Association of *Caveolin-1* polymorphisms with colorectal cancer susceptibility in Taiwan

Running title: Yang MD *et al. Cav-1* polymorphisms in colorectal cancer

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Abstract

AIM: To investigate the association of *Cav-1* polymorphisms with colorectal cancer risk in a central Taiwanese population.

METHODS: Three hundred and sixty-two patients with colorectal cancer and same amount of age- and gender-matched healthy controls recruited were genotyped.

RESULTS: There were significant differences between CRC and control groups in the distributions of their genotypes (P=1.6*10^{-12} and 3.0*10^{-4}) and allelic frequencies (P=2.3*10^{-13} and 4.0*10^{-5}) in the *Cav-1* G14713A (rs3807987) and T29107A (rs7804372) polymorphisms, respectively. As for the haplotype analysis, those who had GG/AT or GG/AA at *Cav-1* G14713A/T29107A showed a 0.68-fold (95% confidence interval=0.48-0.98) decreased risk of CRC compared to those with GG/TT, while those of any other combinations were of increased risk. There were joint effects of *Cav-1* G14713A and T29107A genotype with smoking status on individual CRC susceptibility.

CONCLUSION: This is the first report providing evidence that, *Cav-1* being involved in CRC and may be novel useful genomic markers for early detection of CRC.

Key Words: Caveolin-1, Polymorphism, Colorectal cancer, Carcinogenesis, Smoking.

INTRODUCTION

Colorectal cancer is one of the most grave public health problems. There are nearly one million cases of colorectal cancer diagnosis worldwide each year. The prevalent incidence and age-adjusted mortality of CRC has keeping on increasing in the recent years in Taiwan. In 2008, the incidence and mortality of CRC has occupied the third place among the common cancers. Etiological studies have attributed more than 85% of CRC to several environmental factors [1, 2], and in particular meat consumption, cigarette smoking, exposure to carcinogenic aromatic amines, such as arylamines and heterocyclic amines [3-5].

In the recent years, investigators have got interested to caveolae to define how these lipid domains participate in the pathogenesis of human cancers and what their possible utility may be for the detection and treatment [6]. Caveolae are vesicular invaginations of the plasma membrane, which has been thought to play a critical role in transcytosis, communication between cell surface membrane receptors and intracellular signaling protein cascades such as apoptosis and tumorigenesis [7, 8]. Caveolins are the major structural proteins of caveolae and this family contains three members in mammals, Caveolin-1 (Cav-1), Caveolin-2 and Caveolin-3 [7, 9], in which Cav-1 is the principal structural protein. It has been demonstrated that Cav-1 is down-regulated in sarcoma, lung carcinoma, and ovarian carcinoma [10-12]. However, elevated expression of Cav-1 has been associated with the metastasis of esophageal squamous cell carcinoma and prostate cancer and negatively correlated with patient survival [13, 14]. These findings indicate that the role of Cav-1 may vary considerably, depending on the tissue involved.

The emerging evidence pointing to the role of *Cav-1* in carcinogenesis led us to study whether different alleles of this gene are associated with CRC. Thus, the aims of the current study were to determine the genotypic frequency of six polymorphisms of

the *Cav-1* gene at C239A (rs1997623), G14713A (rs3807987), G21985A (12672038), T28608A (rs3757733), T29107A (rs7804372), and G32124A (rs3807992), and their association with CRC susceptibility. To the best of our knowledge, this is the largest study carried out to evaluate the contribution of *Cav-1* polymorphisms in colorectal oncology.

MATERIALS AND METHODS

Study population and sample collection

The study population consisted of 362 CRC patients and 362 cancer-free control volunteers. Patients diagnosed with CRC were recruited at the outpatient clinics of general surgery between 2002-2008 at the China Medical University Hospital, Taichung, Taiwan, Republic of China. The clinical characteristics of patients, including histological details, were all graded and defined by expert surgeons (Dr. Yang's team). All patients voluntarily participated, completed a self-administered questionnaire and provided peripheral blood samples. An equal number of non-cancer healthy volunteers were selected as controls by matching for age, gender and some indulgences after initial random sampling from the Health Examination Cohort of the hospital. The exclusion criteria of the control group included previous malignancy, metastasized cancer from other or unknown origin, and any familial or genetic diseases. This study was approved by the Institutional Review Board of the China Medical University Hospital and written-informed consent was obtained from all participants.

Genotyping conditions

Genomic DNA was prepared from peripheral blood leucocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed according to our

previous papers [15-23]. Briefly, the following primers were used for *Cav-1* C239A (rs1997623): 5'-GTGTCCGCTTCTGCTATCTG-3' and 5'-GCCAAGATGCAGAAGGAGTT-3'; for *Cav-1* G14713A (rs3807987): 5'-CCTTCCAGTAAGCAAGCTGT-3' and 5'-CCTCTCAATCTTGCCATAGT-3'; for *Cav-1* G21985A (12672038): 5'-GGTGTCAGCAAGGCTATGCT-3' and 5'-CCAGACACTCAGAATGTGAC-3'; for *Cav-1* T28608A (rs3757733): 5'-GCTCAACCTCATCTGAGGCA-3' and 5'-GGCCTATTGTTGAGTGGATG-3'; for *Cav-1* T29107A (rs7804372): 5'-GCCTGAATTGCAATCCTGTG-3' and 5'-ACGGTGTGAACACGGACATT-3'; and for *Cav-1* G32124A (rs3807992): 5'-GGTGTCTTGCAGTTGAATG-3' and 5'-ACGGAGCTACTCAGTGCCAA-3'. The following cycling conditions were performed: one cycle at 94^{o}C for 5 min; 35 cycles of 94^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 30 s; and a final extension at 72^{o}C for 10 min. The PCR products were studied after digestion with *Avr II*, *Bfa I*, *Hae III*, *Tsp509 I*, *Sau3AI* and *Nla III*, restriction enzymes for *Cav-1* C239A (cut from 485 bp C type into 170+315 bp T type), *Cav-1* G14713A (cut from 268 bp A type into 66+202 bp G type), *Cav-1* G21985A (cut from 251+43 bp A type into 153+98+43 bp G type), *Cav-1* T28608A (cut from 298 bp T type into 100+198 bp A type), *Cav-1* T29107A (cut from 336 bp A type into 172+164 bp T type), and *Cav-1* G32124A (cut from 213+142+67 bp A type into 142+118+95+67 bp T type), respectively.

Statistical analyses

Only those matches with all SNPs data (case/control =362/362) were selected

into final analyzing. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *Cav-1* single nucleotide polymorphisms (SNP) in the control subjects from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's Chi-square test or Fisher's exact test (when the expected number in any cell was less than five) was used to compare the distribution of the *Cav-1* genotypes between cases and controls. Cancer risk associated with the genotypes was estimated as odds ratio (ORs) and 95% confidence intervals (CIs) using unconditional logistic regression. Data was recognized as significant when the statistical *P*-value was less than 0.05. To evaluate effect modification by smoking, stratified analyses were conducted for chosen SNPs to compare the association across exposure categories of smoking status (never-smokers and smokers). All statistical tests were performed using SAS, Version 9.1.3 (SAS Institute Inc., Cary, NC, USA) on two sided probabilities.

RESULTS

The frequency distributions of selected characteristics of CRC patients and controls are shown in Table I. These characteristics of patients and controls are all well matched. None of these differences between groups were statistically significant ($P>0.05$) (Table I). The frequencies of the genotypes for the *Cav-1* C239A, G14713A, G21985A, T28608A, T29107A and G32124A between controls and CRC patients are shown in Table II. Genotype distribution of various genetic polymorphisms of *Cav-1* G14713A and T29107A were significantly different between CRC and control groups ($P=1.6*10^{-12}$ and $3.0*10^{-4}$, respectively), while those for *Cav-1* C239A, G21985A, T28608A and G32124A were not significant ($P>0.05$) (Table II). To sum up, the polymorphism of *Cav-1* G14713A and T29107A are associated with CRC risk and

may be a biomarker for CRC early detection. The representative PCR-based restriction analyses for the *Cav-1* G14713A and T29107A polymorphisms were shown in Figure 1.

The frequencies of the alleles for the *Cav-1* C239A, G14713A, G21985A, T28608A, T29107A and G32124A between controls and CRC patients are shown in Table III. The two SNPs of *Cav-1* found to be associated with CRC in Table II, G14713A and T29107A, are also found to be associated with higher CRC susceptibility in their allele frequency analysis here. As for other four SNPs, the distributions of their allele frequencies are not significantly different in controls and CRC patients (Table III).

Considering potential interactions between the two significant SNPs of *Cav-1* gene and CRC susceptibility, the risk of CRC related to haplotype distributions of *Cav-1* G14713A and T29107A were further analyzed (Table IV). Compared with GG/TT haplotype of *Cav-1* G14713A and T29107A, the GG/AT or GG/AA group has a 0.68-fold lower risk of CRC (95% CI=0.48-0.98). Other combinations of AG/TT, AG/AT or AG/AA, AA/TT, and AA/AT or AA/AA conferred 2.78-fold (95% CI=2.04-4.22), 2.02-fold (95% CI=1.28-2.94), 3.48-fold (95% CI=1.86-5.59) and 2.29-fold (95% CI=1.49-3.06) increased risks compared to the GG/TT haplotype, respectively (Table IV).

Since smoking is the predominant risk factor for CRC, the interaction between *Cav-1* genotype and individual smoking habits was also analyzed by stratified individual smoking status (Table V). We noticed that subjects with the hetero- or homozygous AA for *Cav-1* G14713A had higher risks of CRC in both smoker and non-smoker groups, no matter before or after adjusting their age, gender and smoking pack-years. In the case of *Cav-1* T29107A, the homozygous AA had lower risks of CRC in both smoker and non-smoker groups. The heterozygous AT of *Cav-1*

T29107A also had protective effects in smoker group. To sum up, there was an obvious interaction between smoking status and *Cav-1* genotypes in the CRC susceptibility.

DISCUSSION

Although several investigations have shown that *Cav-1* plays a critical role in many tumors [10-14], few data are available which consider *Cav-1* for genetic predisposition to cancers [24, 25]. In 2004, the inactivation of *Cav-1* by mutation models or via reducing its expression was found to involve in the pathogenesis of oral cancer [25]. In that study, the exon 1 and 3 sequences of *Cav-1* were investigated in 74 oral squamous cell carcinomas and 15 oral cancer cell lines, and the expression of *Cav-1* was examined. It was reported that only five mutations (1 missense and 4 silent mutations) of *Cav-1* were identified in so many cases, and they were all found in exon 3 [25]. Since sequencing of exonic and promoter regions had not revealed and variants in *Cav-1* that might have been directly involved in any cancer risk, it is reasonable for us to select intronic single nucleotide polymorphisms (SNPs) from the NCBI database, and to evaluate the role of *Cav-1* polymorphisms, which have never been reported to be associated with CRC risk.

The main finding of this study is that *Cav-1* G14713A (rs3807987) and T29107A (rs7804372) polymorphisms are associated with the susceptibility to CRC (Table II and III), while the other four polymorphisms were not. The combinative analysis about *Cav-1* G14713A (rs3807987) and T29107A (rs7804372) showed that when taking G14713A/T29107A GG/TT haplotype as a reference, those with GG/AT or GG/AA were of lower CRC risk, while those with other haplotypes, including AG/TT, AG/AT or AG/AA, AA/TT, AA/AT or AA/AA, were of 1.93- to 3.22-fold higher risk. The data also supported that A allele of G14713A was risky, and A allele of T29107A

was protective. Although these genetic variations do not direct result in amino acid coding change, it is plausible to suspect the alternative splicing, intervention, modification, determination or involvement of these SNPs influence the expression level or stability of the *Cav-1* protein. In our immunohistochemistry detection of tumor tissue from oral cancer patients, taking the distant parts from the same subjects as internal control, we have found that *Cav-1* was down-regulated in the tumor sites (unpublished data).

Environmental factors such as cigarette smoking were reported to be closely related to CRC carcinogenesis. In this study, the joint effects of *Cav-1* gene and individual smoking behaviors were analyzed, and both significant genetic-environmental interactions were observed in *Cav-1* G14713A (rs3807987) and T29107A (rs7804372) (Table V). The sample size and similar trends of significant data after age- and behavior-adjustments strengthen the accuracy and reliability of our findings, and the frequencies of *Cav-1* polymorphisms variant alleles were similar to those reported in the NCBI website in other Asian population studies. For instance, the minor A allele frequencies of *Cav-1* G14713A are 22.1% in our control group, close to those of 16.7% for Beijing and 22.2% for Tokyo populations in NCBI, which strongly suggest no selection bias for the subject's enrolments in terms of genotypes. The smoking population in our patient group is rather low, so that the data itself and that of matched control group are disadvantageous for us to do the stratified analysis of smoking status (Table V). We agree that it is important to verify our findings in further larger studies and clarify the role of *Cav-1* with more phenotypic and functional evidence in CRC and other cancer. In conclusion, this is the first report to provide evidence for *Cav-1* G14713A and T29107A, but not C239A, G21985A, T28608A, or G32124A, were associated with higher susceptibility to CRC. They both have joint effects with smoking status on CRC susceptibility. The G allele

of *Cav-1* G14713A and the A allele of *Cav-1* T29107A might become potential biomarkers for the CRC early detection, prediction and targets for integrative cancer therapy.

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Figure Legend

Fig 1. PCR-based restriction analysis of the G14713A (A) and T29107A (B) polymorphisms of *Cav-1* gene shown on 3% agarose electrophoresis. M: 100 bp DNA size marker, (A) A/A: indivisible homozygote, A/G: heterozygote, and G/G: divisible homozygote. (B) A/A: indivisible homozygote, A/T: heterozygote, and T/T: divisible homozygote.

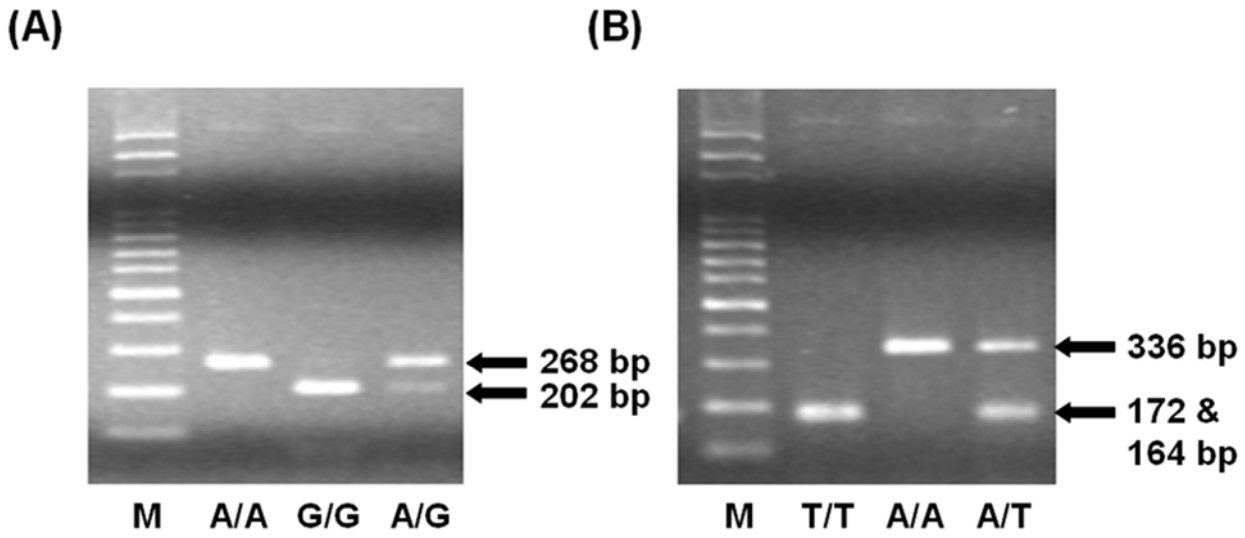

Table I Frequency distributions of characteristics among colorectal cancer patients and controls.

Characteristics	Patients (n = 362)			Controls (n = 362)			*P*
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			64.4 (6.2)			63.8 (5.8)	0.149
Age group (years)							0.932
≦60	93	25.7%		95	26.2%		
＞60	269	74.3%		267	73.8%		
Gender							0.707
Male	209	57.7%		203	56%		
Female	153	42.3%		159	44%		
Habits							
Cigarette smokers	84	23.2%		91	25.1%		0.602
Alcohol drinkers	51	14.1%		44	12.2%		0.509

[a]*P* based on Chi-square test.

Table II. Distribution of *Cav-1* genotypes among colorectal cancer patients and controls

Genotype	Controls	%	Patients	%	P^a
C239A rs1997623					0.3837
CC	355	98.1%	357	98.6%	
AC	7	1.9%	5	1.4%	
AA	0	0.0%	0	0.0%	
G14713A rs3807987					**$1.6*10^{-12}$**
GG	234	64.6%	135	37.3%	
AG	96	26.5%	165	45.6%	
AA	32	8.8%	62	17.1%	
G21985A rs12672038					0.9722
GG	211	58.2%	214	59.1%	
AG	124	34.3%	122	33.7%	
AA	27	7.5%	26	7.2%	
T28608A rs3757733					0.8964
TT	209	57.7%	214	59.1%	
AT	120	33.2%	118	32.6%	
AA	33	9.1%	30	8.3%	
T29107A rs7804372					**0.0003**
TT	179	49.5%	216	59.7%	
AT	120	33.1%	117	32.3%	
AA	63	17.4%	29	8.0%	
G32124A rs3807992					0.8583
GG	179	49.4%	172	47.5%	
AG	144	39.8%	148	40.9%	
AA	39	10.8%	42	11.6%	

[a]*P* based on Chi-square test.

Table III. Distribution of *Cav-1* alleles among colorectal cancer patients and controls

Allele	Controls	%	Patients	%	P^a
C239A rs1997623					0.5621
Allele C	717	99.0%	719	99.3%	
Allele A	7	1.0%	5	0.7%	
G14713A rs3807987					**$2.3*10^{-13}$**
Allele G	564	77.9%	435	60.1%	
Allele A	160	22.1%	289	39.9%	
G21985A rs12672038					0.8064
Allele G	546	75.4%	550	76.0%	
Allele A	178	24.6%	174	24.0%	
T28608A rs3757733					0.6279
Allele T	538	74.3%	546	75.4%	
Allele A	186	25.7%	178	24.6%	
T29107A rs7804372					**$4.0*10^{-5}$**
Allele T	478	66.0%	549	75.8%	
Allele A	246	34.0%	175	24.2%	
G32124A rs3807992					0.5711
Allele G	502	69.3%	492	68.0%	
Allele A	222	30.7%	232	32.0%	

[a]*P* based on Chi-square test.

Table IV Distribution of *Cav-1* G14713A/ T29107A haplotypes among colorectal cancer patients and controls

G14713A/ T29107A haplotype	Controls	%	Patients	%	Crude Odds Ratio (95% CI)[a]	Adjusted Odds Ratio (95% CI)[b]
GG/TT	116	32.0%	81	22.4%	1.00 (Reference)	1.00 (Reference)
GG/AT or GG/AA	118	32.6%	54	14.9%	0.66 (0.43-1.01)	0.68 (0.48-0.98)[c]
AG/TT	47	13.0%	99	27.3%	3.02 (1.93-4.72)[c]	2.78 (2.04-4.22)[c]
AG/AT or AG/AA	49	13.5%	66	18.2%	1.93 (1.21-3.07)[c]	2.02 (1.28-2.94)[c]
AA/TT	16	4.4%	36	9.9%	3.22 (1.68-6.20)[c]	3.48 (1.86-5.59)[c]
AA/AT or AA/AA	16	4.4%	26	7.2%	2.33 (1.17-4.61)[c]	2.29 (1.49-3.06)[c]

[a] 95% CI, 95% confidence interval.

[b] 95% CI, 95% confidence interval, and date were calculated by unconditioned logistic regression and adjusted for age, gender, smoking, alcohol drinking and betel quid chewing behaviors.

[c] Statistically significant.

Table V. Distribution of *Cav-1* G14713A and T29107A genotypes and colorectal cancer after stratification by smoking habit.

SNP/Genotype	Overall			Never smokers			Ever smokers		
	Controls N (%)	Cases N (%)	Adjusted[a] OR (95% CI)[c]	Controls N (%)	Cases N (%)	Adjusted[b] OR (95% CI)[c]	Controls N (%)	Cases N (%)	Adjusted[b] OR (95% CI)[c]
G14713A (rs3807987)									
GG	234 (64.6)	135 (37.3)	1.00 (Ref.[d])	171 (63.1)	107 (38.5)	1.00 (Ref.[d])	63 (69.2)	28 (33.3)	1.00 (Ref.[d])
AG	96 (26.5)	165 (45.6)	2.98 (2.14-4.14)	75 (27.7)	124 (44.6)	2.64 (1.81-3.84)	21 (23.1)	41 (48.8)	4.39 (2.21-8.75)
AA	32 (8.8)	62 (17.1)	3.36 (2.09-5.41)	25 (9.2)	47 (16.9)	3.00 (1.75-5.17)	7 (7.7)	15 (17.9)	4.82 (1.77-13.13)
T29107A (rs7804372)									
TT	179 (49.5)	216 (59.7)	1.00 (Ref.[d])	136 (50.2)	164 (59.0)	1.00 (Ref.[d])	43 (47.3)	52 (61.9)	1.00 (Ref.[d])
AT	120 (33.1)	117 (32.3)	0.79 (0.57-1.11)	89 (32.8)	91 (32.7)	0.84 (0.54-1.21)	52 (34.1)	26 (31.0)	0.40 (0.22-0.76)
AA	63 (17.4)	29 (8.0)	0.37 (0.23-0.58)	46 (17.0)	23 (8.3)	0.40 (0.22-0.71)	17 (18.6)	6 (7.1)	0.28 (0.21-0.79)

[a] Adjusted for age, gender and smoking (pack-years).

[b] Adjusted for age and gender.

[c] OR, odds ratio; CI, confidence interval.

[d] Ref., reference.