Sequencing of DC-SIGN promoter indicates an association between promoter variation and risk of nasopharyngeal carcinoma in cantonese

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Abstract

Background: The dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) is an important pathogen recognition receptor of the innate immune system. DC-SIGN promoter variants play important role in the susceptibility to various infectious diseases. Nasopharyngeal carcinoma (NPC) is a malignancy that is common in southern China and whether DC-SIGN promoter variants have effects on susceptibility to NPC is still unknown. The aim of this study is to ascertain the potential involvement of DC-SIGN promoter single nucleotide polymorphisms (SNPs) in NPC susceptibility.

Methods: We conducted a case control study based on Cantonese population including 444 NPC patients and 464 controls matched on age and sex. The 1041 bp of DC-SIGN promoter region was directly sequenced for all samples. Sequence alignment and SNP search were inspected using DNAStar analysis programs and haplotype frequencies were estimated in Haploview V 4.0. The associations between the SNPs and the risk of NPC were analyzed using chi-square test and non-conditional logistic regression analysis with SPSS 13.0 software.

Results: A total of six variants were observed in the DC-SIGN promoter region and DC-SIGN -139 GG and -939 AA were significantly associated with NPC risk with adjusted Odds Ratios (ORs) of 2.10 (95% confidence interval [CI] = 1.23-3.59; \( P = 0.006 \)) and 2.52 (1.29-4.93; \( P = 0.007 \)) respectively and subjects carrying the risk allele DC-SIGN -871 G had 1.47-fold (95% CI = 1.14-1.90) increased risks of developing NPC (\( P = 0.003 \)). Haplotype analysis revealed that h1 ‘AAAG’ was significantly associated with protection against NPC (OR = 0.69; \( P = 0.0002 \)) and the association was still significant when using 1000 permutation test runs (\( P = 0.001 \)).

Conclusions: Our study indicated that DC-SIGN promoter variants appear to be involved in the susceptibility to NPC and the detailed mechanism of this effect need further studies.

Background

Nasopharyngeal carcinoma (NPC) is rare in most populations around the world but common in China and Southeast Asia, where the incidence can reach 20 to 50 per 100,000 individuals [1-5]. Epstein-Barr virus (EBV) is considered a major risk factor for NPC, and there is a dose-response relationship between EBV antibodies and NPC risk [6,7]. EBV is present in over 90% of the world population [8], most often as a form of in vivo latency in healthy carriers with low copies of episomal virus maintained in resting memory B cells [9-11].

DC-SIGN (Dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin), encoded by CD209 on chromosome 19p13.3, is a C-type lectin that is expressed on subsets of dendritic cells (DCs) and alveolar macrophages [12-15], and functions both as a cell adhesion receptor and as a pathogen recognition receptor [16]. Acting as a pathogen uptake receptor, DC-SIGN could mediate interactions with a plethora of pathogens [17] including bacteria such as Helicobacter pylori [18]; viruses such as HIV-1 [19], Ebola [20,21], Cytomegalovirus [22], Hepatitis-C virus [23,24], Dengue...
virus [20,21,25], and SARS-coV [26], and parasites such as Leishmania pifanoi [27]. Several studies have recently reported on the role of DC-SIGN promoter variants in the susceptibility to or pathogenesis of various infectious diseases, such as dengue fever [25,28], tuberculosis [13,14,29,30], Acquired Immune Deficiency Syndrome (AIDS) [19,31-33], celiac disease [34]. Sakuntabhai et al. [25] reported that the G allele of the variant DC-SIGN -336 was associated with strong protection against dengue fever [35]. In addition, several previous reports suggested that variants in the DC-SIGN promoter conferred protection against tuberculosis [29,30]. However, whether DC-SIGN promoter variants have effects on susceptibility to NPC is still unknown and so far no study has reported on the variants in the DC-SIGN promoter in Cantonese population.

Therefore, we explored the relationship between DC-SIGN promoter polymorphisms and susceptibility to NPC by determining DC-SIGN promoter sequence variation in a case-control study in Cantonese.

Methods

Study subjects

All subjects were unrelated Cantonese population in Guangdong Province, China. Cases were recruited consecutively from December 2005 to October 2006 with pathologically confirmed diagnosis of NPC at the Sun Yat-Sen University Cancer Center (SYSUCC), Guangzhou, China. Total 500 NPC patients were collected and 444 were Cantonese origin living in Southern China. Population controls were cancer-free individuals, randomly selected from individuals who attend annual community-based physical examinations during the same time period. The selection criteria for control subjects included no individual history of cancer, all of them were Cantonese, and matched to NPC cases by age (± 5 years), sex and the time period for blood sample collection. Total 464 controls were involved. All study subjects had signed informed consent agreements before epidemiological data and blood samples were collected by trained SYSUCC staff interviewers.

For both cases and controls, venous blood specimens totalling 5-10 ml were collected from subjects and genomic DNA was then extracted from the lymphocytes using the QIAamp DNA Blood Midi Kit (Qiagen, German) following the manufacturer’s protocol. These procedures were reviewed and approved by the Human Ethics Approval Committee of SYSUCC.

Genotyping of DC-SIGN promoter variants

A region approximately 1,041 bp upstream of the ATG start codon that includes the promoter region was amplified using the following primers: 5’- GCAGTCTTTGGTTCCTTGGAG -3’ for forward primer 1 and 5’-ACTTGCGATGCCTCCTCAGT -3’ for reverse primer 1; 5’-TGCTTGCTGTCCTCATTTTGG-3’ for forward primer 2 and 5’-AGCATACAGAAACCCGTTTGG-3’ for reverse primer 2. Primer 1 delimits the promoter region between nt -602 and 28 [GenBank: NC_000019.9] and amplifies a 630 bp fragment. Primer 2 delimits the promoter region between nt -404 and nt -1041 and amplifies a 638 bp fragment. Polymerase chain reaction (PCR) amplification was performed in a volume of 20 μL as follows: 15.85 μL ddH2O, 2.0 μL 10 × reaction buffer (with Mg2+), 0.5 μL 4 × dNTP (10 mmol L−1), 0.2 μL of each primer (20 μM), 1.25 U Taq DNA polymerase, and 1 μL genomic DNA (20 ng). Touchdown PCR was performed in the model 9700 GeneAmp PCR system (Applied Biosystems, Foster City, CA) with the following conditions: one initial denaturation of 95°C for 5 minutes, and then 5 cycles of 94°C for 30 seconds, 61°C for 30 seconds (-0.5°C every cycle), and 72°C for 45 seconds; then 32 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, followed by one elongation step at 72°C for 10 minutes. The amplified products were analysed by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. PCR amplification was performed using the same conditions for primer 1 and primer 2 as described above.

PCR products were recovered, and further purified. Sequencing reactions were performed using PCR primers and all nucleotide sequences were obtained using the 3730 automated sequencer (Applied Biosystems). Sequence alignment and SNP search were inspected using DNASTar analysis programs (DNASTar, Madison, WI, USA) using the nucleic acid sequences from GeneBank at National Center for Biotechnology Information (NCBI) as the prototype sequence [GenBank: NC_000019.9].

Statistical analysis

To test the different promoter polymorphisms of DC-SIGN gene for a possible distortion in genotypic and allelic frequencies between cases and controls, a chi-square test or Fisher’s exact test was used to compare the genotypic and allelic distribution between cases and controls. In addition, to control confounding factors, unconditional logistic regression analysis was conducted to compare the genotype frequencies between cases and controls by adjusting for age, sex, the level of educational. The analyses were performed in SPSS software for Windows, version 13.0 (SPSS). For power calculation, the QUANTO program (Version 1.2) was used. Haplotype frequencies were estimated using the accelerated expectation maximisation algorithm implemented in Haploview V 4.0 [36]. Haplotype frequencies occurring at <5% were excluded from the analysis. Association testing for the haplotypes was performed using the
The minor allele frequencies (MAFs) of all SNPs presented detailed information on the six variants: -939 G/A, -871 A/G, -336 A/G, -190 A/G, -139 A/G and -116 G/T.

Results
This study included a total of 444 NPC patients and 464 control subjects. The characteristics of study subjects are summarised in Table 1. The mean age (± standard deviation) of the patients was 41.7 ± 9.6 y and 61.0% of the cases were male. For controls, the age was 41.3 ± 10.6 y and 60.1% of subjects were male. There was no statistically significant difference between cases and controls with respect to the frequency distributions of age and the sex distribution was unbiased (all P-values > 0.05). There was significant association between the level of education and NPC susceptibility. In current study, 21.7% of the case patients versus 8.0% of control subjects were illiterate or had an education of primary school (P < 0.001).

Direct sequencing of the promoter region of DC-SIGN revealed the occurrence of six variants: -939 G/A, -871 A/G, -336 A/G, -190 A/G, -139 A/G and -116 G/T. Table 2 presents detailed information on these six variants. The minor allele frequencies (MAFs) of all SNPs evaluated in the DC-SIGN promoter were >0.05 except for -190 A/G (MAF = 0.003) and -116 G/T (MAF = 0.006), and all loci fit Hardy-Weinberg equilibrium expectations (P-values > 0.05).

Table 3 presents the genotypic and allelic frequencies of these six variants among Cantonese population. For DC-SIGN -139 A/G, subjects carrying mutant genotype -139 AG, which means a SNP -139 A/G in heterozygous alleles and -139 GG, which means a SNP -139 A/G in both G alleles, had 1.42-fold (95% CI = 1.07-1.86) and 1.99-fold (95% CI = 1.20-3.30) increased risks of developing NPC, respectively, when compared with those carrying wild genotype -139 AA. After adjusting for age, sex and the level of education, genotype -139 AG and -139 GG had 1.41-fold (95% CI = 1.05-1.88) and 2.10-fold (95% CI = 1.23-3.59) increased risks of developing NPC, respectively, when compared with those carrying wild genotype -139 AA (P trend = 0.005). Furthermore, subjects carrying the risk allele -139 G had 1.42-fold (95% CI = 1.15-1.74) increased risks of developing NPC when compared with those carrying allele -139 A (P = 0.001).

Table 1 Demographic characteristics and socio-economic status of the study population

| Variables                  | Case (n = 444) | Control (n = 464) | P-value* |
|----------------------------|---------------|------------------|---------|
| Gender (%)                 |               |                  |         |
| Male                       | 271 (61.0)    | 279 (60.1)       | 0.78    |
| Female                     | 173 (39.0)    | 185 (39.9)       |         |
| Age(%) mean ± SD (y)       | 41.7 ± 9.6    | 41.3 ± 10.6      | 0.71    |
| ≤30                        | 49 (11.0)     | 61 (13.1)        |         |
| 31-40                      | 163 (36.7)    | 158 (34.1)       |         |
| 41-50                      | 162 (36.5)    | 163 (35.2)       |         |
| 51-60                      | 51 (11.5)     | 60 (12.9)        |         |
| 61+                        | 19 (4.3)      | 22 (4.8)         |         |
| Level of education         |               |                  |         |
| Illiteracy or primary school| 94 (21.7)     | 37 (8.0)         | <0.001  |
| High school                | 268 (61.8)    | 255 (55.1)       |         |
| University or above        | 72 (16.6)     | 171 (36.9)       |         |

*P-values were calculated using the chi-square test.
Table 2 The minor allele frequencies and Hardy-Weinberg equilibrium tests of 6 variants in DC-SIGN gene

| Polymorphism   | NCBI rs number | Position | Genotype | Gene region | MAF § | PH-W¶ | % Geno -typed |
|---------------|----------------|----------|----------|-------------|-------|--------|---------------|
| DC-SIGN -116  | rs2287886      | –        | G/T      | Promoter    | 0.006 | 1.00   | 100.0         |
| DC-SIGN -139  | rs2287886      | –        | A/G      | Promoter    | 0.275 | 0.64   | 100.0         |
| DC-SIGN -190  | rs4804803      | –        | A/G      | Promoter    | 0.003 | 1.00   | 100.0         |
| DC-SIGN -336  | rs735239       | –        | A/G      | 5'flanking  | 0.154 | 0.58   | 100.0         |
| DC-SIGN -871  | rs735239       | –        | A/G      | 5'flanking  | 0.222 | 0.71   | 100.0         |

† The chromosome position listed here is taken from the NCBI database dbSNP build 130.
‡ First allele is major allele, the second is minor allele.
§ MAF denotes Minor Allele Frequency.
¶ PH-W represents the P value of Hardy-Weinberg equilibrium tests.

Table 3 Association between DC-SIGN promoter variants and NPC

| SNP         | Genotype | Case | Control | Unadjusted OR (95% CI)* | Unadjusted P-value* | Adjusted OR (95% CI) # | Adjusted P-value # |
|-------------|----------|------|---------|-------------------------|---------------------|------------------------|--------------------|
| DC-SIGN -116| GG       | 440  | 457     | ref                     | ref                 |                        |                    |
|             | GT       | 4    | 7       | 0.59 (0.17-2.04)        | 0.40                | 0.57 (0.16-2.01)       | 0.38               |
|             | TT       | 0    | 0       | –                      | –                   | –                      | –                  |
| Allele G    | 884      | 921  | ref     |                         |                     |                        |                    |
|             | T        | 4    | 7       | 0.60 (0.17-2.04)        | 0.40                | –                      | –                  |
| DC-SIGN -139| AA       | 212  | 268     | ref                     | ref                 |                        |                    |
|             | AG       | 188  | 168     | 1.42 (1.07-1.86)        | 0.01                | 1.41 (1.05-1.88)       | 0.02               |
|             | GG       | 44   | 28      | 1.99 (1.20-3.30)        | 0.007               | 2.10 (1.23-3.59)       | 0.006              |
| Allele A    | 612      | 704  | ref     |                         |                     |                        |                    |
|             | G        | 276  | 224     | 1.42 (1.15-1.74)        | 0.001               | –                      | –                  |
| DC-SIGN -190| AA       | 441  | 462     | ref                     | ref                 |                        |                    |
|             | AG       | 3    | 2       | 1.57 (0.26-9.45)        | 0.68                | 1.57 (0.24-10.36)      | 0.64               |
|             | GG       | 0    | 0       | –                      | –                   | –                      | –                  |
| Allele A    | 885      | 926  | ref     |                         |                     |                        |                    |
|             | G        | 3    | 2       | 1.57 (0.26-9.42)        | 0.68                | –                      | –                  |
| DC-SIGN -336| AA       | 365  | 396     | ref                     | ref                 |                        |                    |
|             | AG       | 77   | 63      | 1.33 (0.92-1.91)        | 0.13                | 1.43 (0.98-2.09)       | 0.07               |
|             | GG       | 2    | 5       | 0.43 (0.08-2.25)        | 0.45                | 0.57 (0.11-3.07)       | 0.51               |
| Allele A    | 807      | 855  | ref     |                         |                     |                        |                    |
|             | G        | 81   | 73      | 1.18 (0.85-1.64)        | 0.34                | –                      | –                  |
| DC-SIGN -871| AA       | 301  | 352     | ref                     | ref                 |                        |                    |
|             | AG       | 127  | 104     | 1.43 (1.06-1.93)        | 0.02                | 1.44 (1.05-1.98)       | 0.03               |
|             | GG       | 16   | 8       | 2.34 (0.99-5.54)        | 0.05                | 1.97 (0.80-4.88)       | 0.14               |
| Allele A    | 729      | 808  | ref     |                         |                     |                        |                    |
|             | G        | 159  | 120     | 1.47 (1.14-1.90)        | 0.003               | –                      | –                  |
| DC-SIGN -939| GG       | 251  | 301     | ref                     | ref                 |                        |                    |
|             | AG       | 161  | 148     | 1.31 (0.99-1.72)        | 0.06                | 1.28 (0.95-1.71)       | 0.11               |
|             | AA       | 32   | 15      | 2.56 (1.36-4.83)        | 0.003               | 2.52 (1.29-4.93)       | 0.007              |
| Allele G    | 663      | 750  | ref     |                         |                     |                        |                    |
|             | A        | 225  | 178     | 1.43 (1.15-1.79)        | 0.002               | –                      | –                  |

* Odds ratios and P-values were calculated using the chi-square test.
# Odds ratios and P-Values were calculated by adjusting for age, sex, educational level.
However, when using 1000 permutation test runs, there was no significant association between haplotype $h_2$ ‘GAGA’ and NPC phenotype ($P = 0.23$).

**Discussion**

This report on the distribution of genetic polymorphisms in the DC-SIGN promoter in the Cantonese population revealed that DC-SIGN -139 GG and -939 AA were significantly associated with increased risk of NPC and the risk allele DC-SIGN -871 G was significantly associated with NPC susceptibility (Table 3). Haplotype analysis revealed that $h_1$ ‘AAAG’, which contains all four wild-type SNPs (-139 A, -336 A, -871 A and -939 G), was associated with a significantly decreased risk of NPC and $h_2$ ‘GAGA’ was significantly associated with the NPC phenotype (Table 4). Moreover, we found two new variants in Cantonese population, -116 G/T and -190 A/G, although the MAFs for both were low (0.6% and 0.3%, respectively).

In current study, the MAFs of four common SNPs (-139 A/G, -336 A/G, -871 A/G and -939 G/A) in the promoter region of DC-SIGN in Cantonese population were similar to that in 45 unrelated Han Chinese in Beijing, China (HCB) http://www.ncbi.nlm.nih.gov/projects/SNP/snp. In addition, Kashima et al. [37] found that the allelic frequency of DC-SIGN -332 A was 10.7% by sequencing 28 Asians; however, this SNP was neither detected by Barreiro et al. in Asians [30] nor by Koizumi et al. in Japanese individuals [31]. Equally, this SNP is not detected in individuals of Cantonese in current study, which indicates that DC-SIGN -332 A is not widespread in most Asians. Two other SNPs in the promoter region, DC-SIGN -745 G/T and -201 G/T, were not present in current cohort, though they were observed exclusively in the Zimbabwean population [38]. The allelic distribution of DC-SIGN genes differs widely in populations from different ethnic groups, presumably the result of selective pressure exerted by prevalent pathogens in these geographically distinct regions. NPC is rare in most populations around the world but common in China and Southeast Asia and this could be reflected in SNP frequencies. DC-SIGN, a protein expressed on the surface of DCs, has recently received considerable attention in research on AIDS [33], dengue [25,35], tuberculosis [29,30] and Ulcerative Colitis [39]. Previous studies have indicated that DC-SIGN -336 G is associated with protection against dengue disease in Thailand population [25] and tuberculosis disease in sub-Saharan Africa individuals [29]. It may mainly due to the location of the DC-SIGN -336 SNP 214 bp upstream of the major transcription site, affecting a Sp1-like binding site and further modulating DC-SIGN transcriptional activity [25]. However,
Barreiro and colleagues [30] found that -336 A and -871 G variants conferred protection against tuberculosis in Eurasian populations. Meriem Ben-Ali et al. [40] found no association between DC-SIGN promoter variation and susceptibility to tuberculosis in Tunisian patients. These contrasting results may be due to significant differences in the distribution of DC-SIGN alleles in different ethnic populations [38]. Different population genetic backgrounds as well as differences in linkage disequilibrium (LD) patterns can be at the basis of the conflicting results. In current study, there was no observable association between DC-SIGN -336 SNP and NPC susceptibility in the Cantonese population.

The mechanism of involvement of mutant DC-SIGN -139 and 939 in the pathogenesis of NPC remains unknown. Previous studies have already demonstrated a higher frequency of allele DC-SIGN -139 A in individuals not infected with HIV compared with infected patients [32]. In another study, allele -139 G was found to be associated with the rapid progression of AIDS in a population of Japanese haemophiliacs [31]. In current study, we found that the frequencies of DC-SIGN -139 GG and -939 AA were significantly higher in NPC patients compared with healthy controls. One potential mechanism for this effect may involve differential inducible expression of DC-SIGN on blood DCs as a result of these two polymorphisms, but this remains to be demonstrated. DC-SIGN -139 is located close to one of the binding sites of the transcription factor AP-1 in the promoter region of DC-SIGN, and we could speculate that the substitution of one nucleotide close to this site may change the level of expression of DC-SIGN and further contribute to the progression of NPC.

As for DC-SIGN -939, it is yet to be determined whether this variant will affect the expression of DC-SIGN.

EBV is an important etiological agent of NPC and establishes persistent infections by employing multiple strategies to evade host immune responses. Consistent with the critical function of DCs in anti-viral immunity, myriad viruses are known to infect different subsets of DCs and to affect their differentiation, survival, and migration and/or T cell stimulatory capacity [41-44]. However, no studies have been performed so far to determine whether DC-SIGN is the EBV receptor. EBV has been observed to infect DC-SIGN positive cells such as immature DCs [45], monocytes [46-48] and some macrophages [49-51]. Furthermore, Li et al. [45] showed that EBV infection inhibited DC development from monocyte precursors, and further showed that immature DCs that become resistant to EBV-induced apoptosis still support virus entry [50]. Guerreiro-Cacais AO et al reported that EBV-infected macrophages could facilitate dissemination of EBV within the oral mucosal epithelium [50]. Recently, DC-SIGN could serve as putative receptor for secretory IgA (SlgA) on immature DCs by binding to high mannose glycoprotein on SlgA protein has been reported [52]. Sixbey JW et al have demonstrated that EBV-SlgA complex promoted EBV infection of epithelial cells through secretory component-mediated IgA transport [53]. We hypothesize that DC-SIGN expressed on immature DCs may recognize EBV-SlgA complex though binding to SlgA and thus promote EBV infection of immature DCs. This link DC-SIGN to EBV infection of immature DCs was need further confirmed and the study is on the way in our Lab.

We would like to point out that the sample size of the current study was not large enough and these results need to be validated in larger samples. Despite limitations, the current study represents the first comprehensive genetic association study examining the relationship between DC-SIGN promoter genetic variants and NPC risk in a case-control study and supplying genetic data of DC-SIGN promoter polymorphism in Cantonese population.

**Conclusions**

Our study shows that the mutant genotypes -139 GG and -939 AA detected in the promoter region of the DC-SIGN gene were involved in NPC susceptibility, and further studies are necessary to demonstrate the role of DC-SIGN promoter polymorphisms in the function of DC-SIGN as well as their effect on EBV infection.

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**Authors’ contributions**

Designed the study: WHJ and MSZ; collected samples and experiments: WHJ, QSF, L2C, WSL, YFX, JQD, WLL, YXZ; performed the data analysis: YFX; writing the manuscript: YFX, WHJ and MSZ. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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