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ORIGINAL RESEARCH

CD226 gene polymorphisms are associated with non-small-cell lung cancer in the Chinese Han population

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Background: The immunoglobulin-like glycoprotein *CD226* (DNAX accessory molecule-1) represents receptor-activating cytotoxic T lymphocyte and natural killer cells taking part in tumor surveillance, the pathogenesis of inflammation, and autoimmune disorders. The aim of the present study is to analyze the association between polymorphisms rs763361 and rs727088 in the *CD226* gene and their impact on the pathogenesis of non-small-cell lung cancer (NSCLC).

Materials and methods: Polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLP) were used to genotype the single nucleotide polymorphisms (SNPs) rs763361 and rs727088 of the *CD226* gene in 302 NSCLC patients and 389 ethnicity matched healthy controls.

Results: The frequencies of the T allele and TT genotype of rs763361 (T allele odds ratio [OR] 1.42, 95% confidence interval [CI] 1.14–1.77; TT genotype OR 2.73, 95% CI 1.70–4.39), as well as the G allele and GG genotype of rs727088 (G allele OR 1.89, 95% CI 1.50–2.39; GG genotype OR 4.62, 95% CI 2.31–9.20) in the NSCLC patients were significantly higher than that of normal controls, indicating that both of these two SNPs as risk factors were associated with NSCLC ($P<0.05$). Results of stratified analysis revealed that the polymorphism of rs727088 was associated with lymph node invasion and clinical stage cancer ($P<0.05$). However, there was no association between SNP rs763361 and clinical characteristics.

Conclusion: Our results demonstrated that *CD226* gene polymorphisms (T allele of rs763361 and G allele of rs727088) as risk factors were associated with NSCLC.

Keywords: *CD226*, NSCLC, polymorphism, DNAX

Introduction

Non-small-cell lung cancer (NSCLC) accounts for 80% of all lung cancers and is a leading cause of cancer mortality worldwide.[1,2] Tobacco smoking is the most well-established risk factor for lung cancer. Another important risk factor is environmental exposure to chemical carcinogens or occupational carcinogens. Moreover, genetic factors also play a crucial role in the pathogenesis of NSCLC.[1,2]

CD226, also known as DNAX accessory molecule 1 (DNAX1) or platelet and T cell antigen 1, is a transmembrane glycoprotein belonging to the immunoglobulin superfamily and contains two IG-like domains in its extracellular portion. It is highly conserved among humans, gibbons, monkeys, and mice, and its gene has been mapped to human chromosome 18.[3–6] In humans, CD226 is expressed on the majority of natural killer (NK) cells, T cells, and monocytes, and on a small subset of B cells.[5]

Recently, rs763361 (C/T polymorphism), the non-synonymous (Gly307Ser) coding variant that is located at 18q22.3 in the *CD226* gene, was first correlated to type 1

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diabetes susceptibility, and has been shown to be associated with multiple autoimmune diseases and systemic sclerosis (SSc).[7,8] In addition, the rs763361 glycine-to-serine substitution could interfere in the phosphorylation of CD226 at 322Tyr and 329Ser residues, and may modulate the downstream signaling pathway via these posttranslational modifications.[9,10] Furthermore, a three-variant haplotype in *CD226* gene (rs763361-rs34794968-rs727088) was found to be associated with systemic lupus erythematosus in recent research, and the authors suggested that rs727088 may be the single nucleotide polymorphism (SNP) with a functional influence on CD226 transcription levels.[11] Another study performed on SSc subjects proposed that genetic variants, including CD226 rs763361, rs727088, and rs3479968 did not influence SSc susceptibility, but that a CD226 three-variant haplotype did cause genetic predisposition to SSc-related pulmonary fibrosis.[12] Additionally, two earlier studies reported that CD226 polymorphism was associated with susceptibility to gastric cancer and cervical squamous cell carcinoma.[13,14]

Considering the important role of CD226 and its ligands in diseases, it is necessary to investigate whether CD226 is a candidate susceptible gene to cancers. However, to our knowledge, there is no report about the relationship between CD226 polymorphisms and lung cancer. Therefore, in the present study, we attempted to clarify the association between *CD226* gene polymorphisms (rs763361 and rs727088) and NSCLC, and we have found that *CD226* gene polymorphisms (rs763361 and rs727088) are associated with NSCLC.

Materials and methods

Study subjects

A total of 302 unrelated Chinese patients with NSCLC were recruited from the West China First University Hospital, Sichuan University from January 2010 to January 2012. The patients (age, mean ± standard deviation [SD], 60.29±10.11 years; male/female ratio, 216/86) consisted of 138 adenocarcinomas, 138 squamous cell carcinomas, and 26 other types of cancer, including adenosquamous carcinomas and large-cell carcinomas. All patients were adjuvant therapy-free. The control group consisted of 389 healthy subjects (age, mean ± SD, 61.71±12.14 years; male/female ratio, 278/111) from a routine health survey in the same hospital. Control subjects were genetically unrelated and without any personal or family history of cancer or other serious disease. The diagnosis of NSCLC was confirmed by histological examination of tissues from biopsy. Data on stages were presented according to the International Union Against Cancer's tumor node metastasis system, and differentiation and histological type were measured according to the World Health Organization classification for NSCLC.[15] The study design and procedure were approved by the institutional review board of West China Hospital, and all patients completed a written informed consent before enrollment.

Determination of genotypes

Genomic DNA of each subject was extracted from 200 µL ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood samples with a DNA isolation kit from BioTeke Corporation (Peking, People's Republic of China), and the procedure was performed according to the manufacturer's instructions. The primers were established with the primer-introduced restriction analysis (PIRA) polymerase chain reaction (PCR) designer (http://primer1.soton.ac.uk/primer2.html). The rs763361 primers, which were used for amplification, were 5′-TTTGGTCTGCGAGAGAAGGT-3′ and 5′-TGCCTGCATTTATGAGAGGT-3′, and the primers used for amplification of the rs727088 were 5′-TCCCTCCCAAATTTCTACCC-3′ and 5′-CCATCCCAGGTCTAGCCTTA-3′.

The PCR reactions were performed in a total volume of 25 µL, composed of 2.5 µL 10× PCR buffer, 1.5 mmol/L of $MgCl_2$, 0.15 mmol/L of deoxyribonucleotide triphosphates, 0.5 µmol/L of each primer, 100 ng of genomic DNA, and 1 U of Taq DNA polymerase. The PCR conditions for both SNPs were 94°C for 4 minutes, followed by 32 cycles of 30 seconds at 94°C, 30 seconds at 62°C, and 30 seconds at 72°C, with a final elongation at 72°C for 10 minutes. PCR products were digested overnight with specific restriction enzymes (*Msp* I for rs763361 and *Bcc* I for rs727088), were then separated with a 6% polyacrylamide gel, and were stained with 1.5 g/L of argent nitrate. For the rs763361 polymorphism, allele C was divided into two fragments of 79 bp and 126 bp; allele T was uncuttable, and the fragment was 205 bp; for the rs727088 polymorphism, allele A was cuttable, yielding two fragments of 31 bp and 208 bp; allele G was uncuttable, and the fragment was 239 bp. The genotypes were confirmed by the DNA sequencing analysis (BigDye® Terminator v3.1 Cycle Sequencing Kit; Thermo Fisher Scientific, Waltham, MA, USA). Approximately 20% of the samples were randomly selected to perform the repeated assays, and the results were 100% concordant.

Statistical analysis

Genotype and allele frequencies of the two SNPs were calculated by directed counting. The chi-square test was used to compare the genotype and allele frequencies between two groups and to evaluate the Hardy–Weinberg equilibrium. Odds ratios (OR) and respective 95% confidence intervals (95% CIs)

were reported to evaluate the effects of any difference between alleles and genotypes. Data analyses and summarizations were conducted using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL, USA). Values of $P<0.05$ (2-sided) were considered to be statistically significant. The study power was calculated by using the Quanto 1.1.1 program (http://hydra.usc.edu/gxe).

Results

Comparison of *CD226* gene polymorphism between patients and controls

The two *CD226* SNPs were successfully genotyped in 302 NSCLC patients and 389 control subjects. The genotype distributions for SNP rs763361 and SNP rs727088 were in Hardy–Weinberg equilibrium ($P\geq0.05$) in both patients and controls. Three genotypes of both SNPs were identified, and the genotypes were confirmed by DNA sequencing analysis. Allele and genotype frequencies of these two SNPs were calculated and are summarized in Table 1. As shown in Table 1, significantly increased NSCLC risk was found to be associated with the T allele of SNP rs763361 ($P=0.001$; OR =1.42; 95% CI =1.14–1.77), and with the G allele of SNP rs727088 ($P<0.001$; OR =1.89; 95% CI =1.50–2.39).

Association between CD226 polymorphisms and clinicopathologic characteristics of NSCLC patients

Results of stratified analyses by clinicopathological features including sex, age, histological classification, tumor size, lymph node invasion, metastasis, and clinical stage for the two SNPs are presented in Tables 2 and 3. The rs763361 locus did not show any differences among stratified patient groups, whereas the frequencies of allele and genotype of the rs727088 locus were significantly different between patient groups with different stages of lymph node invasion ($P=0.024$ and $P=0.042$, respectively, for allele and genotype) and clinical stage ($P=0.033$). A significantly higher frequency of allele G was observed in patients with lymph node invasion at the N2 stage, and logistic-regression analysis revealed that allele G was associated with lymph node invasion (OR =1.784; 95% CI =1.077–2.954).

The study power has been performed with the Quanto 1.1.1 program. Under a dominant genetic model, at the 0.05 level of significance with the two-sided test for the two polymorphisms, our current study had 94.99% and 96.80% power, respectively, for the rs763361 and the rs727088 polymorphisms to detect an effect with a relative risk of 1.8 in the group of NSCLC patients and in the group of healthy controls.

Discussion

Our present study provided the first evidence that both the T/C of rs763361 and the G/A of rs727088 were associated with NSCLC. The allele T of rs763361 and the allele G of rs727088 were remarkably correlated with NSCLC risk, indicating that CD226 may be used as a risk candidate biomarker for NSCLC susceptibility.

As an adhesion molecule and as a triggering receptor in NK and cytotoxic T lymphocyte (CTL) cells,[5] CD226 is important for NK cell-mediated recognition of several human tumors, including myeloma, neuroblastoma, and Ewing's sarcoma.[16–19] Adhesion molecules act directly as signaling molecules, initiating intracellular pathways that trigger activation and adhesiveness of CTL cells. They also

Table 1 Genotype and allele distribution of two single-nucleotide polymorphism loci in patients with non-small-cell lung cancer and normal controls

SNP genotype/allele	Patients n=302 (%)	Controls n=389 (%)	*P*-value	OR (95% CI)
rs763361 genotype				
CC	84 (27.8)	151 (38.8)	Ref	Ref
CT	166 (55.0)	194 (49.9)	0.012[a]	1.98 (1.42–2.75)
TT	52 (17.2)	44 (11.3)	<0.001[a]	2.73 (1.70–4.39)
Allele				
C	334 (55.3)	496 (63.8)	0.001[a]	1.42 (1.14–1.77)
T	270 (44.7)	282 (36.2)		
rs727088 genotype				
AA	109 (36.1)	218 (56.0)	Ref	Ref
AG	163 (54.0)	158 (40.6)	<0.001[a]	2.06 (1.50–2.83)
GG	30 (9.9)	13 (3.3)	<0.001[a]	4.62 (2.31–9.20)
Allele				
A	381 (63.1)	594 (76.3)	<0.001[a]	1.89 (1.50–2.39)
G	223 (36.9)	184 (23.7)		

Notes: [a]Statistically significant.
Abbreviations: OR, odds ratio; CI, confidence interval; SNP, single-nucleotide polymorphism; n, number of patients or controls; Ref, reference.

Table 2 Association between clinical characteristics of patients with non-small-cell lung cancer and polymorphism of locus rs763361

Characteristics	Patients, n	Genotype, n (%)			*P*-value	Allele no (%)		*P*-value	OR (95% CI)
		CC	CT	TT		C	T		
Sex									
Female	86	26 (30.2)	51 (59.3)	9 (10.5)	0.161	103 (59.5)	69 (40.1)	0.155	1.30 (0.91–1.86)
Male	216	57 (26.4)	115 (53.2)	42 (19.4)		229 (53.5)	199 (46.5)		
Age									
≤60 years	171	47 (27.5)	95 (55.6)	29 (16.9)	0.830	189 (55.3)	153 (44.7)	0.984	0.99 (0.72–1.38)
>60 years	131	37 (28.2)	71 (54.2)	23 (17.6)		145 (55.3)	117 (44.7)		
Histological type									
ADC	138	44 (31.9)	75 (54.3)	19 (13.8)		163 (59.1)	113 (40.9)		
SCC	138	33 (23.9)	77 (55.8)	28 (20.3)	0.190	143 (51.8)	133 (48.2)	0.087	1.34 (0.96–1.88)
Others	26	7 (26.9)	14 (53.8)	5 (19.2)	0.733	28 (53.8)	24 (46.2)	0.485	1.24 (0.68–2.24)
Differentiation									
Poor	204	59 (28.9)	111 (54.4)	34 (16.7)	0.809	229 (56.1)	179 (43.9)	0.554	1.11 (0.79–1.56)
Moderate–well	98	25 (25.5)	55 (56.1)	18 (18.4)		105 (53.6)	91 (46.4)		
Tumor size									
T1	34	11 (32.4)	17 (50.0)	6 (17.6)		39 (57.4)	29 (42.6)		
T2	192	53 (27.6)	106 (55.2)	33 (17.2)	0.828	212 (55.2)	172 (44.8)	0.743	1.09 (0.65–1.84)
T3	51	15 (29.4)	27 (52.9)	9 (17.6)	0.955	57 (55.9)	45 (44.1)	0.850	1.60 (0.57–1.97)
T4	25	5 (20.0)	16 (64.0)	4 (16.0)	0.512	26 (52.0)	24 (48.0)	0.563	1.24 (0.58–2.59)
Lymph node									
N0	156	43 (27.6)	83 (53.2)	30 (19.2)		170 (54.1)	144 (45.7)		
N1	107	36 (33.6)	56 (52.3)	15 (14.0)	0.407	128 (59.8)	86 (40.2)	0.197	0.79 (0.56–1.13)
N2	38	5 (13.2)	26 (68.4)	7 (18.4)	0.147	36 (47.4)	40 (52.6)	0.289	1.31 (0.79–2.17)
Metastasis									
M0	285	78 (27.4)	157 (55.1)	50 (17.5)	0.709	313 (54.9)	257 (45.1)	0.435	0.75 (0.37–1.54)
M1	17	6 (35.3)	9 (52.9)	2 (11.8)		21 (61.8)	13 (38.2)		
Clinical stage									
I + II	223	67 (30.0)	116 (52.0)	40 (17.9)	0.209	250 (56.1)	196 (43.9)	0.577	0.89 (0.62–1.28)
III + IV	79	17 (21.5)	50 (63.3)	12 (15.2)		84 (53.2)	74 (46.8)		

Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; OR, odds ratio; CI, confidence interval.

act as accessory molecules, sustaining cellular contacts that are necessary for the NK cell receptors and T cell receptors to engage their cognate ligands and to deliver intracellular signals.[7]

NK cells display cytolytic activity against virus-infected cells and a wide variety of tumors of different histotypes.[20] A role for NK cells in therapeutic intervention is becoming more apparent, such as in cases of melanoma, acute myeloid leukemia, and multiple myeloma.[20–22] Emerging evidence suggests that CD226 plays an important role in T cell- and NK cell-mediated immunity to a variety of tumors expressing CD226 ligands.[16,17,23–25] Many tumor cell lines of epithelial tissue origin express CD155 and/or CD112, which, as the main ligands for CD226, are lysed in vitro by NK cells and CTL cell lines in a CD226-dependent manner.[5] The expression of CD155 and/or CD112 has also been detected in tumor samples from patients with metastatic neuroblastoma, some of which were shown to be susceptible to CD226-mediated lysis by activation of NK cells.[17,26] In some vitro studies, it has been shown that CD226 triggers NK cell-mediated killing of a range of tumor cells expressing CD155 and CD112.[16,17,24,27,28] NKT cells, $\gamma\delta$T cells, and CD4 T cells express CD226 when activated.[18,29–31] CD226 binds to CD155 and to CD112, both of which have been found to be upregulated on tumors. CD155 is widely expressed on normal cells and is overexpressed on many tumor types.[16,17,23,27,29–32] Moreover, some tumors overexpress CD155 and release it as a soluble molecule,[33] possibly to block CD226 and to prevent recognition of tumor cells.[20] One study demonstrated that the CD226–CD155 interaction is crucial for recognition, and through resting allogeneic NK cells, it can kill freshly isolated human ovarian carcinoma cells.[23] Hence, these results may provide inspiration for the design of future protocols of adoptive NK cell- and antibody-based immunotherapies for ovarian carcinoma and possibly for other human tumors.[23,34] Furthermore, the generation of autoreactive T cells may be regulated by CD226–CD155 interactions, emphasizing the importance of CD226 in the immune response.[35]

The importance of CD226 in tumor immunity on both NK cells and T cells has been shown in vivo.[24] In murine models, two independent research groups have reported an increased risk of tumor development in mice lacking either the CD226 or NKG2G receptor.[36,37] Compared with wide-type mice, CD226-deficient mice developed significantly more CD226 ligand-expressing fibrosarcoma and papilloma tumors in

Table 3 Association between clinical characteristics of patients with non-small-cell lung cancer and polymorphism of locus rs727088

Characteristics	Patients, n	Genotype n (%)			*P*-value	Allele n (%)		*P*-value	OR (95% CI)
		AA	AG	GG		A	G		
Sex									
Female	86	34 (39.5)	43 (50.0)	9 (10.5)	0.677	111 (64.5)	61 (35.5)	0.640	1.09 (0.76–1.58)
Male	216	75 (34.7)	120 (55.6)	21 (9.7)		270 (65.5)	162 (37.5)		
Age									
≤60 years	170	63 (37.1)	91 (53.5)	16 (9.4)	0.895	217 (63.8)	123 (36.2)	0.667	1.08 (0.77–1.50)
>60 years	132	46 (34.8)	72 (54.5)	14 (10.6)		164 (62.1)	100 (37.9)		
Histological type									
ADC	1,381	49 (35.5)	75 (54.3)	14 (10.1)		173 (62.7)	103 (37.3)		
SCC	138	47 (34.1)	77 (55.8)	14 (10.1)	0.967	171 (62.0)	105 (38.0)	0.861	1.03 (0.73–1.46)
Others	26	13 (50.0)	11 (42.3)	2 (7.7)	0.376	37 (71.2)	15 (28.8)	0.364	0.68 (0.36–1.30)
Differentiation									
Poor	204	72 (35.3)	111 (54.4)	21 (10.3)	0.898	255 (62.5)	153 (37.5)	0.670	0.93 (0.65–1.32)
Moderate–well	98	37 (37.8)	52 (53.1)	9 (9.2)		126 (64.3)	70 (35.7)		
Tumor size									
T1	34	16 (47.1)	16 (47.1)	2 (5.9)		48 (70.6)	20 (29.4)		
T2	192	72 (37.5)	101 (52.6)	19 (9.9)	0.509	245 (63.8)	139 (36.2)	0.280	1.36 (0.78–2.39)
T3	51	15 (29.4)	28 (54.9)	8 (15.7)	0.161	58 (56.9)	44 (43.1)	0.070	1.82 (0.95–3.51)
T4	25	6 (24.0)	18 (72.0)	1 (4.0)	0.156	30 (60.0)	20 (40.0)	0.230	1.60 (0.74–3.45)
Lymph node									
N0	157	55 (35.0)	87 (55.4)	15 (9.6)		197 (62.7)	117 (37.3)		
N1	107	48 (44.9)	51 (47.7)	8 (7.5)	0.270	147 (68.7)	67 (31.3)	0.167	0.77 (0.53–1.12)
N2	38	6 (15.8)	25 (65.8)	7 (18.4)	0.042[a]	37 (48.7)	39 (51.3)	0.024[a]	1.78 (1.08–2.95)
Metastasis									
M0	285	103 (36.1)	156 (54.7)	26 (9.1)	0.142	362 (63.5)	208 (36.5)	0.371	1.37 (0.68–2.76)
M1	17	6 (35.3)	7 (41.2)	4 (23.5)		19 (55.9)	15 (44.1)		
Clinical stage									
I + II	223	90 (40.4)	113 (50.7)	20 (9.0)	0.033[a]	193 (55.8)	153 (44.2)	0.986	0.99 (0.68–1.46)
III + IV	79	19 (24.1)	50 (63.3)	10 (12.7)		88 (55.7)	70 (44.3)		

Note: [a]Statistically significant.
Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; OR, odds ratio; CI, confidence interval.

response to the chemical carcinogens methylcholanthrene and 7,12-dimethylbenz[a]anthracene.[37] These results verified the notion of CD226 plays a crucial role in immune surveillance of tumor development.[34] In addition, one recently published study showed that DNAM-1 was expressed by Vg9Vd2 T cells, and Nectin-like-5 but not Nectin-2 was involved in DNAM-1-dependent gammadelta T-cell functions.[38] Another point is that activated NK cell receptors play a role in $\gamma\delta$T cell cytotoxicity, and monoclonal antibody-mediated masking experiments have revealed that cytotoxicity and interferon-γ production by human $\gamma\delta$T cells in response to hepatocellular carcinomas was CD226-dependent.[22,38] Therefore, CD226 may act as a tumor surveillance receptor in NK cells.

Interestingly, our present study demonstrated that SNP rs763361 of allele T and SNP rs727088 of allele G were associated with NSCLC, and with lymph node invasion and clinical stage cancer. More importantly, we have provided the first useful evidence that the *CD226* gene is associated with NSCLC in the Chinese population. CD226 may be used as a predictor for monitoring cancer, and more importantly, a possible immunotherapy target, which may be useful in clinical applications. Further studies with larger sample sizes in populations of different ethnicities are necessary to confirm our findings.

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Disclosure

The authors report no conflicts of interest in this work.

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