.81 (0.59–1.10)	0.18
P for trend			0.16	
H139R (exon 4)				
HH	485 (70.8)	525 (67.5)	1.00 (referent)	
HR	182 (26.6)	224 (28.8)	0.88 (0.70–1.11)	0.28
RR	18 (2.6)	29 (3.7)	0.70 (0.38–1.28)	0.25
P for trend			0.14	
Imputed phenotype[b]				
Low	367 (53.6)	414 (53.2)	1.00 (referent)	
Intermediate	223 (32.6)	246 (31.6)	1.06 (0.84–1.33)	0.64
High	95 (13.9)	118 (15.2)	0.92 (0.67–1.25)	0.59
P for trend			0.79	

[a]Adjusted for sex, age, residence area, and cigarette smoking.
[b]Imputed phenotype was based on the number of high-activity alleles (113Y and 139R): low, 0–1; intermediate, 2; and high, 3–4 [13].

repeated the analysis excluding 16 cases and 40 controls aged <40 yr because younger cases may have included more cases of familial colorectal cancer. The results were essentially the same as those described above; mean ages (SD) were 60.8 yr (8.4) in the cases and 60.1 yr (9.0) in the controls ($P = 0.12$).

The OR of colorectal cancer associated with the combined genotypes of the *EPHX1*, Y113H, and H139R are shown in Table 2. There were 56 samples with combinations of the *EPHX1* Y113H and H139R genotypes which were not captured by the imputed phenotype according to the Smith and Harrison's method [35]. Only one of those having the 113HH genotype was a variant homozygote of the 139RR genotype. As compared with the composite genotypes of 113HH and 139HH (very slow imputed phenotype activity) [35], the combined genotypes of the 113YY and 139HH (intermediate imputed phenotype activity) showed a statistically significant increase in the OR ($P = 0.04$). However, the interaction between the two polymorphisms was far from the statistical significance ($P = 0.23$; Table 2).

In the analysis stratified by smoking, neither of the polymorphisms nor the imputed EPHX1 phenotype was related to colorectal cancer risk in each stratum (Table 3). The results were the same when stratified into never-smoking and ever-smoking

Table 2. Adjusted ORs for the Combination of Genotypes of the *EPHX1* Y113H and H139R Polymorphisms

Y113H	H139R		
	HH	HR	RR
HH			
N[a]	94/123	20/20	1/0
OR (95% CI)[b]	1.00 (referent)	1.39 (0.70–2.75)	—
YH			
N[a]	253/271	84/115	5/10
OR (95% CI)[b]	1.26 (0.91–1.73)	0.99 (0.67–1.47)	0.71 (0.23–2.17)
YY			
N[a]	138/131	78/89	12/19
OR (95% CI)[b]	1.48 (1.02–2.13)[c]	1.17 (0.78–1.77)	0.88 (0.40–1.93)

[a]Number of cases/controls.
[b]Adjusted for sex, age, residence area, and cigarette smoking.
[c]$P = 0.04$.
Imputed phenotypes according to Smith and Harrison [35]: rapid, 113YY/139RR or 113YY/139HR; normal, 113YY/139HH or 113YH/139HR; slow, 113YH/139HH; and very slow, 113HH/139HH.

Table 3. Effect Modification of Cigarette Smoking on Colorectal Cancer Risk Associated With the *EPHX1* Polymorphisms

	Cigarette-yr					
	<400			≥400		
Genotype	*N*[a]	OR (95% CI)[b]	*P*-value	*N*[a]	OR (95% CI)[b]	*P*-value
Y113H (exon 3)						
YY	144/164	1.00 (referent)		84/75	1.40 (0.92–2.12)	0.11
YH	213/274	0.88 (0.66–1.18)	0.39	129/122	1.32 (0.91–1.91)	0.15
HH	59/89	0.77 (0.52–1.16)	0.21	56/54	1.24 (0.78–1.97)	0.36
P for trend		0.21			0.62	
P for interaction		0.67				
H139R (exon 4)						
HH	284/352	1.00 (referent)		201/173	1.55 (1.15–2.11)	0.005
HR	120/152	0.99 (0.74–1.32)	0.92	62/72	1.15 (0.77–1.73)	0.49
RR	12/23	0.62 (0.30–1.27)	0.19	6/6	1.38 (0.43–4.42)	0.59
P for trend		0.40			0.25	
P for interaction		0.56				
Imputed phenotype[c]						
Low (0–1)	209/278	1.00 (referent)		158/136	1.67 (1.19–2.33)	0.003
Intermediate (2)	146/166	1.18 (0.88–1.57)	0.27	77/80	1.40 (0.94–2.08)	0.10
High (3–4)	61/83	0.96 (0.66–1.41)	0.84	34/35	1.43 (0.84–2.43)	0.19
P for trend		0.83			0.46	
P for interaction		0.41				

[a]Number of cases/controls.
[b]Adjusted for sex, age, and residence area.
[c]Based on the number of 113Y and 139R alleles shown in parentheses.

groups (data not shown). Alcohol intake did not modify the associations with the *EPHX1* Y113H ($P = 0.36$), H139R ($P = 0.16$) or the imputed EPHX1 phenotype ($P = 0.06$). On the other hand, BMI showed statistically significant effect modifications on the associations with the Y113H and the imputed EPHX1 phenotype (Table 4). The 113Y allele (fast allele) and the imputed EPHX1 phenotype were positively associated with colorectal cancer risk among individuals with high BMI (≥25.0 kg/m^2), but not among those with low BMI (<25.0 kg/m^2).

As regards the gene-gene interactions, there was a statistically significant interaction between H139R and the *GSTM1/GSTT1* genotypes (Table 5). The OR decreased progressively with an increasing number of the 139R allele (fast allele) in the null genotype of both *GSTM1* and *GSTT1*. Neither the *CYP1A1*2A* nor the *CYP1A1*2C* showed measurable interactions with the *EPHX1* polymorphisms (data not shown).

DISCUSSION

In this study, neither of the *EPHX1* Y113H and H139R polymorphisms nor the combination of these polymorphisms predicting EPHX1 activity was associated with the risk of colorectal cancer. The present findings are consistent with the results from four [20–23] of the six previous studies [18–23] that showed no association between either of the *EPHX1* polymorphisms and colorectal cancer. While cigarette smoking and alcohol intake showed no effect modification on the association with the *EPHX1* genotype, there were statistically significant effect modifications of BMI and the *GSTM1/GSTT1* null genotypes.

Two British case–control studies reported contradictory findings on the association between the *EPHX1* Y113H polymorphism and colorectal cancer. The *EPHX1* 113HH genotype was associated with a 3.8-fold increase in the risk of colorectal cancer in a small case–control study [18], but was associated with a 32% decrease in the risk in another larger study [19]. However, genotype distribution of the *EPHX1* Y113H polymorphism differed substantially and was not in Hardy–Weinberg equilibrium in these two studies. The 113HH genotype accounted for 21.8% of the controls in the latter study [19] and 6.4% of the controls in the former [18]. Deviation from the Hardy–Weinberg equilibrium was highly significant in the latter ($P < 0.001$) and marginally significant in the former ($P = 0.04$). The PCR-RFLP method used in the two studies may have caused an error in genotyping the Y113H polymorphism due to the presence of a synonymous polymorphism (AAG to AAA) at codon 119 [36]. The reverse primer in these studies contained this polymorphism, and

Table 4. Effect Modification of Body Mass Index on Colorectal Cancer Risk Associated With the *EPHX1* Polymorphisms

	Body mass index (kg/m^2)					
	<25.0			≥25.0		
Genotype	*N*[a]	OR (95% CI)[b]	*P*-value	No.[a]	OR (95% CI)[b]	*P*-value
Y113H (exon 3)						
YY	158/197	1.00 (referent)		70/42	2.17 (1.39–3.38)	0.001
YH	239/290	1.02 (0.77–1.34)	0.91	103/106	1.21 (0.85–1.72)	0.28
HH	90/105	1.03 (0.72–1.47)	0.87	25/38	0.80 (0.46–1.40)	0.44
P for trend		0.79			0.001	
P for interaction		0.004				
H139R (exon 4)						
HH	348/395	1.00 (referent)		137/130	1.20 (0.90–1.60)	0.21
HR	129/175	0.83 (0.63–1.09)	0.18	53/49	1.28 (0.84–1.95)	0.25
RR	10/22	0.53 (0.24–1.15)	0.11	8/7	1.38 (0.49–3.89)	0.54
P for trend		0.04			0.51	
P for interaction		0.18				
Imputed phenotype[c]						
Low (0–1)	270/306	1.00 (referent)		97/108	1.01(0.73–1.40)	0.94
Intermediate (2)	151/187	0.94 (0.71–1.23)	0.65	72/59	1.46 (0.99–2.16)	0.06
High (3–4)	66/99	0.75 (0.52–1.07)	0.11	29/19	1.87 (1.01–3.43)	0.05
P for trend		0.11			0.02	
P for interaction		0.01				

[a]Number of cases/controls.
[b]Adjusted for sex, age, residence area, and cigarette smoking.
[c]Based on the number of 113Y and 139R alleles shown in parentheses.

Table 5. Combinations of the *EPHX1* and *GST* Polymorphisms and Colorectal Cancer Risk

	GSTM1 + *GSTT1*					
	Non-null			Null		
Genotype	*N*[a]	OR (95% CI)[b]	*P*-value	*N*[a]	OR (95% CI)[b]	*P*-value
Y113H (exon 3)						
YY	167/182	1.00 (referent)		61/57	1.12 (0.74–1.72)	0.59
YH	254/297	0.91 (0.69–1.19)	0.49	88/99	0.93 (0.65–1.33)	0.68
HH	81/110	0.76 (0.53–1.09)	0.13	34/33	1.07 (0.63–1.82)	0.79
P for trend		0.13			0.73	
P for interaction		0.64				
H139R (exon 4)						
HH	357/416	1.00 (referent)		128/109	1.33 (0.99–1.79)	0.06
HR	131/161	0.95 (0.72–1.25)	0.72	51/63	0.92 (0.62–1.38)	0.69
RR	14/12	1.35 (0.61–2.97)	0.46	4/17	0.29 (0.10–0.89)	0.03
P for trend		0.86			0.003	
P for interaction		0.01				
Imputed phenotype[c]						
Low (0–1)	270/323	1.00 (referent)		97/91	1.26 (0.90–1.76)	0.17
Intermediate (2)	162/188	1.07 (0.82–1.40)	0.62	61/58	1.27 (0.85–1.89)	0.25
High (3–4)	70/78	1.09 (0.76–1.57)	0.64	25/40	0.74 (0.43–1.26)	0.27
P for trend		0.51			0.13	
P for interaction		0.11				

[a]Number of cases/controls.
[b]Adjusted for sex, age, residence area, and cigarette smoking.
[c]Based on the number of 113Y and 139R alleles shown in parentheses.

the heterozygous 113YH genotype may have been misclassified as the homozygous 113HH genotype due to failure of the 113Y allele to be amplified. The present study was based on the validated Taqman assay as used in the previous four studies [20–23] that showed no association between the Y113H polymorphism and colorectal cancer risk. Genotype distribution was in Hardy–Weinberg equilibrium in the four studies [20–23] and in the present study.

A somewhat decreased risk of colorectal cancer among those with the 139RR genotype in the present study is consistent with the finding in the Physicians' Health Study, in which the OR for 139HR and 139RR compared with 139HH were 0.68 (95% CI 0.47–0.97) and 0.94 (95% CI 0.38–2.32), respectively, the OR for 139HR and 139RR combined being 0.70 (75% CI 0.49–0.99). However, no such association was replicated in the Nurses' Health Study [20] and the other three [18,19,21].

It was found in the present study that 4% of the whole sample was not included in any of the four class categories of the imputed EPHX1 phenotype as classified by the Smith and Harrison's method [35]. Likewise, 5% sample had been unclassified in a study in Germany [37]. Ulrich et al. [28] also noted that several genotype combinations were not captured by this method.

Two studies addressed the interaction between the *EPHX1* polymorphisms and smoking on colorectal cancer risk to find no effect modification of smoking on the association with the *EPHX1* polymorphisms singly or in combination in the United States [20,22]. The present study also found no interaction between smoking and the *EPHX1* polymorphisms in a Japanese population. Previously, none has addressed the effect modifications of alcohol intake and BMI on colorectal cancer risk associated with the *EPHX1* polymorphisms. The present study showed that the associations of the *EPHX1* polymorphisms with colorectal cancer differed by BMI. High BMI is known to be related with increased risk of colorectal cancer [38], and was the case in the present study population [30]. The present findings are very intriguing, but we could not prepare a prompt explanation for an elevated risk associated with the 113Y allele (fast allele) among those with high BMI. Another interesting finding was a decreased risk associated with the 139R allele in individuals with the *GSTM1/GSTT1* null genotypes. The null genotypes of the *GSTM1* and *GSTT1* genes are associated with the loss of enzyme activity [39]. Given the dual role of the EPHX1 enzyme on the activation and detoxification of carcinogenic hydrocarbons [10], it is possible that the 139R allele (fast allele) is protective in the absence of the GSTM1/GSTT1 enzyme activity.

The use of community controls and the large number of subjects were strengths of the present study. The other strength was that the study subjects consisted of an ethnically homogenous population of Japanese, and concern over population stratification was negligible. There were several weaknesses to be discussed. The participation rate in terms of genotyping was rather low (65% in cases and 56% in controls). However, it is unlikely that participation had been affected by genetic polymorphisms under study. As reported previously [40], older persons (particularly among the controls) and women were less likely to give consent for genotyping, but there was no difference between those who gave consent and those who did not in terms of smoking, residence area, and alcohol intake.

In conclusion, neither of the *EPHX1* polymorphisms nor the imputed phenotype activity was associated with colorectal cancer risk. Cigarette smoking did not modify the associations with the *EPHX1* polymorphisms, nor did alcohol intake. The observed interactions with BMI and the *GSTM1/GSTT1* null genotypes remain to be consolidated in further studies.

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ORIGINAL ARTICLE

Differences in the expression of epithelial–mesenchymal transition related molecules between primary tumors and pulmonary metastatic tumors in colorectal cancer

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Abstract
Purpose Epithelial–mesenchymal transition (EMT) is a key event in cancer metastasis. This study immunohistochemically examined the expression of EMT-related molecules in both primary colorectal cancer and pulmonary metastases, and analyzed the expression pattern.
Methods Ten patients with colorectal cancer that underwent surgical resections for both the primary tumor and metastatic pulmonary tumors were included. The expression status of EMT-related molecules was examined using immunohistochemical staining.
Results Nine of the 10 cases maintained the expression of both E-cadherin and β-catenin in the primary site. The expression of E-cadherin and β-catenin in the pulmonary metastatic site was preserved in 10 and 12 out of 15 metastatic lesions, respectively. The EMT-related transcription factor, Twist, was positively expressed in all 10 cases, Smad interacting protein 1 (Sip1) in 9, Snail in 4 and Slug in 3 of the primary sites. On the other hand, staining for Twist, Sip1 and Snail at the metastatic pulmonary site, was negative in all 10 cases.
Conclusion The expression of EMT-related transcription factors in metastatic pulmonary tumors from colorectal cancer decreased in comparison to the primary tumors. These findings suggested that the expression status of EMT-related transcription factors might play an important role in the implantation of metastatic foci.

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Keywords EMT · Pulmonary metastasis · Colorectal cancer · Snail · Slug

Introduction

Metastasis is frequently a fatal step in the progression of solid malignancies. Metastatic progression is a complex, multistep process that entails local invasion, followed by the dissemination of the malignant cells, and finally re-establishment at distant sites and thus resulting in the formation of metastasis.

Epithelial–mesenchymal transition (EMT) is one of the major molecular mechanisms that induces tumor invasion and metastasis [1]. EMT allows cancer cells to become motile and to invade the stroma surrounding the initial neoplastic focus, thereby facilitating intravasation of tumor cells into the blood or lymphatic vessels, and thus leading to dissemination to distant sites [2]. There have been several reports on the role of EMT in the progression of cancer, and in the acquisition of either invasive characteristics or a poor prognosis in several types of cancer [1, 2].

One of the hallmarks of EMT is the functional loss of E-cadherin. E-cadherin is a molecule that mediates homophilic cell–cell adhesion and tissue homeostasis in the normal epithelia [3], and has been shown to suppress invasion in many tumor cell types [4, 5]. The extracellular domain of E-cadherin interacts with the E-cadherin expressed on adjacent cells, and the intracellular domain binds directly to β-catenin. β-Catenin forms a multiprotein complex and binds to the cytoskeleton. This cadherin/catenin/cytoskeleton complex maintains cell–cell adhesion, cell shape and polarity, and regulates cell migration [2]. The major proteins implicated in the transcriptional repression of E-cadherin include the zinc finger proteins

Snail and Slug, Smad interacting protein 1 (SIP1), and a basic helix-loop-helix (bHLH) protein called Twist [6–8]. These proteins repress E-cadherin expression through binding E-boxes in the human E-cadherin promoter [9]. The reduction of E-cadherin expression caused by these transcriptional factors consequently induces the tumor cells to increase their motility and invasiveness [1, 2].

The disseminated tumor cells must proliferate at the distant metastatic site to colonize the host tissue and to expand the neoplastic mass. Dissociated single carcinoma cells have either a reduced expression or no expression of E-cadherin, but they regain a normal membranous E-cadherin expression upon reaching the peritumoral stroma or the distant metastatic sites, and then aggregate newly in new solid islands. This probably provides an advantage for tumor growth in new tissue microenvironments. This reverse transition, called the mesenchymal to epithelial transition (MET), also plays an important role in cancer metastasis [2, 10].

Colorectal cancer is one of the most common solid tumors in the world, and the lung is the most common extra-abdominal sites of distant metastases from colorectal cancer. A metastasectomy of the lung is reported to improve the prognosis of colorectal cancer [11]. Mohri reported that the reduced expression of E-cadherin in colorectal cancer tissue is significantly associated with an increased incidence of tumor recurrence after apparently curative resection, and that it is a significant prognostic factor independent of other clinicopathological features [12]. Furthermore, the expression of various EMT-related transcriptional factors such as Snail and Slug is implicated in the progression of colorectal cancer [13, 14]. These EMT-related molecules may thus play an important role in the progression of metastatic colorectal cancer.

The present study immunohistochemically examined the expression status of EMT-related molecules in both primary colorectal cancer and the pulmonary metastatic colorectal cancer, and analyzed the expression patterns of these molecules and their influence on the postoperative prognosis after pulmonary metastasectomy.

Materials and methods

Patients

The present study included 10 patients with colorectal cancer that underwent surgical resections for both the primary tumor and metastatic pulmonary tumors at Kyushu University Hospital. The 10 patients underwent a pulmonary metastasectomy between 1998 and 2008 according to the generally accepted criteria [15]. They included 8 males and 2 females ranging from 50 to 70 years of age. One patient underwent surgical resection of metastatic pulmonary tumors three times, and three patients did so twice. These patients' characteristics are shown in Table 1. Written informed consent for the comprehensive use of the pathological materials was obtained from all the patients.

Reagents and antibodies

This study used antibodies against Twist (H-81; Santa Cruz Biochemistry Inc., Santa Cruz, CA, USA), Snail (E-18; Santa Cruz Biochemistry Inc.), Sip1 (L-20; Santa Cruz Biotechnology Inc.), Slug (G-18; Santa Cruz Biotechnology, Inc.), E-cadherin (NCH-38, HECD-1; TaKaRa, Kyoto, Japan), N-cadherin (M3613, clone 6G111; Dako, Kyoto, Japan), vimentin (M0725, cloneV9; Dako) and β-catenin (610154; BD Biosciences, Franklin Lakes, NJ, USA). The Envision system® (Dako) was applied for Twist, E-cadherin, N-cadherin, vimentin and β-catenin, while the simple stain peroxidase method (Nichirei Biosciences Inc., Tokyo, Japan) and the avidin–biotin–peroxidase complex technique (Nichirei Biosciences Inc.) were applied for Snail and Sip1, and Histofine Simple Stain MAX-PO(G)® (Nichirei Co. Ltd.) was applied for Slug.

Immunohistochemistry

The resected specimens were fixed in 10 % formalin and embedded in paraffin and 3-μm-thick sections were prepared. The sections were stained for Twist (1:100), Snail (1:100), Sip1 (1:50), Slug (1:100), E-cadherin (1:500), N-cadherin (1:25), vimentin (1:25) and β-catenin (1:200). The deparaffinized sections were incubated in 0.03 % (v/v) hydrogen peroxide in ethanol for 30 min at room temperature to quench endogenous peroxidase activity and then were incubated with the primary antibody. The secondary antibodies as supplied were applied and the chromogen was developed with the immersion of the slides in a diaminobenzidine–H_2O_2 substrate. The slides were counterstained in Mayer's hematoxylin, dehydrated and mounted. Nonimmune serum was used at the same dilution instead of the primary antibodies in every run, as a negative control. Positive controls were also stained for each staining batch.

The expression was independently evaluated by two of the authors (H.K and T.Y.) using a blind protocol design (the observers had no information on the patients' clinical outcomes or any other clinicopathological data). E-cadherin and β-catenin were observed at the intercellular junctions and with partly reduced expression in the cytoplasm of some of the tumor cells and N-cadherin was observed in the cell membrane. Vimentin was localized in the stromal structure of the normal or neoplastic tissues as well as the cytoplasm of the neoplastic tissues. Twist and Sip1 expression was detected in the cytoplasm and/or nuclei of the cancer cells,

Table 1 The patients' characteristics

Case	Age	Sex	Pathological staging	Degree of differentiation	Period from first operation to pulmonary metastectomy (months)
Case 1	63	M	IIIa	Moderately	26
Case 2	69	M	IV	Moderately	12
Case 3	50	M	IIIb	Moderately	24
Case 4	70	M	II	Moderately	32
Case 5	61	F	IIIb	Moderately	33
Case 6	61	M	IV	Moderately	88[a]
					88[a]
Case 7	61	M	I	Well	32
					51
Case 8	70	M	II	Moderately	59
Case 9	59	M	IIIb	Moderately	19
Case 10	61	F	IIIb	Moderately	18[b]
					18[b]
					18[b]

[a] This case was simultaneously detected to have two metastatic pulmonary tumors on the ipsilateral side of the lung

[b] This case was simultaneously detected to have two metastatic pulmonary tumors on the ipsilateral side of the lung and one metastatic tumor in the contralateral lung

whereas Snail expression was detected only in the cytoplasm. The Slug expression was detected in the cytoplasm and/or perinuclear regions of the cancer cells.

The staining intensity of Twist and Sip1 was scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong) and the extent of staining was scored as 0 (0 %), 1 (1–25 %), 2 (26–50 %), 3 (51–75 %) or 4 (76–100 %) according to the percentage of the positive staining areas in relation to the total tumor area. The sum of the intensity and extent scores was used as the final staining score (0–7) as previously reported [16]. Tumors having a final staining score of 4 or higher were considered to have a positive expression. Specimens that exhibited staining of Snail, Slug, N-cadherin and vimentin in >10 % of the tumor cells were classified as positive, and the others were considered negative, as previously indicated [17, 18]. Paraffin-embedded tissues from normal liver (Twist, Sip1, Snail, N-cadherin and vimentin) or lung (Slug) of the homogenous immunophenotype were included as positive controls. Three categories were established for evaluation of E-cadherin and β-catenin expression, based on the percentage of cancer cells that maintained membranous staining: <5 % positively stained cells were classified as 'negative', 5–50 % were classified as 'reduced' and >50 % stained cells as 'preserved', as previously indicated [19]. Paraffin-embedded tissues from the normal colon epithelium of the homogenous immunophenotype were included as positive controls for these antigens.

Statistical analysis

Comparisons among the various EMT-related molecules were performed using the Chi-square test. Each patient's survival time was calculated beginning from the date of the pulmonary metastasectomy until the date of death or the date of the last follow-up, for the analyses of the overall survival. The univariate survival analyses were estimated by the Kaplan–Meier method, and the differences among the groups were analyzed by the log-rank test. All the statistical analyses were performed using the Stat View version 5.0 statistical software package (SAS Institute Inc, Cary, NC, USA). A two-sided p value <0.05 was considered to be statistically significant.

Results

Expression of EMT-related molecules in primary and pulmonary metastatic tumors

Representative immunostaining patterns of various EMT-related molecules in both the primary site and the pulmonary metastatic site are shown in Figs. 1 and 2, respectively. The immunostaining results are presented case by case in Table 2 and are summarized in Table 3. Nine of the ten cases preserved both E-cadherin and β-catenin in the primary site. The expression of E-cadherin and β-catenin in the pulmonary metastatic site was preserved in 10 and 12 out of the 15 metastatic lesions, respectively. Neither N-cadherin nor vimentin were positively expressed in the cancer cells in any specimens from either the primary or metastatic sites.

Twist was expressed at the primary site in all 10 cases, Sip1 in 9, Snail in 4 and Slug in 3 cases. On the other hand, Twist, Sip1 and Snail were negative in all the metastatic pulmonary tumor specimens from the 10 cases. Slug was

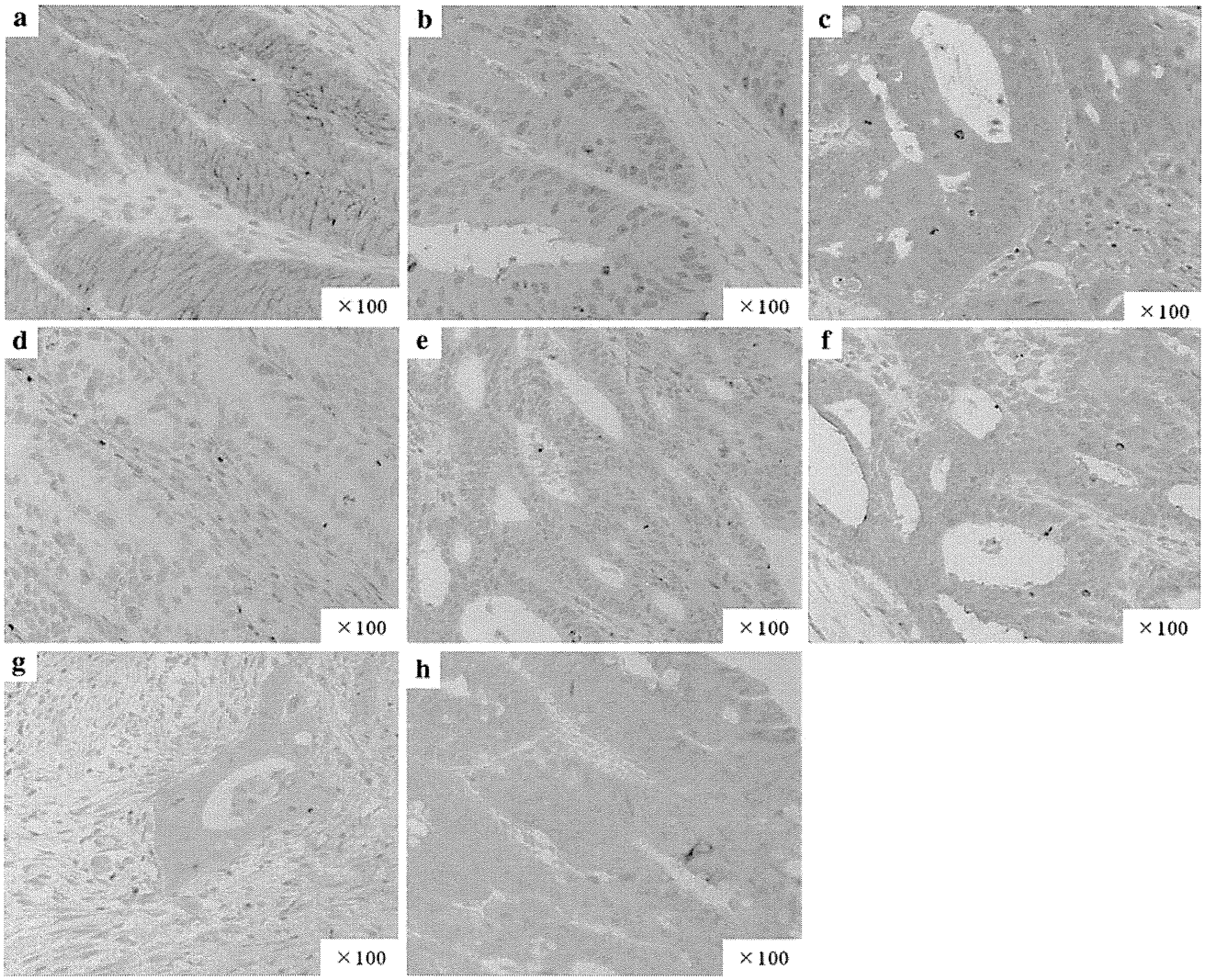

Fig. 1 Immunohistochemical staining of E-cadherin (**a**), β-catenin (**b**), N-cadherin (**c**), vimentin (**d**), Twist (**e**), Sip1 (**f**), Snail (**g**) and Slug (**h**) in primary colorectal cancer specimens

only positive in 6 of 15 specimens, all of which were from the 3 (cases 6, 7 and 10) cases where the primary tumor expressed Slug (Table 2). In addition, no relationship was observed between the expression pattern of E-cadherin and its repressor molecules.

Relationship between the expression status of E-cadherin and its repressor molecules

The relationship between E-cadherin expression and the expression of its repressor molecules in the pulmonary metastatic tumors was analyzed. Twist, Sip1 and Snail were negative in all the pulmonary metastatic tumors of the 10 cases. Only Slug was expressed in 7 pulmonary metastatic tumors from 3 cases (Table 2), and the expression was significantly associated with a reduction in the E-cadherin expression ($p = 0.003$).

Influence of Snail and Slug expression on survival after pulmonary metastasectomy

Both Twist and Sip1 were positive in almost all the primary sites, while the expression of Snail and Slug was heterogeneous among those cases. The prognostic significance of the expression of Snail and Slug in the primary site was analyzed. The overall survival after pulmonary metastasectomy in Snail-positive cases was significantly worse than that of Snail-negative cases (Fig. 3). No survival difference was associated with the expression of Slug ($p = 0.60$).

Discussion

The present study first examined the expression levels of EMT-related molecules in both primary site tumors and

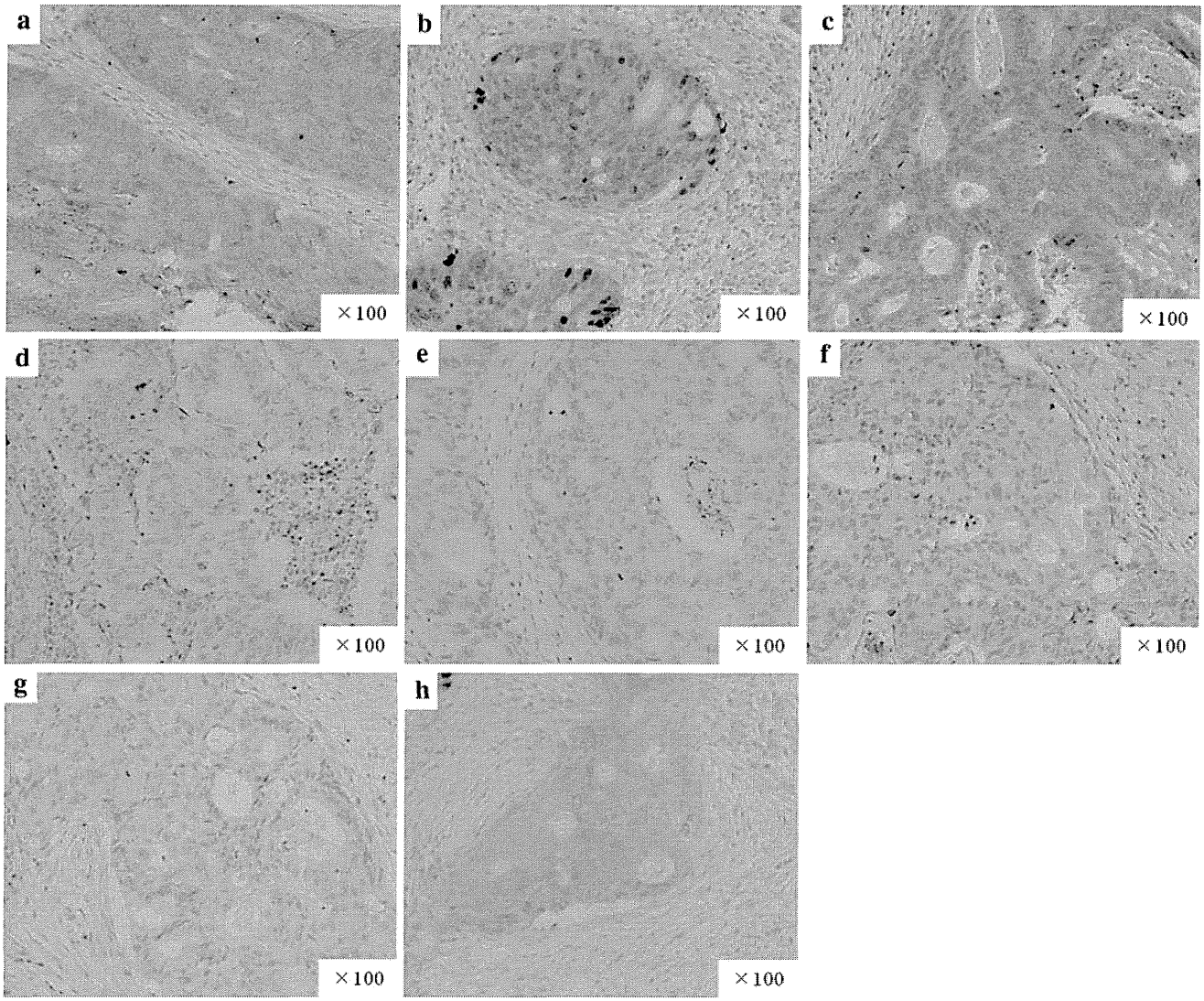

Fig. 2 Immunohistochemical staining of E-cadherin (**a**), β-catenin (**b**), N-cadherin (**c**), vimentin (**d**), Twist (**e**), Sip1 (**f**), Snail (**g**) and Slug (**h**) in metastatic pulmonary tumor specimens

pulmonary metastatic tumors from primary colorectal cancer. The metastatic pulmonary tumors comparatively preserved the expression of both E-cadherin and β-catenin in the primary site, while the expression levels of various EMT-related transcription factors were significantly different from the primary site tumor. The expression of Twist, Sip1 and Snail was frequently observed in primary site tumor cells, while they were infrequently observed in the metastatic site tumor cells. These results support the hypothesis that metastasized tumor cells might lose the mesenchymal signature derived from the EMT once they are implanted in distant organs. Therefore, tumor cells downregulate E-cadherin repressors, such as Twist, Sip1, Snail once they have arrived at distant organs, to promote the reacquisition of epithelial characteristics and allow the cells to proliferate.

There is speculation that the expression status of E-cadherin, a key molecule involved in the EMT, is preserved or overexpressed in metastatic foci, while the E-cadherin expression is reduced in the primary tumor. The metastatic foci may re-express E-cadherin to promote cell-to-cell adhesion, to establish new colonies, and the expression of its repressors may be decreased. There have been only a few studies that have so far addressed this subject. Kowalski et al. [20] reported that distant metastases express E-cadherin more strongly than the primary tumor in breast cancer. Ikeguchi et al. [21] found an increased expression of adhesion molecules, such as E-cadherin, in metastatic lymph nodes or liver tumors in comparison with the primary tumor in about half of patients with colorectal cancer that they examined. The present study also compared the expression status of

Table 2 The immunohistochemical scores of EMT-related molecules

Case	Site	% of cancer cells that preserved staining for E-cadherin	% of cancer cells that preserved staining for β-catenin	Twist	Sip1	Snail	Slug
Case 1	Colon	100 (preserved)	59 (preserved)	6	6	−	−
	Lung	100 (preserved)	80 (preserved)	0	2	−	−
	Lung	82 (preserved)	68 (preserved)	3	0	−	−
Case 2	Colon	65 (preserved)	75 (preserved)	5	7	+	−
	Lung	73 (preserved)	57 (preserved)	0	0	−	−
Case 3	Colon	60 (preserved)	93 (preserved)	7	6	−	−
	Lung	77 (preserved)	73 (preserved)	2	0	−	−
Case 4	Colon	100 (preserved)	64 (preserved)	4	5	−	−
	Lung	100 (preserved)	90 (preserved)	0	0	−	−
Case 5	Colon	90 (preserved)	84 (preserved)	5	6	−	−
	Lung	78 (preserved)	74 (preserved)	0	0	−	−
Case 6	Colon	82 (preserved)	78 (preserved)	5	6	−	+
	Lung	21 (reduced)	65 (preserved)	3	0	−	+
	Lung	33 (reduced)	57 (preserved)	0	0	−	+
Case 7	Colon	100 (preserved)	88 (preserved)	5	2	−	+
	Lung	19 (reduced)	12 (reduced)	0	0	−	+
	Lung	70 (preserved)	63 (preserved)	0	0	−	+
Case 8	Colon	82 (preserved)	95 (preserved)	7	5	+	−
	Lung	66 (preserved)	68 (preserved)	3	0	−	−
Case 9	Colon	91 (preserved)	90 (preserved)	6	4	+	−
	Lung	64 (preserved)	44 (reduced)	0	0	−	−
Case 10	Colon	46 (reduced)	100 (preserved)	6	4	+	+
	Lung	38 (reduced)	58 (preserved)	0	0	−	+
	Lung	12 (reduced)	22 (reduced)	0	0	−	+
	Lung	75 (preserved)	77 (preserved)	0	2	−	+

Table 3 The relationship between the expression of EMT-related molecules in the primary tumors and pulmonary metastatic tumors

	E-cadherin		β-catenin		N-cadherin		Vimentin	
	Preserved	Reduced	Preserved	Reduced	Positive	Negative	Positive	Negative
Colon	9	1	10	0	0	10	0	10
Lung	10	5	12	3	0	15	0	15
	Twist		Sip1		Snail		Slug	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Colon	10	0	9	1	4	6	3	7
Lung	0	15	0	15	0	15	6	9

E-cadherin between the metastatic pulmonary tumor and the primary colorectal cancer, but recognized no significant differences between them. Slug was the only EMT-related transcription factor examined that was expressed at the both primary site and the pulmonary metastatic site, and this expression was associated with a reduction of E-cadherin at the metastatic site. Some cases showed that E-cadherin expression was preserved even when Slug was expressed. These results indicate that positive expression of EMT-related molecules does not necessarily mean the loss of E-cadherin by individual cancer cells. Furthermore, the expression patterns of these molecules are heterogeneous in the same tumor, and there is no relationship between the expression patterns of E-cadherin and its repressor molecules.

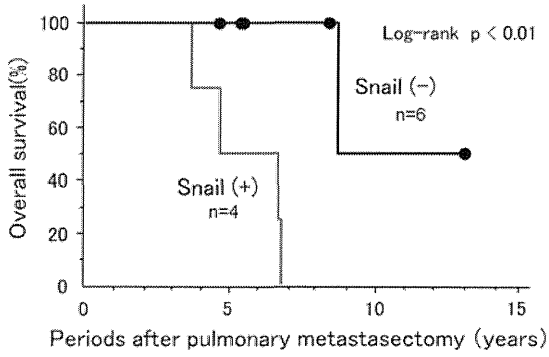

Fig. 3 Overall survival after pulmonary metastasectomy according to the expression status of Snail at the primary site

EMT-derived migratory cancer cells typically establish secondary colonies at distant sites that appear to be histologically similar to the primary tumor from which they arose. Therefore, it is thought that they no longer exhibit the mesenchymal phenotypes associated with metastasizing carcinoma cells. Reconciling this behavior with the proposed role of the EMT as a facilitator of metastatic dissemination requires the additional concept that metastasizing cancer cells must shed their mesenchymal phenotype via a MET during the course of secondary tumor formation [22]. The tendency of disseminated cancer cells to undergo MET likely reflects the local microenvironment that they encounter after extravasation into the parenchyma of a distant organ, quite possibly the absence of the heterotypic signals they experienced in the primary tumor that were responsible for inducing EMT in the first place [1, 23, 24]. These considerations indicate that the induction of EMT is likely to be an important mechanism central to the progression of carcinomas to a metastatic stage, and implicates MET during the subsequent colonization process.

Slug was the only EMT-related transcription factor examined that was associated with the reduction of E-cadherin in the pulmonary metastatic tumors. On the other hand, the prognosis after pulmonary metastasectomy was not associated with the expression of Slug, but was associated with the expression of Snail in the primary tumors. Roy et al. [13] reported that Snail is overexpressed in colorectal cancer tissue, and there is a trend for colorectal cancer with metastatic ability to overexpress Snail more frequently. Shioiri et al. [14] reported the positive expression of Slug to be significantly associated with the Dukes stage and distant metastasis, and that Slug expression is an independent prognostic factor. The roles of both Snail and Slug in the tumor progression and the metastasis of primary colorectal cancer is considered to be heterogeneous among patients, and many of the steps in the metastatic process still require further investigation to determine their full implications.

In conclusion, metastatic tumor cells might be able to establish secondary colonies and gain a proliferative ability through their decreased expression of EMT-related transcription factors and the subsequent reacquisition of epithelial characteristics.

Conflict of interest None.

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Estrogen receptor-β gene polymorphism and colorectal cancer risk: Effect modified by body mass index and isoflavone intake

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Estrogen receptor (ER)-β signaling has generally been implicated in protection against colorectal cancer. The ER-β gene cytosine-adenine (*ESR2* CA) repeat polymorphism was reported to be associated with colorectal cancer, although showing contradicting results probably caused by ethnicity or age distribution of the subjects. We investigated the association between this polymorphism and the colorectal cancer risk in a community-based case-control study in Japan (685 cases/778 controls), including only subjects younger than 75. The effect modifications of the body mass index (BMI) and isoflavone intake were also examined. *ESR2* CA repeat polymorphism was determined by polymerase chain reaction using fluorescein-labeled primers. CA repeat alleles were classified into short (S) allele (<22 repeats) and long (L) allele (≥22 repeats). Subjects were divided into three genotype groups (SS/SL/LL). The risk of colon cancer, but not of rectal cancer, was increased with an increasing number of L alleles among postmenopausal women; age-adjusted odds ratio (OR) for SL and LL genotypes compared with the SS genotype were 1.78 and 2.91, respectively (trend $p = 0.002$). Increased risks of colon cancer associated with the L allele were more evident among postmenopausal women with low BMI (<25 kg m^{-2}) or with high isoflavone intake. Such associations were not observed among men or premenopausal women. Having longer *ESR2* CA repeat increases colon cancer risk among postmenopausal women younger than 75, possibly with modification of BMI and isoflavone intake. Aging and estrogenic condition may be important in the colon cancer pathogenesis associated with *ESR2* CA repeat polymorphism.

Epidemiology

Key words: estrogen receptor-β CA repeat polymorphism, colorectal cancer, body mass index, isoflavone, age
Abbreviations: BMI: body mass index; CA: cytosine-adenine; CI: confidence interval; DNA: deoxyribonucleic acid; ER: estrogen receptor; HRT: hormone replacement therapy; IGF: insulin-like growth factor; MET: metabolic equivalents; MSI: microsatellite instability; OR: odds ratio; PCR: polymerase chain reaction; SHBG: sex hormone binding globulin
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What's new?

Long and short cytosine-adenine (CA) repeat polymorphisms in the estrogen receptor-beta gene (*ESR2*) have been associated with increased colon cancer risk in postmenopausal women of different ages and ethnicities. In the present study, long *ESR2* CA repeats were found to increase risk in postmenopausal Japanese women under age 75. Low BMI and high isoflavone intake augmented risk, indicating that aging as well as estrogen modification may influence *ESR2* CA repeat polymorphism colon cancer pathogenesis.

A large number of *in vitro* and *in vivo* studies have suggested that estrogen may confer a decreased risk of colorectal cancer.[1,2] Estrogens suppress the proliferation of colorectal cancer cells,[1] and hormone replacement therapy (HRT) has consistently been related to a decreased risk of colorectal cancer[3,4]; however, the evidence is not necessarily consistent. Endogenous estrogen levels have been shown to be positively associated with colorectal cancer risk among postmenopausal women,[5,6] and it was recently reported that reproductive history putatively leading to greater exposure to endogeneous estrogens was related to an increased risk of colorectal cancer in postmenopausal women.[7] Moreover, estrogen concentrations were higher in colon cancerous tissues than in nonneoplastic tissues in colon cancer patients, and their prognosis was poorer when intratumoral estrogen concentrations were higher.[8]

Colorectal epithelial cells frequently express a second estrogen receptor, ER-β, but not the classic receptor, ER-α, and ER-β signaling has generally been implicated as protective in colorectal carcinogenesis.[1,2] Dinucleotide (cytosine-adenine, CA) repeat polymorphism at intron 5 of the ER-β gene (*ESR2*), which was first reported for a Japanese population,[9] has been reported to be associated with bone mineral density,[10–12] menopausal symptoms[13,14] and Alzheimer's disease.[15] In a previous study on an autopsy series of elderly Japanese (mean age, men, 79; women, 82), colon cancers occurred more frequently among women having a shorter *ESR2* CA repeat, although this was not true for men.[16] In contrast, Slattery *et al.* reported an increased risk of colon cancer among women with long CA repeats in a community-based case-control study in the United States (mostly composed of Caucasians).[17] Among the differences in the study settings between the two studies, ethnic difference (Caucasians versus Japanese) and difference of age distribution seemed to be most essential. To elucidate which influenced the discrepancy, we examined the association between the *ESR2* CA repeat polymorphism and the risk of colorectal cancer in a community-based case-control study in Japan, which was composed of subjects younger than 75. We also addressed effect modifications of the body mass index (BMI) and isoflavone intake. High BMI is known to be associated with elevated production of nonovarian estrogens,[18] and isoflavones have an estrogenic property, showing higher binding affinity for ER-β than ER-α.[2]

Material and Methods

The Fukuoka Colorectal Cancer Study was a case-control study of Japanese residents living in Fukuoka City and three adjacent areas. The study protocol was approved by the ethics committees of the Kyushu University Faculty of Medical Sciences and the participating hospitals. Informed consent was obtained from each subject. Details of the methods have been reported previously.[19]

Subjects

The cases were a consecutive series of patients with histologically confirmed incident colorectal adenocarcinomas who were admitted to two university hospitals or six affiliated hospitals for surgical treatment from September 2000 to December 2003. Eligible cases were aged 20–74 years at the time of diagnosis; lived in the study area; had no prior history of partial or total removal of the colorectum, familial adenomatous polyposis or inflammatory bowel disease; and were mentally competent to give informed consent and to complete the interview. Of the 1,053 eligible cases, 840 (80%) participated in the interview, and 685 (65%) gave informed consent for genotyping. Colon and rectal cancer cases numbered 384 and 290, respectively; 11 cases had cancers at both sites.

Eligibility criteria for controls were the same as those of the cases except for having no diagnosis of colorectal cancer at the time of selection. A total of 1,500 control candidates were selected by two-stage random sampling using residential registry, and were invited to participate in the study by mail. Among them, 1,382 were found to be eligible. Of these, 833 persons (60%) participated in the survey and 778 (56%) gave informed consent for genotyping.

In the analysis of the interaction between genotype and isoflavone intake, we excluded those who were in the top 1% or bottom 1% of total energy intake within each stratum of sex and age (<55, 55–64 and ≥65 years) in all subjects.

Interview

Research nurses interviewed cases and controls in person regarding dietary and nondietary lifestyle factors.[19,20] The index dates were the date of the onset of symptoms or screening for cases, and the time of interview for controls. Anthropometric questions inquired about height (cm) and weight (kg), current and 10 years earlier. BMI, weight per height^2 (kg m^{-2}), 10 years earlier was used in the analysis because current BMI was unrelated to risk.[21]

The dietary interview ascertained consumption frequencies and portion sizes of 148 food/dish items on average over the past year by using a computer-assisted interview.[20] Isoflavone

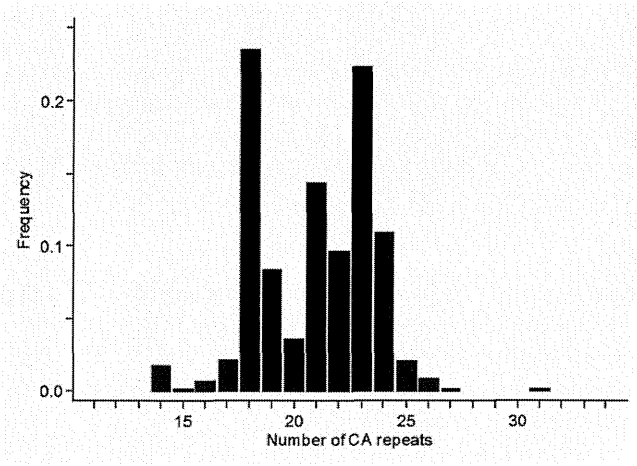

Figure 1. Distribution pattern of the number of CA repeats of *ESR2* in controls. Two major peaks are observed at 18 and 23.

intake was estimated from the average daily intake of nine soy food items, as described in detail previously.[22] Energy-adjusted intake of isoflavone estimated by the dietary interview was found to be fairly valid in comparison with the intake based on the 28-day diet record over 1 year among 28 control subjects.[22]

Other lifestyle factors considered as potential confounders were habitual alcohol consumption 5 years prior to the index date, cumulative exposure to cigarette smoking until the beginning of the previous decade of age, type of job (sedentary or non-sedentary), and leisure-time physical activity as expressed by metabolic equivalents (MET)-hours per week. Women who had been menopausal for 3 years or longer were classified as postmenopausal. Those with a history of hysterectomy were regarded as postmenopausal if they were aged 55 years or older.

Genotyping

Deoxyribonucleic acid (DNA) was extracted from buffy coat using a commercial kit (QIAGEN, Hilden, Germany), and DNA concentrations were adjusted to 50 ng μL^{-1}. Microsatellite polymorphisms were determined by polymerase chain reaction (PCR) using fluorescein-labeled oligonucleotide primers designed to amplify the polymorphic (CA)n repeat in the human ER-β gene (*ESR2* gene accession number, ID 2100; *ESR2* CA repeat polymorphism, rs3223460). The forward primer was labeled with NED fluorescent dye and used together with the tailed reverse primer (5′-NED-TCCCTGCTACCTTTGTGGAC-3′, 5′-CAGCATGGGACAC-CACTG-3′). The size of the fluorescence-labeled PCR product was determined by electrophoresis using an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA), and alleles were assigned with Gene Mapper software (Applied Biosystems). Using the same cutoff as in previous Japanese studies,[13,16] we designated the allele with CA repeats < 22 as S allele and ≥ 22 as L allele. The subjects were divided into three genotypes, *i.e.*, SS, SL and LL.

Statistical methods

Statistical analyses were performed in men and women separately. Logistic regression analysis was used to estimate the odds ratio (OR) and 95% confidence interval (CI) for the association between the *ESR2* CA repeat genotype and colorectal cancer risk. The age at the index date was used in the analysis, and was regarded as age at onset in the cases. Statistical adjustment was made for age, residence area (Fukuoka City or adjacent areas), smoking (0, 1–399, 400–799 or ≥800 cigarettes per year), alcohol intake (0, 0.1–0.9, 1.0–1.9 or ≥2.0 U day^{-1}), BMI 10 years before (<22.5, 22.5–24.9, 25.0–27.4 or ≥27.5 kg m^{-2}), job (sedentary or nonsedentary), leisure-time physical activity (0, 1–15.9 or ≥16 MET-hours per week) and parental colorectal cancer. Results from the multivariate adjustment did not differ from those with adjustment for age alone, and the latter are presented. The trend of increasing (or decreasing) OR with the number of L alleles was tested by the Wald statistic for an ordinal variable representing the number of L alleles. The interaction between the genotype and a covariate was evaluated using the product term of an ordinal variable for the L alleles and a dichotomous variable for a factor of interest. Statistical significance was declared if the two-sided p value was <0.05. Statistical analyses were carried out using SAS version 9.2 (SAS Institute, Cary, NC).

Results

The number of *ESR2* CA repeats was distributed from 14 to 31 with two large peaks at 18 and 23 among the controls (Fig. 1). Age-adjusted OR of colorectal cancer increased modestly with an increasing number of the L allele in women (trend $p = 0.09$), but not in men. In women, a more evident increase in the OR associated with the L allele was observed for colon cancer (trend $p = 0.002$), while no such association was observed for rectal cancer. Neither colon nor rectal cancer showed a measurable association with *ESR2* CA repeats genotype in men (Table 1). There were 42 patients with onset at < 45 years old (15 females and 27 males), who may have had hereditary and/or HNPCC-related colorectal cancers. The analysis excluding such cases showed almost the same results. For instance, the age-adjusted OR of colon cancer for the SL and LL genotypes compared with the SS genotype were 1.77 (95% CI 1.07–2.95) and 2.53 (95% CI 1.40–4.59), respectively (trend $p = 0.002$) in women, and the corresponding OR for men were 0.81 (95% CI 0.55–1.20) and 1.03 (95% CI 0.65–1.65), respectively (trend $p = 0.96$).

When stratified by menopausal status (Table 2), an increased risk of colorectal cancer associated with the L allele was observed among postmenopausal women (trend $p = 0.03$), but not among premenopausal women (trend $p = 0.88$). An increased risk among postmenopausal women was evident only for colon cancer (colon cancer, trend $p = 0.002$; rectal cancer, trend $p = 0.95$). In premenopausal women, the

Table 1. *ESR2* CA repeat polymorphism and colorectal cancer risk by sex

Genotype[1]	*N* (%) of controls	*N*	Colorectal cancer; OR (95% CI)[2]	*N*	Colon cancer; OR (95% CI)[2]	*N*	Rectal cancer; OR (95% CI)[2]
Women							
SS	95 (33.0)	72	1.00 (referent)	30	1.00 (referent)	41	1.00 (referent)
SL	141 (49.0)	127	1.20 (0.81–1.77)	80	1.83 (1.11–3.00)	44	0.72 (0.44–1.18)
LL	52 (18.1)	60	1.53 (0.94–2.47)	41	2.53 (1.41–4.52)	19	0.84 (0.44–1.60)
Trend			$p = 0.09$		$p = 0.002$[3]		$p = 0.44$
Men							
SS	124 (25.3)	112	1.00 (referent)	68	1.00 (referent)	42	1.00 (referent)
SL	265 (54.1)	215	0.89 (0.65–1.21)	110	0.74 (0.51–1.08)	101	1.12 (0.73–1.70)
LL	101 (20.6)	99	1.06 (0.73–1.55)	55	0.96 (0.62–1.50)	43	1.24 (0.75–2.05)
Trend			$p = 0.81$		$p = 0.77$		$p = 0.39$
Interaction[4]			$P = 0.24$		$P = 0.007$[3]		$P = 0.26$

[1]The S allele was defined as < 22 CA repeats and the L allele as ≥ 22 CA repeats. [2]Age-adjusted odds ratio (95% confidence interval). [3]Significant. [4]Interaction between sex and genotype, based on the Wald statistic for the product term of sex and an ordinal variable (scores 0, 1, and 2) representing the number of L alleles.

Table 2. *ESR2* CA repeat polymorphism and colorectal cancer risk by menopausal status among women

Genotype[1]	*N* (%) of controls	*N*	Colorectal cancer; OR (95% CI)[2]	*N*	Colon cancer; OR (95% CI)[2]	*N*	Rectal cancer; OR (95% CI)[2]
Premenopause							
SS	29 (31.9)	26	1.00 (referent)	9	1.00 (referent)	17	1.00 (referent)
SL	45 (49.5)	42	1.13 (0.56–2.26)	26	1.94 (0.79–4.78)	15	0.59 (0.25–1.40)
LL	17 (18.7)	13	0.88 (0.35–2.21)	9	1.78 (0.58–5.41)	4	0.39 (0.11–1.39)
Trend			$P = 0.88$		$p = 0.27$		$p = 0.11$
Postmenopause							
SS	66 (33.5)	46	1.00 (referent)	21	1.00 (referent)	24	1.00 (referent)
SL	96 (48.7)	85	1.25 (0.78–2.02)	54	1.78 (0.98–3.23)	29	0.75 (0.40–1.42)
LL	35 (17.8)	47	1.89 (1.06–3.37)	32	2.91 (1.46–5.80)	15	1.05 (0.48–2.29)
Trend			$p = 0.03$[3]		$p = 0.002$[3]		$p = 0.95$
Interaction[4]			$p = 0.14$		$p = 0.43$		$p = 0.13$

[1]The S allele was defined as < 22 CA repeats and the L allele as ≥ 22 CA repeats. [2]Age-adjusted odds ratio (95% confidence interval). [3]Significant. [4]Interaction between menopausal status and genotype, based on the Wald statistic for the product term of menopausal status and an ordinal variable (scores 0, 1, and 2) representing the number of L alleles.

exclusion of the early-onset cases did not change the results for either colorectal (trend $p = 0.46$) or colon cancer (trend $p = 0.39$), but slightly accentuated the decreased OR of rectal cancer for the SL and LL genotypes (trend $p = 0.05$). In men, the overall and site-specific risks of colorectal cancer showed no measurable difference in the association with the polymorphism between those aged <55 and ≥55 years (data not shown).

Effect modifications of BMI and isoflavone intake on the association with *ESR2* CA repeat polymorphism are summarized in Tables 3 and 4, respectively. Increased risks of colon cancer associated with the L allele were more pronounced among women, especially of postmenopausal status, with low BMI (Table 3) and with high isoflavone intake (Table 4). The interaction among postmenopausal women was nearly statistically significant with respect to BMI ($p = 0.07$) and isoflavone intake ($p = 0.07$). Neither BMI nor isoflavone intake modified the association between *ESR2* CA polymorphism and colon cancer risk among premenopausal women and among men. The analyses based on continuous variables also suggested effect modifications of BMI ($p = 0.09$) and isoflavones ($p = 0.08$) for colon cancer in postmenopausal women.

Discussion

The L allele of the *ESR2* CA repeat was associated with an increased risk of colon cancer, but not of rectal cancer, among postmenopausal women. An increased risk of colon cancer with the L allele among postmenopausal women was influenced by BMI and isoflavone intake.