Single nucleotide polymorphism of the *AXIN2* gene is preferentially associated with human lung cancer risk in a Japanese population

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Abstract. The *AXIN2* gene, a negative regulator gene of Wnt/ß-catenin signaling, is a putative tumor suppressor gene on human chromosome 17q24. In the genomic locus on which the *AXIN2* gene is located, allelic loss and rearrangement were frequently detected in many cancers. An association between human cancer risk and a single nucleotide polymorphism (SNP) at codon 50 of the *AXIN2* gene, encoding either proline (CCT) or serine (TCT), remains undefined. We, therefore, investigated the distribution of the SNP at codon 50 in 110 healthy controls and 160 patients with non-small-cell lung cancer, 113 patients with colorectal cancer, and 63 patients with head and neck cancer. We found that the frequency of the homozygous T/T (Ser/Ser) genotype was significantly less in lung cancer patients (5.0%) than in healthy controls (13.6%) (p=0.005). As compared with the C/C (Pro/Pro) genotype of the controls, lung cancer patients with the T/T genotype showed reduced risk of cancer; the adjusted odds ratio (OR) for patients with the homozygous T/T (Ser/Ser) genotype was 0.31 (95% confidence interval (CI), 0.12-0.79). The association was particularly strong in lung cancer patients with lung adeno-carcinoma (LAD) (adjusted OR, 0.24; 95% CI, 0.07-0.81), with well-differentiated grade cancer (adjusted OR, 0.12; 95% CI, 0.01-0.99) and with moderately-differentiated grade cancer (adjusted OR, 0.18; 95% CI, 0.04-0.85). These results suggest that the *AXIN2* Pro50Ser SNP is associated with development of lung cancer as a protective SNP, while an association between the *AXIN2* SNP and risk of colorectal cancer and of head and neck cancer was not observed. This is the first report to show an association between the *AXIN2* SNP and lung cancer risk.

Introduction

The axis inhibition protein 2 (*AXIN2*) gene contains 10 coding exons spanning more than 2.5 kb at human chromosome 17q24, and encodes an 843 amino acid protein (1,2). The gene product is a negative regulator of Wnt/ß-catenin signaling (3-7) and acts as a tumor suppressor by limiting the deregulation of Wnt signaling that is commonly observed in many cancers (8,9). The Wnt signaling pathway plays important roles in vertebrate development, cellular proliferation, differentiation and carcino-genesis (4,5,10), and mutations of the pathway components have been observed in a vast number of human cancers, including colon cancer (11,12), melanoma (13), medullo-blastoma (14,15), hepatocellular carcinoma (16,17) and ovarian and uterine cancers (18-20). Wnt is a secreted glycoprotein that binds to Frizzled receptors and regulates the stabilization and localization of ß-catenin (21-24). In the absence of the Wnt signal, ß-catenin is subjected to phosphorylation and subsequent degradation by action of a multiprotein destruction complex (25,26). When cells receive Wnt signal through the receptors, ß-catenin is stabilized and imported into the nucleus, and binds to transcriptional factors of the TCF/LEF family that regulate the expression of Wnt target genes including *c-Myc*, *cyclin D1*, *PPAR-δ*, *MMP-7* and *AXIN2* genes (27-31). There-fore, the regulation of ß-catenin phosphorylation or stability is central to the Wnt signaling pathway, and it is a crucial role in carcinogenesis in which the stabilized and accumulated ß-catenin stimulates the downstream target genes (25,26). The multiprotein destruction complex on Wnt signaling pathway is organized by glycogen synthase kinase (GSK-3ß), APC and AXIN (4,6,7). Especially, in the destruction complex, AXIN serves as a scaffold protein facilitating the phosphorylation of ß-catenin by GSK-3ß and plays a crucial role (6,9,32-34).

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Therefore, the aberrant expression or mutation of these genes affects the development of cancers. Many reports on highly frequent allelic loss and rearrangement in the human chromosome 17q23-24 region, on which the *AXIN2* gene is located, in numerous cancers provide evidence that AXIN2 protein plays a role as a tumor suppressor (8,9).

Genetic polymorphisms of the tumor suppressor gene (TSG) have been analyzed to determine the susceptibility to several cancers, including lung (35-38), breast (39), cervical (40), prostate (41) and nonmelanoma skin cancers (42). The *AXIN2* gene is known to have a single nucleotide polymorphism (SNP) at codon 50 encoding either proline (CCT) or serine (TCT), while the function of this SNP and its molecular mechanism has not been fully clarified. We conducted a pilot study to ascertain whether the AXIN2 SNP, Pro50Ser, is associated with cancer-incidence, and showed significant correlation with lung cancer. This is, to our knowledge, the first report providing evidence for an association between the *AXIN2* polymorphism and cancer risk.

Materials and methods

Subjects. The case groups were comprised of 160 patients with non-small-cell lung cancer [lung adenocarcinoma (LAD), n=105; lung squamous carcinoma (LSC), n=49; adeno/squamous-cell carcinoma, n=3; large cell carcinoma, n=3] (108 men, 52 women; mean age, 66.4 years), 113 patients with colorectal cancer, and 63 patients with head and neck cancer. All the patients were treated at Okayama University Hospital (Okayama, Japan) and had been histologically diagnosed for each cancer subtype. Clinicopathological staging and histological classification of cancers were made according to the criteria of the UICC Tumor-Node-Metastasis Classification of Malignant Tumors (TNM), 6th edition, 2002, (ICD-O C34 for lung; ICD-O C18-C20 for colon and rectum; ICD-O C0-C13, C30, C31 for head and neck cancer). The hospital accepts patients from Okayama and ten other prefectures in western Japan. Each of the 110 healthy controls was selected by computer-aided randomization from among five individuals matched in smoking habit, gender and age (±5 years) for each lung cancer patient, all of which were from the subjects of cohort studies on a Japanese general population older than 40 years of age in a town near Saitama Cancer Center. The residents of this town are not closed genetically or demographically, i.e. its population has increased because of population influx from other areas with a social increase rate of ~5% every year for 15 years. Written informed consent was obtained from all cancer patients and controls. The study was approved by The Bioethics Committee of Okayama University Medical School.

DNA extraction. Genomic DNA of cancer patients was isolated from a non-cancerous region of the resected specimens or from mononuclear cells of peripheral blood by the standard method of proteinase K digestion and phenol-chloroform extraction. Genomic DNA of healthy controls was extracted from peripheral lymphocytes.

Genetic analysis. The *AXIN2* polymorphism was detected by a polymerase chain reaction-restriction fragment length poly-

morphism (PCR-RFLP) technique. According to the published sequence of the human *AXIN2* gene, we designed two primers (forward primer 5'-AAG GGC CAG GTC ACC AAA CAC ATG-3', and reverse primer 5'-CAC CGG GTC AGA GGG GAA TC-3') to amplify a 110-bp fragment in exon 1 of the *AXIN2* gene. Reaction mixture (20 μl) contained 0.2 mM of each dNTP, 1X PCR buffer, 8 pmol of each primer, 20 ng of genomic DNA and 0.5 unit of TaqDNA polymerase (Takara, Kyoto, Japan). The PCR amplification was initiated by a denaturing step at 94˚C for 3 min, followed by 40 cycles at 94˚C for 30 sec, 64˚C for 1 min, 72˚C for 1 min, and a final extension step at 72˚C for 7 min. For RFLP analysis, each PCR product was digested with *Afl* III restriction endnuclease (New England Biolabs, Beverly, MA) at 37˚C overnight. The digested products were then subjected to electrophoresis on 3% agarose gel, stained with ethidium bromide and visualized on UV transilluminator. The allele types were determined as follows: a single 110-bp fragment for the C/C (Pro/Pro) genotype, two fragments of 87- and 23-bp for the T/T (Ser/Ser) genotype and three fragments of 110-, 87- and 23-bp for the C/T (Pro/Ser) genotype. To confirm the allele types, some PCR products were processed using the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3100 sequencer (Applied Biosystems).

Statistical analysis. We compared the allele frequencies of the CCT/TCT (Pro/Ser) polymorphism in the *AXIN2* gene between the healthy control group and the patient groups with non-small-cell lung cancer, colorectal cancer, and head and neck cancer. Distribution of the *AXIN2* genotype (C/C, C/T, T/T) in the patients and controls was tested for adherence to the Hardy-Weinberg equilibrium. All statistical inquiries were conducted by SPSS software Ver.12.0 (SPSS Inc., Tokyo, Japan). The Chi-square test was used to compare genotype distribution between cancer patients and healthy controls. Odds ratio (OR) and 95% confidence interval (95% CI) were adjusted for age, gender and smoking status using an unconditional logistic regression model. The relationship between genotype and clinicopathological characteristics was examined by the Chi-square test. A p-value of <0.050 was considered to be statistically significant.

Results

Assessment of cancer risk by AXIN2 genotyping. Representative polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns of the CCT/TCT (Pro/Ser) genotypes in codon 50 of the *AXIN2* gene are shown in Fig. 1. A single fragment at 110-bp characterizes the major C/C (Pro/Pro) genotype. Two fragments at 87- and 23-bp characterize the T/T (Ser/Ser) genotype, while the fragment at 23-bp is not visible clearly. We confirmed that each PCR mixture contained no non-specific bands by electrophoresis on 3% agarose gel before digestion with *Afl* III (data not shown). We studied a total of 446 individuals; 110 healthy controls, 160 lung cancer, 113 colorectal cancer and 63 head and neck cancer patients. The frequencies of the three genotypes in the *AXIN2* gene are shown in Table I. The frequencies of the genotypes C/C, C/T and T/T found in controls were 38.2, 47.3 and 13.6%, respectively (the genotype of one individual
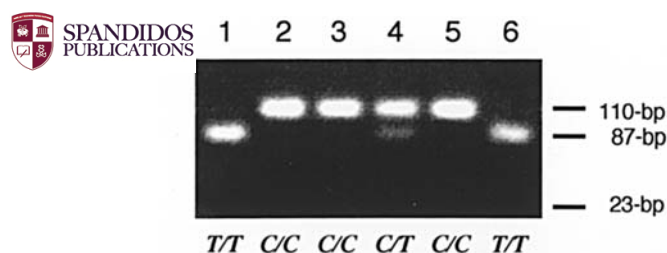
Figure 1. PCR-RFLP analysis of the *AXIN2* SNP at codon 50. PCR product was digested with *Afl* III restriction enzyme, and electrophoresed on 3% agarose gel. A single 110-bp fragment shows the C/C (Pro/Pro) genotype, two fragments of 87 and 23 bp show the T/T (Ser/Ser) genotype and three fragments of 110, 87 and 23 bp show the C/T (Pro/Ser) genotype. Case number and genotypes are shown at the top and bottom, respectively.

could not be identified because of unsuccessful PCR), and fitted the Hardy-Weinberg equilibrium with allele frequencies of 0.62 (C) and 0.38 (T). For lung cancer patients, the frequencies of the three *AXIN2* genotypes, C/C, C/T and T/T, were 50.6, 44.4 and 5.0%, respectively, and the result fitted the Hardy-Weinberg equilibrium with allele frequencies of 0.73 (C) and 0.27 (T). As compared with the controls with the C/C genotype, lung cancer patients with the homozygous T/T genotype exhibited a significantly decreased risk with odds ratio (OR) of 0.28 [95% confidence interval (CI), 0.11-0.70], suggesting that the homozygous Ser/Ser (T/T) genotype has a reduced risk of lung cancer development as a protective SNP. The characteristics of the 160 lung cancer patients and 110 healthy controls are shown in Table II. There were no significant differences in gender and age between the lung cancer patients and healthy controls. Pack-year equivalents were used for smoking status (we could not obtain the smoking status for 5 of 160 patients). There were also no significant differences in smoking status. As compared with the controls with the C/C genotype, we found that lung cancer patients with the homozygous T/T genotype exhibited a significantly decreased risk with OR of 0.31 (95% CI, 0.12-0.79) adjusted for age,

Table I. *AXIN2* genotypes in healthy controls and lung cancer, colorectal cancer and head and neck cancer patients.

AXIN2	Patients	Controls	p-value[a]	OR (95% CI)	
genotype	n (%)	n (%)		Crude	Adjusted[b]
Lung cancer					
C/C	81 (50.6)	42 (38.2)		1 (Reference)	1 (Reference)
C/T	71 (44.4)	52 (47.3)	0.189	0.71 (0.42-1.19)	0.69 (0.41-1.17)
T/T	8 (5.0)	15 (13.6)	0.005	0.28 (0.11-0.70)	0.31 (0.12-0.79)
Unknown	0 (0.0)	1 (0.90)			
Total	160	110			
Allele frequencies			0.010		
C	233 (72.8)	136 (62.0)			
T	87 (27.2)	82 (38.0)			
Colorectal cancer					
C/C	54 (47.7)	42 (38.2)		1 (Reference)	1 (Reference)
C/T	44 (38.9)	52 (47.3)	0.148	0.66 (0.37-1.16)	0.64 (0.35-1.18)
T/T	15 (13.4)	15 (13.6)	0.548	0.78 (0.34-1.77)	0.85 (0.35-2.06)
Unknown	0 (0.0)	1 (0.90)			
Total	113	110			
Allele frequencies			0.282		
C	152 (67.3)	136 (62.0)			
T	74 (32.7)	82 (38.0)			
Head and neck cancer					
C/C	25 (39.7)	42 (38.2)		1 (Reference)	1 (Reference)
C/T	29 (46.0)	52 (47.3)	0.849	0.94 (0.48-1.83)	0.94 (0.42-2.13)
T/T	9 (14.3)	15 (13.6)	0.987	1.00 (0.38-2.64)	0.88 (0.27-2.89)
Unknown	0 (0.0)	1 (0.90)			
Total	63	110			
Allele frequencies			0.953		
C	79 (62.7)	136 (62.0)			
T	47 (37.3)	82 (38.0)			

[a]p-values were for the difference in genotype frequencies between patients and controls and were calculated by Chi-square test. [b]Adjusted for age, gender and smoking status. Patients whose smoking status was not known were omitted when ORs were calculated.

Table II. Characteristics of lung cancer patients and healthy controls.

	Patients (n=160) n (%)	Controls (n=110) n (%)	p-value[a]
Gender			0.449
Male	108 (67.5)	79 (71.8)	
Female	52 (32.5)	31 (28.2)	
Age (years±SD)	66.4±8.28	66.5±9.04	
Smoking habit			0.126
Non-smoker	50 (31.3)	26 (23.6)	
Smoker	105 (65.6)	84 (76.4)	
Unknown	5 (3.1)	0 (0.0)	
<20 pack-years	5 (4.8)	14 (16.7)	
≥20 pack-years	98(93.3)	70 (83.3)	
Unknown	2 (1.9)	0 (0.0)	

[a]p-values were calculated by Chi-square test.

gender and smoking status (Table I). Although lung cancer patients with the heterozygous C/T genotype also showed a decreased risk with OR of 0.69 (95% CI, 0.41-1.17), it was not significant statistically (Table I). We found no association of the *AXIN2* SNP with colorectal cancer and head and neck cancer (Table I).

Association between AXIN2 genotype and clinicopathological characteristics in lung cancer patients. When we analyzed the relationship of SNP genotype with gender and smoking habit, males or smokers showed decreased cancer risk in the homozygous T/T genotype as compared with the controls with the C/C genotype (Table III). Table III also shows the relationship between *AXIN2* genotype and clinicopathological parameters in lung cancer patients. Concerning histological cell type, we found that lung adenocarcinoma patients with the homozygous T/T genotype exhibited a significantly decreased risk with OR of 0.24 (95% CI, 0.07-0.81; p=0.021). Concerning differentiation grade, patients with well- and moderately-differentiated lung cancer exhibited a decreased risk with ORs of 0.12 (95% CI, 0.01-0.99; p=0.050) and 0.18 (95% CI, 0.04-0.85; p=0.030), respectively, in the homozygous T/T

Table III. Association between *AXIN2* genotype and clinicopathological characteristics of lung cancer patients.

	Genotype				OR (95% CI)[a]	
Characteristics	C/C (%)	C/T (%)	T/T (%)	Total	C/T	T/T
Controls	42 (38.2)	52 (47.3)	15 (13.6)	109		
Lung cancer	81 (50.6)	71 (44.4)	8 (5.0)	160	0.69 (0.41-1.17)	0.31 (0.12-0.79)[b]
Gender						
Male	53 (49.1)	48 (44.4)	7 (6.5)	108	0.70 (0.37-1.32)	0.33 (0.12-0.99)[b]
Female	28 (53.8)	23 (44.2)	1 (2.0)	52	0.66 (0.26-1.72)	0.21 (0.02-2.45)
Smoking habit						
Smoker	52 (49.5)	46 (43.8)	7 (6.7)	105	0.78 (0.42-1.47)	0.35 (0.13-0.99)[b]
Non-smoker	27 (54.0)	22 (44.0)	1 (2.0)	50	0.64 (0.24-1.71)	0.19 (0.02-2.45)
Unknown	2 (40.0)	3 (60.0)	0 (0.0)	5		
Histological cell type						
LAD	54 (51.4)	47 (44.7)	4 (3.9)	105	0.69 (0.38-1.23)	0.24 (0.07-0.81)[b]
LSC	23 (46.9)	22 (44.9)	4 (8.2)	49	0.81 (0.39-1.68)	0.44 (0.13-1.54)
Other	4 (66.7)	2 (33.3)	0 (0.0)	6		
Differentiation grade						
Well	27 (52.9)	23 (45.0)	1 (2.1)	51	0.73 (0.35-1.52)	0.12 (0.01-0.99)[b]
Moderate	33 (52.4)	28 (44.4)	2 (3.2)	63	0.67 (0.35-1.30)	0.18 (0.04-0.85)[b]
Poor	16 (44.4)	15 (41.6)	5 (14.0)	36	0.75 (0.33-1.72)	0.78 (0.24-2.57)
Unknown	4 (44.4)	5 (55.6)	0 (0.0)	9		
Clinical stage						
0.I.II	60 (53.1)	46 (40.7)	7 (6.2)	113	0.59 (0.34-1.04)	0.35 (0.13-0.94)[b]
III.IV	15 (41.7)	20 (55.6)	1 (2.7)	36	1.05 (0.49-2.26)	0.18 (0.02-1.45)
Unknown	2 (40.0)	3 (60.0)	0 (0.0)	5		

[a]OR was adjusted for age, gender and smoking status. [b]p-value <0.05. p-value was calculated by Chi-square test.
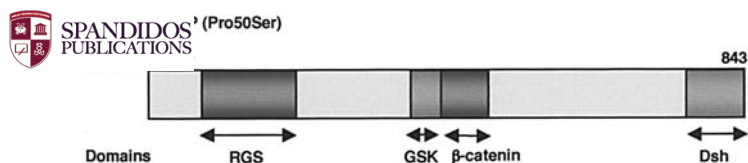
Figure 2. Schematic representation of AXIN2 protein. SNP (Pro50Ser) is shown at NH3-terminal of AXIN2 protein. The predicted interaction domains of AXIN2 protein are depicted in gray. RGS, regulator of G protein signaling domain (AA81-200); GSK, glycogen synthase kinase 3ß (AA372-413); ß-catenin, ß-catenin binding site (AA414-476); Dsh, disheveled homology domain (AA761-843). AA No., amino acid number.

genotype. Nine patients whose differentiation information was not known were omitted when p-value was calculated. Concerning clinical stage, patients in stage 0+I+II showed a decreased risk with OR of 0.35 (95% CI, 0.13-0.94; p=0.037) in the homozygous T/T genotype. Five patients whose TNM stage information was not known were omitted when p-value was calculated.

Discussion

In the present study, we examined whether an SNP (Pro50Ser) in the *AXIN2* gene is associated with risk of developing non-small-cell lung cancer, colorectal cancer, and head and neck cancer. We found a significant difference in genotype distribution between lung cancer patients and healthy controls in Japanese. The major genotype population in the controls is the heterozygous Pro/Ser (C/T) genotype (47.3%); however, it is the homozygous Pro/Pro (C/C) genotype in lung cancer (50.6%) and colorectal cancer patients (47.7%). The frequency of the homozygous Ser/Ser (T/T) genotype in lung cancer patients (5%) is lower than that of the controls (13.6%), while the frequency of the homozygous Ser/Ser (T/T) genotype in colorectal cancer (13.4%) and head and neck cancer patients (14.3%) is almost the same as that of controls (13.6%). These results suggest a tendency towards lung cancer risk of the homozygous Pro/Pro (C/C) genotype and reduced lung cancer risk of the homozygous Ser/Ser (T/T) genotype. Statistically, lung cancer patients with the homozygous Ser/Ser (T/T) genotype showed a significantly reduced risk of cancer (adjusted OR, 0.31; 95% CI, 0.12-0.79) as a protective SNP. We recognize that this distribution of genotype in lung cancer patients does not seriously deviate from the general Japanese population because Japan is an almost racially homogeneous nation and Okayama has had population influxes from other areas such as Tokyo and Osaka (urban cities representing Japan) and the Chugoku and Shikoku Districts (around Okayama).

There are some reports on the function of AXIN protein (3-7,32), and several functional motifs of AXIN have been identified (Fig. 2) (1,17,21,22). The region of AXIN involved in the APC-binding site shows significant homology to members of the regulators of G-protein signaling (RGS) family (3,6). AXIN, a tumor suppressor protein, is a component of Wnt signaling pathway. A large complex of AXIN, APC, ß-catenin, GSK3ß and several other proteins regulates Wnt signaling through the regulation of cytoplasmic localization of ß-catenin (7,32,33). Both AXIN and APC are known to be critical for ß-catenin regulation, and truncated mutations in APC that eliminate the AXIN-binding site result in human cancer, suggesting that the binding avidity of AXIN protein for APC effects carcinogenesis. The RGS domain (amino acids 81 to 200) participates in the important function of AXIN2 protein as a tumor suppressor (13). The *AXIN2* SNP at codon 50 must effect the avidity and be associated with carcino-genesis because the SNP (Pro50Ser) is located very closely to the RGS domain (Fig. 2). However, it is unknown why the association of the *AXIN2* SNP with cancer risk was observed only in patients with lung cancer, but not in patients with head and neck cancer, or colorectal cancer. A larger population may be required to analyze the association of the SNP with head and neck cancer, and colorectal cancer.

In conclusion, our study suggests that this AXIN2 SNP, Pro/Ser at codon 50, is associated with development of lung cancer in a Japanese population. This is, to our knowledge, the first pilot study showing a significant relationship between the AXIN2 Pro50Ser SNP and risk of lung cancer development. Our sample size is small but the findings are statistically significant. Further study with sufficiently larger populations and functional analysis of this polymorphism is required to clarify the unsolved issue. We expect this work to contribute towards developing a novel strategy for early diagnosis and prevention of lung and other cancers in the near future.

Acknowledgements

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