Fine mapping candidate loci for nasopharyngeal carcinoma in southern Chinese specifically linked to Epstein-Barr virus aetiopathogenesis

Y Li 李 艷
L Fu 付 利
AM Wong
YH Fan 范彥輝
MX Li 李淼新
JX Bei
WH Jia 賈衛華
YX Zeng 曾益新
D Chan 陳振勝
KM Cheung 張文智
P Sham 沈伯松
D Chua 蔡清澳
XY Guan 關新元
YQ Song 宋又強

Key Messages

1. Nasopharyngeal carcinoma (NPC) is a malignancy of epithelial origin.
2. The aetiology of NPC is complex and includes multiple genetic and environmental factors.
3. Genetic factors for NPC were detected on chromosome 6p regions.

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The University of Hong Kong:
Department of Biochemistry
Y Li, YH Fan, MX Li, D Chan, YQ Song
Department of Clinical Oncology
L Fu, AM Wong, D Chua, XY Guan
Department of Orthopaedics and Traumatology
KM Cheung
Department of Psychiatry
P Sham
State Key Lab of Oncology in Southern China & Department of Experimental Research, Sun Yat-sen University Cancer Centre, Guangzhou, China
JX Bei, WH Jia, YX Zeng

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Principal applicant and corresponding author:
Dr Song You-Qiang
L3-63, Lab Block, 21 Sassoon Road,
Pokfulam, Hong Kong SAR, China
Tel: (852) 2819 9245
Fax: (852) 2855 1254
Email: songy@hku.hk

Introduction

Nasopharyngeal carcinoma (NPC) is of epithelial origin. Its aetiology is complex and comprises multiple genetic and environmental factors. There are distinct geographical and ethnic differences in its incidence. In Southeast Asians, particularly from the Chinese province of Guangdong, susceptibility to NPC is nearly 100-fold higher than in most persons from European countries. Thus, NPC is regarded as the 'Cantonese' cancer, with incidences ranging from 10 to 50 cases per 100 000 inhabitants in this region.[1] This NPC epidemic also shows familial aggregation.

Genetic linkage studies and the candidate-gene–based approach have been used to identify NPC susceptibility genes.[2] Notably, chromosome 6 super loci containing the human leukocyte antigen (HLA) system has been linked to the pathogenesis of NPC.[3-5] Two genome-wide association studies (GWAS) to scan the whole human genome for disease susceptibility loci reported an increased susceptibility in southern Chinese.[3,5]

The linkage of NPC to 6p21.3 provides a genetic basis for a more thorough linkage analysis for disease susceptible loci in different populations. We studied the NPC-associated genetic markers using case-control analysis. The top 15 NPC genes within the linkage region were chosen from PubMed references, and then tag single nucleotide polymorphisms (tag SNPs) within the genes were selected from the HapMap CHB database. In total, 233 tag SNPs on chromosome 6p were selected to test whether they were associated with NPC in the southern Chinese.

Methods

This study was conducted from January 2009 to December 2010. Ethics approval for this study and written informed consent from all participants were obtained. The disease group included 360 patients of southern Chinese descent from Guangdong with pathologically confirmed diagnosis of NPC. Their mean±standard deviation (SD) age was 46.4±11.2 years; 72% were males. The control group included 360 southern Chinese subjects with degenerate disc disease. Their mean±SD age was 41.4±8.9 years; 66% were females.

The SNPs were selected based on the candidate gene. According to the degenerate disc disease study, the whole genome scan data had 17 313 SNPs genotyped on chromosome 6. Focusing solely on the genes located in the candidate region identified by the meta-analysis of the top candidate genes, 2730 SNPs remained. Only 233 tag SNPs were selected for genotyping.

The MassARRAY Assay genotyping method (Sequenom) was used to genotype according to the manufacturer's protocol. The genotyping of 12 significant SNPs from a Taiwanese group was conducted using ABI Taqman SNP genotyping assays. Human pre-designed Taqman probes were provided by

the Taiwanese group. Real-time data were analysed using the SDS 2.3 application provided by ABI.

To examine candidate gene expression, 20 primary NPC biopsies and adjacent normal tissues at the resection margins were collected immediately after surgical resection at Queen Mary Hospital in Hong Kong. The three NPC cell lines used (CNE2, SUNE1, and C666-1) were maintained in RPMI-1640 medium and supplemented with 10% foetal bovine serum. An immortalised nasopharyngeal epithelial cell line (NP69) was also cultured.

Total RNA from the cell lines was extracted using the Trizol reagent and following the manufacturer's protocol. The Transcriptor High Fidelity cDNA Synthesis Kit was used to synthesise cDNA.

For quantitative PCR analysis, cDNA was subjected to amplification with the SYBR Green PCR Kit using primers for NEDD9 and GABBR1. Human 18S rRNA was used as the endogenous control. The threshold cycle was determined in real time using an ABI PRISM 7700 Sequence Detector.

The association analyses were performed by the PLINK and Haploview 4.2 method. The haplotype structure was also analysed by PLINK using the three-SNP sliding window option. Multiple testing was performed with 10 000 permutations and/or with Bonferroni correction. LocusZoom was used to generate the association plot.

Results

Genetic association study of 6p SNPs in southern Chinese

The overall genotyping call rate was ≥ 97.8%. Genotyped SNPs were arranged according to their physical locations on chromosome 6 and allelic associations ($-\log^{10}$ P values). The most significant association was found for SNP rs2076483 ($P=3.36 \times 10^{-5}$). Two adjacent SNPs, rs2267633 ($P=4.49 \times 10^{-5}$) and rs29230 ($P=1.43 \times 10^{-4}$), located at the 6p23.31 region also showed high significance, suggesting that this region was significantly associated with NPC (data not shown).

Haplotype analysis of the GABBR1 and HLA-A gene regions

The most significantly associated haplotypes—AAA ($P=6.46 \times 10^{-5}$) and GGG ($P=1.0 \times 10^{-4}$)—were located within GABBR1 and comprised three significant SNPs (rs2267633, rs2076483, and rs29230). Haplotype AAA of GABBR1 had a highly significant P value of 6.46×10^{-5}. This indicates that individuals carrying the AAA haplotype could be more susceptible to NPC than GGG carriers. In contrast, the haplotype GG composed of rs2517713 and rs2975042 within the HLA-A gene showed a protective effect against NPC ($P=7.0 \times 10^{-4}$), whereas the haplotype TT exhibited high risk of NPC disease (TT, P=0.0014). Multiple testing correction was conducted with 10 000 permutations; haplotypes AAA (P=0.0008) and GGG (P=0.0010) of GABBR1 and haplotypes GG (P=0.0072) and TT (P=0.0134) of HLA-A all survived the multiple testing.

Using the three-SNP sliding windows, haplotypes AAA and GGG formed by significant SNPs (rs2267633, rs2076483, and rs29230) reached statistical significance ($P=7.610 \times 10^{-5}$ and $P=7.614 \times 10^{-5}$, respectively). Two SNPs haplotypes formed by rs2517713 and rs2975042 were GG and TT, with P values of 0.00078 and 0.00078, respectively, and were even more significant than Haploview results.

Loss of heterozygosity and micro-deletions at 6p as detected by SNP genotyping

The high resolution of the SNP array and the large sample size enabled us to monitor the small DNA copy number changes occurring in NPC. To identify the micro-deletions at 6p in NPC, the frequency of the homozygous genotype in controls and cases should be determined first. For each SNP marker, the ratio of homozygous frequency between the cases and the controls (T/N ratio) was calculated. Using a threshold T/N ratio of >1.0, 19 loci that reached statistical significance were considered loci liable to frequent loss of heterozygosity (LOH). The micro-deleted region was defined when three or more adjacent SNP markers were considered frequent LOH loci. Three micro-deletions were identified at 6p25-24, 6p21.31, and 6p21.3 (Table). The small deletions on 6p affected several genes, including glucosaminyl (N-acetyl) transferase 2, I-branching enzyme (GCNT2), (NEDD9), and GABBR1 (Table). The genes at 6p21.3 were pseudogenes and thus not further studied. The GCNT2 gene has never been linked to cancer development. NEDD9 and GABBR1 were the most promising potential candidate genes. We examined their mRNA expression levels in different cell lines and tissues.

Examination of mRNA expression of candidate NPC susceptibility genes

To study the two candidate genes for the development of NPC, mRNA expression was characterised by quantitative real-time PCR in three NPC cell lines (CNE2, SUNE1, and C666-1) in the immortalised normal nasopharyngeal epithelial cell line NP69, and in 11 primary NPC tissue samples with adjacent normal tissue. Compared to the normal nasopharyngeal epithelial cell line NP69, the SUNE1 and C666-1 cells demonstrated lower NEDD9 mRNA expression. Conversely, the CNE2 cell line displayed an estimated 4-fold increase in NEDD9 expression (data not shown). Compared with adjacent non-tumour tissue, 10 of 11 NPC tumour biopsy samples showed a significant downregulation of NEDD9 (P=0.015; data not shown). Moreover, GABBR1 was downregulated in all three NPC cell lines (data not shown) and in eight of 11 tumour biopsy tissues (data not shown). The GABBR1 gene showed a marginally significant association between the NPC tumour and non-tumour tissue specimens (P=0.059).

Table. Summary of frequent loss of heterozygosity loci at 6p detected by single nucleotide polymorphism (SNP) array

SNP ID*	Location	Homozygous frequency ratio of cases/controls	P value	Gene
rs2085575	6p25.3-24.3	1.2073538	0.004	F13A1
rs3024317	6p25.3-24.3	1.1449631	0.0181	F13A1
rs4960294	6p25	1.1457735	0.0298	RREB1
rs6597256	6p25	1.1318706	0.017	RREB1
rs267184	6p24-23	1.1431448	0.0253	BMP6
rs504083	6p24.2	1.1581754	0.0281	GCNT2
rs1318748	6p24.2	1.1529571	0.0371	GCNT2
rs11759513	6p25-24	1.1893557	0.0232	NEDD9
rs2137873	6p23	1.1861716	0.011	ATXN1
rs235147	6p23	1.1638418	0.03	ATXN1
rs236949	6p23	1.1905564	0.0047	ATXN1
rs2143083	6p22.3-22.2	1.3092179	0.0001	ALDH5A1
rs2267633	6p21.31	1.1809524	0.0033	GABBR1
rs2076483	6p21.31	1.2159952	0.0007	GABBR1
rs29230	6p21.31	1.1642882	0.0007	GABBR1
rs2517713	6p21.3	1.1925186	0.0109	HCP5P3
rs9260734	6p21.3	1.2099734	0.0033	HCG2P6
rs3869062	6p21.3	1.1733857	0.0101	HCG2P6
rs5009448	6p21.3	1.1841842	0.0164	MICD

* SNP markers at micro-deleted region in bold

Discussion

Multiple loci within 6p21.3 were associated with NPC susceptibility. Using samples from southern China, we found significant allelic and haplotype associations with NPC. Consistent with other reports,[3-5] the HLA-A region was significantly associated with NPC. The most significant SNPs were similar to those found in a Taiwanese GWAS.[3] The subjects analysed were all southern Chinese and the MAFs were similar; such deviation might be due to genetic heterogeneity.

GABBR1 underscores a possible role in the aetiology of NPC

Two candidate genes located within the micro-deleted regions, NEDD9 at 6p25-24 and GABBR1 at 6p21.31, were absent or downregulated at the mRNA expression level in primary NPC tumours and NPC cell lines. Although the sample size (n=11) used for quantitative real-time PCR analysis was not sufficient for statistical calculation, the quantitative PCR results did show alterations in gene expression levels. The different expression changes were from the copy number variations in the tumour DNA, and no direct link to the micro-deletion was detected when comparing the normal DNA in cases and controls. However, the different expression levels of NEDD9 and GABBR1 between PNC tumours and normal tissues indicated the importance of both genes in NPC development. A population-based study to determine whether micro-deletion in normal DNAs can also reduce GABBR1 expression is warranted. This study provides the first evidence that the NEDD9 gene is subject to down-regulation at the transcriptional level due to copy number loss in NPC tumours.

The Taiwan GWAS was the first study to associate GABBR1 with NPC and reported elevated GABBR1 protein expression in NPC tumour tissues (compared with the adjacent normal epithelial cells).[3] In another GWAS conducted in Guangzhou,[5] the strong association within the HLA regions on 6p was validated. In addition, three new NPC susceptibility loci were detected on 3q26, 9p21, and 13q12, and novel risk genes were also identified. Other candidate genes and cancer genesis mechanisms could underlie the NPC pathogenic process. In view of the high prevalence of NPC in the southern Chinese population, future studies on NPC should focus on novel pathogenic loci to discover new tumourigenic genes and provide clinical targets for treatment.

Conclusions

Significant single-marker associations were found for SNPs rs2267633 ($P=4.49 \times 10^{-5}$), rs2076483 ($P=3.36 \times 10^{-5}$), and rs29230 ($P=1.43 \times 10^{-4}$). Multiple chromosome 6p susceptibility loci contributed to the risk of NPC.

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