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Polymeric immunoglobulin receptor polymorphisms and risk of nasopharyngeal cancer

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Abstract

Background: Epstein-Barr virus (EBV) associated nasopharyngeal cancer (NPC) is an important squamous cell cancer endemic in Southeast Asia and the Far East and can be considered a multifactorial genetic disease. This research explores potential associations between nasopharyngeal epithelial EBV receptor and NPC susceptibility. To prove the hypothesis, we evaluated two candidate genes, complement receptor 2 (*CR2*) and polymeric immunoglobulin receptor (*PIGR*) by using 4 SNPs, *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T, *PIGR*1093G→A and *PIGR*1739C→T, to genotype 175 cases and 317 controls, divided into Thai, Chinese and Thai-Chinese based on their respective ethnic origins.

Results: The results obtained indicated that *PIGR* is an NPC susceptibility gene. The risk association pertaining to each ethnic group was detected for homozygous *PIGR*1739C with a significant ethnic group adjusted OR (95%CI) of 2.71(1.72–4.23) and $p < 0.00001$. Haplotype of the two missense *PIGR* SNPs, 1093G→A and 1739C→T, and sequence analyses have confirmed the role of the nucleotide *PIGR*1739 and excluded possibility of an additional significant nonsynonymous NPC susceptibility SNP.

Conclusions: We present genetic evidence leading to hypothesize a possibility of PIGR to function as the EBV nasopharyngeal epithelium receptor via IgA-EBV complex transcytosis failure. The *PIGR*1739C→T is a missense mutation changing alanine to valine near endoproteolytic cleavage site. This variant could alter the efficiency of PIGR to release IgA-EBV complex and consequently increase the susceptibility of populations in endemic areas to develop NPC.

Background

Nasopharyngeal cancer (NPC [MIM 161550]) constitutes an endemic multifactorial genetic disease [1]. Whereas the disease is quite rare in the Western world, it occurs at high

frequencies in Southern China, Southeast Asia, and among the Greenland Inuit. The highest aged-adjusted incidence has been reported in South China to be 30–50/100,000. Intermediate incidence has been noted in South East Asia with a ratio of 3/100,000 among Thais and 10/100,000 among Thais of Chinese extraction, respectively [2]. Regarding environmental carcinogenesis, both viral and chemical carcinogens contribute to NPC with Epstein Barr virus (EBV) as the most important etiological factor. The single clonally derived viral genome can be found in all endemic NPC cells [3–5]. On the other hand, several reports indicate that consuming salty fish or preserved food and thus concentrating chemical carcinogens such as nitrosamine, can promote the development of NPC [6,7]. Interestingly, two NPC susceptibility genes, *HLA* and cytochrome P450 2E1 (*CYP2E1*), were discovered based on their hypothesized relationship with environmental factors contributing to NPC etiology. HLA was first studied in Singapore as this gene would be responsible for patients' immune response to cancer or EBV infected cells [8]. In particular HLA A2 and B46 conferred a high relative risk as to NPC development according to various reports in Asia [9]. In addition, NPC development was proposed to correlate with patients' history of consuming preserved food and hence *CYP2E1*, which metabolises nitrosamine, was studied revealing a risk association in Taiwan [10] and Thailand [11]. This research aims at exploring the genetic aspect of EBV entry into the nasopharyngeal epithelium (NE). This process not only requires specific host factors but also differentiates between the mechanisms of EBV carcinogenesis originating in epithelial and/or lymphoid cells. Whereas EBV-associated Burkitt's lymphoma is prevalent in Africa, populations demonstrating a higher risk for developing NPC do not show any significant increase in the lymphoma incidence [12].

The mechanism of EBV entry into the NE has not yet been conclusively elucidated but at least two receptors, complement receptor type 2 (CR2) and polymeric immunoglobulin receptor (PIGR), have been proposed. CR2 is an integral membrane glycoprotein to which EBV can bind and thus infect B-lymphocytes [13]. EBV can infect recombinant epithelial cells expressing CR2 especially when in contact with virus-producing lymphocytes [14,15]. In addition, there has been a study reporting CR2 expression in embryonic NE cell by RT-PCR [16]. As for PIGR, the protein can be discovered on NE, interestingly as a pneumoccocus receptor, [17] where it mediates endocytosis and transcytosis of IgA-EBV complexes to deliver EBV into the luminal surface [18]. The viral translocation process can fail and consequently cause EBV infection if the epithelium loses its polarity or has mutated *PIGR* [19,20]. In addition to NE, EBV was reported to enter NPC in vitro depending on the presence of viral specific IgA [21]. Interestingly, serology studies indicate indirectly that PIGR is involved in NPC development. High EBV-IgA titers can be detected in a significant portion of NPC patients and the test has significant predictive role in diagnosing the disease [22]. Based on specific endemic distribution, NPC susceptibility genes can be hypothesized to originate from ancestor alleles. Hence, this study has been designed candidate-gene approaches for studying complex genetic traits [23]. The specific aim has been to explore whether *CR2* or *PIGR* might be NPC susceptibility genes by determining the risk association between their DNA polymorphisms and haplotypes among NPC patients and controls from several individuals of high-risk ethnic origin.

Results

PIGR *and* CR2 *SNPs NPC case-control study*

The study explored the risk of association between *CR2* or *PIGR* DNA polymorphisms and NPC phenotype. Both the 175 patients and 317 controls were separated into Thai, Chinese and Thai-Chinese, according to their grandparents' ethnic origin so that each group would have a higher probability of shared ancestors. The genotype of four SNPs, *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T,*PIGR*1093G→A and *PIGR*1739C→T, were investigated. *CR2*IVS2-848C→T located near exon 1, 2 [24], and *PIGR*IVS3-156G→T in intron 3 [25], have previously been published as RFLP polymorphisms. *PIGR*1093G→A and *PIGR*1739C→T, on exon 5 and 7, respectively, were selected from a SNP database http://www.ncbi.nlm.nih.gov/SNP/. *PIGR*1093G→A displays an amino acid alteration from glycine to serine and *PIGR*1739C→T from alanine to valine, respectively. No missense SNP from *CR2* has recently been reported by genbank. The amplicons of *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T and *PIGR*1739C→T were genotyped by PCR-RFLP, and *PIGR*1093G→A by ARMS (fig 1).

Upon comparison between the frequency of these alleles in patients and controls of identical ethnic origin, Thai or Chinese, a significant risk association to *PIGR*1739C→T was detected but neither to *PIGR*IVS3-156G→T nor *PIGR*1093G→A (table 1). The OR (95%CI) of *PIGR*1739C→T in the Thai group was 1.70(1.03–2.82) and 2.35(1.11–5.07) among Chinese NPC cases with 1739C as the susceptible allele. When we analyzed all three ethnic groups and used Mantel-Haenszel stratification method to correct for ethnic matched control, the difference between crude and adjusted OR of *PIGR*1739C→T was less than 15%. The significant race adjusted OR (95%CI) is 2.26(1.51–3.25) with the *p* value was less than 0.0001. The NPC susceptibility conferred by *PIGR* requires homozygous 1739C to increase the likelihood of NPC development, with the race adjusted OR(95%) = 2.71(1.72–4.23) and $p < 0.00001$ (table 2). The similar significant contribution can be demonstrated with all subgroups (table 2). Regarding *CR2*, *CR2*IVS2-848C→T

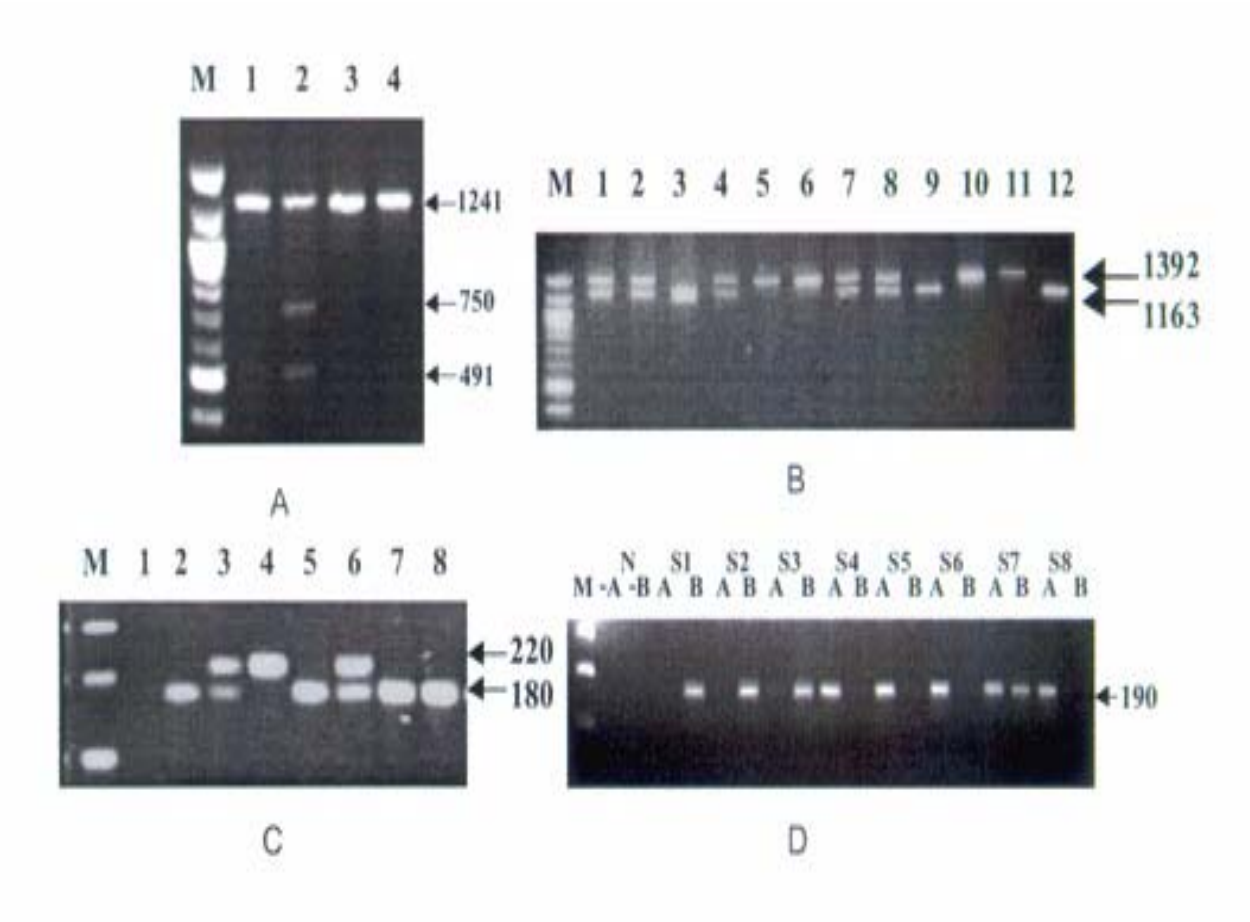

Figure 1
SNPs analysis of *CR2* and *PIGR*. (A) The *CR2*IVS2-848C→T was distinguishable by *TaqI* restriction. Digestion of the 1241 bp amplicon yielded two DNA fragments, 750 and 491 bp. Lane 1, 3 and 4 were homozygous -/- and lane 2 was heterozygous +/-. (B-D) *PIGR* polymorphisms was investigated by PCR-RFLP and ARMS. (B) RFLP analysis of 1392 bp *PIGR*IVS3-156G→T PCR products with *PvuII* digestion yielded 1163 and 229 bp DNA fragments. Lane 1,2,4,7 and 8 were heterozygous +/-, samples 3,9 and 12 were homozygous +/+, and samples 5,6,10 and 11 were homozygous -/-. (C) RFLP of *PIGR*1739C of the 220 bp PCR product was analyzed by *HgaI* digestion and yielded two fragments of 180 and 40 bp, respectively, whereas 1739T remained as 220 bp DNA product. Heterozygous CT yielded 220,180 and 40 bp fragments. Negative control in lane1; homozygous CC in lanes 2,5,7 and 8; heterozygous CT in lanes 3 and 6, and homozygous TT in lane 4. (D) *PIGR*1093G→A was detected by ARMS. Negative control in lanes 1 and 2 for primer sets A and B. Samples S1, 2, 3, were homozygous 1093G. Samples S4, 5, 6 were homozygous 1093A, and S7 was heterozygous.

demonstrated no considerable correlation with NPC when analyzing Thai, Chinese or including all cases. Nevertheless significant OR (95%CI) = 1.80(1.14–3.03) with $p < 0.05$ could be demonstrated upon adjusting for age, sex and ethnic of all cases (table 1). This data suggests that the role of *CR2* as an NPC susceptibility gene need further evaluation while the role of *PIGR* is crucial.

Haplotype analysis of two missense PIGR *SNPs, 1093 G→A and 1739 C→T*

To further elucidate the relevance of the two missense SNPs as for NPC development, genotype data of 1093G→A and 1739C→T were tabulated into four haplotypes, 1093G-1739T (GT), 1093G-1739C (GC), 1093A-1739T (AT), and 1093A-1739C (AC) (table 3). The

Table 1: *CR2* and *PIGR* polymorphisms in NPC cases and control subjects.

	THAI		CHINESE		TOTAL	
	case	control	case	control	case	control
Number of tests	110	104	42	107	175	317
CR2IVS2-848C→T						
+/+	0	1	1	1	2	2
+/-	34	20	7	21	48	63
-/-	76	83	34	85	125	252
+ allele frequency	0.15	0.11	0.11	0.11	0.15	0.11
Crude OR (95%CI)	1.55(0.84–2.85)		1.00(0.41–2.39)		1.48(0.98–2.22)[1]	
Ethnic group adjusted OR (95%CI)					1.47(0.96–2.26)	
age, sex and ethnic group adjusted OR (95%CI)					1.80(1.14–3.03)[1]	
*PIGR*IVS3-156G→T						
+/+	32	30	9	42	49	114
+/-	61	58	26	48	99	155
-/-	17	16	7	17	27	48
+ allele frequency	0.57	0.57	0.52	0.62	0.56	0.60
Crude OR (95%CI)	1.00(0.67–1.50)		0.68(0.40–1.17)		0.84(0.64–1.11)	
Ethnic group adjusted OR (95%CI)					0.88(0.66–1.18)	
Age, sex and ethnic group adjusted OR (95%CI)					0.93(0.67–1.29)	
*PIGR*1093G→A						
GG	53	48	17	52	79	142
GA	49	46	24	42	82	137
AA	8	10	1	13	14	38
G allele frequency	0.70	0.68	0.69	0.68	0.69	0.66
Crude OR (95%CI)	1.11(0.72–1.71)		1.04(0.58–1.86)		1.10(0.83–1.47)	
Ethnic group adjusted OR (95%CI)					1.03(0.76–1.40)	
Age, sex and ethnic group adjusted OR (95%CI)					0.96(0.68–1.36)	
*PIGR*1739C→T						
CC	79	58	32	60	132	170
CT	26	40	9	38	37	130
TT	5	6	1	9	6	17
C allele frequency	0.84	0.75	0.87	0.74	0.86	0.74
Crude OR (95%CI)	1.70(1.03–2.82)[1]		2.35(1.11–5.07)[1]		2.14(1.49–3.09)[3]	
Ethnic group adjusted OR (95%CI)					2.26(1.51–3.25)[3]	
Age, sex and ethnic group adjusted OR (95%CI)					2.06(1.36–3.30)[2]	

Total is Thai, Chinese and Thai-Chinese cases and controls. *CR2*IVS2-848C→T and *PIGR*IVS3-156G→T allele+ and allele- are digested and not digested with the restriction enzyme, respectively. G and A are nucleotide at *PIGR*1093, and C and T are nucleotide at *PIGR*1739, respectively. OR (95%CI) = odd ratios and 95% confidence interval of allele CR2+, *PIGR*IVS3-156G, *PIGR*1093G, *PIGR*1739C when compared with the other alleles of the same SNPs. [1] $p < 0.05$, [2] $p < 0.001$, [3] $p < 0.0001$

frequency of each haplotype based on its ethnic group was estimated by the EH program and distribution was compared between groups. Among the controls, there were no relevant difference between the Thai and the Chinese, yet the *p* values for the cases and the controls of both subgroups and the total were below 0.05 and 0.005, respectively (table 3).

Comparison among haplotypes confirmed 1739C→T as NPC susceptible mutation and excluded any other particular *PIGR* allele (table 3). First, whereas 1739C→T did, no haplotype was associated with significant OR in all Thai, Chinese and Thai-Chinese populations. Second, there were haplotypes with 1739C as susceptible alleles such as GC in the Chinese and total and 1739T as protective alleles such as AT in the Thai, GT in the Chinese and both haplotypes in the total population. In addition, there was no significant haplotype with 1739C as a protective allele or 1739T associated with significant higher relative risk. Finally, the relationship between each haplo-

Table 2: Risk of nasopharyngeal carcinoma associated with *PIGR*1739C→T genotype according to different models of inheritance.

	THAI OR (95%CI)	CHINESE OR (95%CI)	TOTAL OR (95%CI)	ETHNIC GROUP ADJUSTED OR (95%CI)	AGE, SEX AND ETHNIC GROUP ADJUSTED OR (95%CI)
C codominance, T wild type					
CC	1.63(0.42–6.55)	4.80(0.57–105.53)	2.20(0.79–6.44)	2.59(0.85–7.48)	2.53(0.68–13.43)
CT	0.78(0.18–3.35)	2.13(0.22–50.65)	0.81(0.27–2.48)	1.08(0.34–3.53)	1.24(0.23–8.00)
TT	1.00	1.00	1.00	1.00	1.00
C dominance, T wild type					
TT	1.00	1.00	1.00	1.00	1.00
CC or CT	1.29(0.33–5.04)	3.77(0.46–81.87)	1.60(0.58–4.62)	1.96(0.66–5.70)	1.99(0.51–10.40)
C recessive, T wild type					
CT or TT	1.00	1.00	1.00	1.00	1.00
CC	2.02(1.10–3.17)[1]	2.51(1.05–6.09)[1]	2.65(1.73–4.08)[3]	2.71(1.72–4.23)[3]	2.42(1.51–4.29)[2]

CC and TT are the homozygous C and T at nucleotide 1739, respectively. CT is the heterozygous at nucleotide 1739. Total is Thai, Chinese and Thai-Chinese cases and controls. OR (95%CI) is odd ratios and 95% confidence interval between allele and compared allele, [1] $p < 0.05$, [2] $p < 0.001$, [3] $p < 0.00001$

Table 3: Haplotype frequencies of *PIGR*1093–1739, crude OR and ethnic group adjusted OR.

Haplotype	THAI		CHINESE		TOTAL		ETHNIC GROUP ADJUSTED
	case	control	case	control	case	control	
GC frequency	0.556169	0.525147	0.590097	0.447343	0.543478	0.451985	
OR (95%CI)	1.13(0.76–1.68)		1.18(1.05–3.12)		1.37(1.04–1.79)		1.36(1.02–1.80)
GT frequency	0.148376	0.171968	0.100379	0.234900	0.043478	0.175374	
OR (95%CI)	0.84(0.49–1.46)		0.35(0.14–0.80)		0.64(0.43–0.93)		0.55(0.37–0.84)
AC frequency	0.280194	0.224853	0.278951	0.286302	0.413043	0.283864	
OR (95%CI)	1.34(0.85–2.13)		0.95(0.52–1.72)		1.23(0.91–1.66)		1.28(0.93–1.76)
AT frequency	0.015260	0.078032	0.030573	0.031455	0.000001	0.088777	
OR (95%CI)	0.17(0.04–0.62)		1.10(0.22–4.85)		0.12(0.03–0.40)		0.26(0.09–0.63)
case vs case		0.085					
case vs control	<0.05[a]		<0.05[b]		<0.005[c]		
control vs control		0.192					

Each haplotype frequency was calculated by the Estimating Haplotype-frequencies (EH) software program[23]. Total is Thai, Chinese and Thai-Chinese cases and controls. OR (95%CI) = odd ratios and 95% confidence interval between the tested haplotype and the other three alleles, The OR (95%CI) calculation used the estimated number from each haplotype number of cases and controls. The number of haplotypes in the total category were calculated from the summation of estimated haplotype number from each ethnic. GC, AC, GT, and AT are 1093G-1739C, 1093A-1739C, 1093G-1739T, and 1093A-1739T haplotypes, respectively. Case vs case = p value comparing haplotype frequency between Thai case and Chinese case. Case vs control = p value comparing haplotype frequency between case and control in each ethnic. a is Thai, b is Chinese and c is total. Control vs control = p value comparing haplotype frequency between Thai control and Chinese control.

Table 4: Ethnic group adjusted odd ratios between each *PIGR*1093–1739 haplotype.

	GC	AC	GT	AT
GC	1.00	0.97(0.70–1.36)	1.93(1.24–2.91)	4.26(1.68–11.85)
AC		1.00	2.02(1.23–3.23)	4.31(1.76–13.18)
GT			1.00	2.18(0.83–7.34)
AT				1.00

Ethnic group adjusted odd ratios were calculated from the number of cases and controls of each haplotype in the first column by comparing with haplotype listed in the upper row. Numbers before () are odd ratios and within () are 95% confidence interval. GC, AC, GT, and AT are 1093G-1739C, 1093A-1739C, 1093G-1739T, and 1093A-1739T haplotypes, respectively.

type was measured and the data obtained supported the importance of 1739C→T (table 4). Whereas the ORs of the same 1739 nucleotides, between GC and AC or GT and AT, were not statistically significant, both GC and AC haplotypes conveyed a higher OR than GT and AT.

PIGR *sequences of NPC patients*

By complete sequencing the coding region of the *PIGR* gene from 16 NPC patients with homozygous 1739C and upon comparison with the genomic DNA sequence AC098935 and AL359089 as well as with *PIGR* SNPs reported in genbank, the 2 SNPs at base pairs 1093 and 1739 were confirmed. No additional nonsynonymous mutation was identified. However, there were six positions of new synonymous SNPs, IVS1-59G→A, IVS1-35G→A, IVS4-3C→T, 373C→T, 549G→A, and 1773C→T.

Discussion

The SNPs and haplotypes case control association study implicated that the probability to develop NPC might altered by DNA variation of *PIGR*, with nucleotide 1739 playing this crucial part. *PIGR* has been assigned to 1q31 and comprises of 11 exons [18]. The 1093G→A and 1739C→T are located on exon 5 and 7, respectively. The physical distance between nucleotide *PIGRIVS3*-156, 1093 and 1739 were 25 and 2.5 kb, respectively. Thus the three SNPs were closely linked but they distinctly contributed to NPC development. Whereas *PIGR*1739C→T exhibited a remarkably significant relative risk of NPC development among all Thai and Chinese populations, association of *PIGR*IVS3-156G→T and 1093G→A was not shown. Our data have provided an example that conclusions drawn from association studies aimed at identifying a susceptibility gene without related functional SNP should be interpreted with extreme caution [23]. If 1739C→T had not been chosen for this study, the importance of *PIGR* as an NPC susceptibility gene would have been misconstrued.

The manner, in which this *PIGR*1739C→T alters cellular movement of the IgA complex and consequently increases the possibility of EBV associate NPC development, remains to be elucidated. The polymorphism is a missense mutation altering the amino acid alanine to valine. The codon is located on exon 7, which is adjacent to the endoproteolytic cleavage site of the PIGR extracellular domain [29,30]. As a result, homozygous 1739C of the PIGR of individuals from high-risk ethnic origin may alter efficiency to release IgA-EBV complex and consequently, their NEs would have higher possibility to be infected by EBV. Future functional study is needed to elucidate how the polymorphisms in relationship with EBV biology alter the NPC susceptibility.

It is important to note that the 1739C→T mutation can also be found in other lower risk ethnic groups, such as Caucasian [30]. Hence, this *PIGR* nucleotide, despite a remarkably association with NPC, cannot account for its unique endemic distribution. On the other hand, this gene may be critical only for members of high-risk populations, to develop NPC as a consequence of other endemic genetic and/or environmental risk factors. Additionally, it is also interesting to find out whether the SNP on *PIGR* is a risk-associated factor affecting the disease susceptibility in the Caucasian population where NPC is rare and not 100% EBV related.

PIGR plays a crucial role in mucosal immunity not only against EBV, but also pneumococcal infection by facilitating transporting polymeric immunoglobulin transport across the mucosal epithelium. Human PIGR can bind to a major pneumococcal adhesion and enhanced pneumococcal adherence and invasion [17]. In other words, two important human pathogens, EBV and pneumococcus, employ the same human antibody transport protein, PIGR, to cause two common human diseases at the same tissue type, NE. The manner in which these two organisms interact with host (DNA) variation in vivo and what are the consequences might be on a global evolutionary scale will be very interesting subject for further investigation.

Conclusions

The aim of this research was to explore risk associations between polymorphisms of two hypothesized nasopharyngeal epithelial EBV receptors, *CR2* and *PIGR*, and NPC susceptibility The results obtained indicated that *PIGR* is an NPC susceptibility gene. The risk association pertaining to each ethnic group was detected for homozygous *PIGR*1739C with a significant ethnic group adjusted OR (95%CI) of 2.71(1.72–4.23) and $p < 0.00001$. This suggested genetic evidence leading to hypothesize a possibility of PIGR as the epithelial receptor for EBV via IgA-EBV complex transcytosis failure. More importantly, the *PIGR*1739C→T is a missense mutation changing alanine to valine near endoproteolytic cleavage site. This variant could alter the efficiency of PIGR to release IgA-EBV complex and consequently increase the susceptibility of populations in endemic areas to develop NPC.

Methods

Sample collection

After having obtained the subjects' informed consent as to the purpose of the study blood samples were collected by venipuncture from NPC patients and controls. The patients and controls were interviewed and then separated into three groups, Thai, Chinese, and Thai-Chinese, respectively, based on the ethnic origins of their grandparents. If their ancestors, including their great grandparents, originated from China, they were considered Chinese. On the other hand, if their ancestors originated from Thailand, they were defined as Thai. In addition, if their ancestors originated from Thailand and China, they were defined as Thai-Chinese. Conclusively, 104 Thai, 107 Chinese and 106 Thai-Chinese were enrolled in the control group whereas the NPC patients comprised 110 Thai, 42 Chinese and 23 Thai-Chinese. The 175 patients were recruited at King Chulalongkorn Memorial Hospital between 1994 and 2001. They were permanent residents in Bangkok or the central part of Thailand. All cases were Thai, Chinese or Thai-Chinese. The tumors of every patient was histologically ascertained as NPC type II or III, according to WHO classification. The 317 controls were healthy blood donors from Thai Red Cross Society, locating in the King Chulalongkorn Memorial Hospital. All had permanent resident in Bangkok or the central part of Thailand and those previously diagnosed with NPC or belonging to an ethnic group other than Thai, Chinese or Thai-Chinese had been excluded. The male:female ratio among the cases was 1.8:1 and among the controls 1.6:1, respectively. Their DNA was isolated by proteinase K and incubated overnight at 50°C, followed by phenol/chloroform extraction and ethanol precipitation.

SNP's information

Four SNPs, *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T,*PIGR*1093G→A and *PIGR*1739C→T, were chosen. *CR2*IVS2-848C→T located in intron 2 [24] and *PIGR*IVS3-156G→T in intron3, [25] had previously been published as RFLP polymorphisms, whereas *PIGR*1093 and *PIGR*1739 were selected from a SNPs database. http://www.ncbi.nlm.nih.gov/SNP/

PCR-RFLP

PCR reactions were performed, using Perkin-Elmer/DNA Thermal cycle480, in a total volume of 50 μl to amplify *CR2*IVS2-848C→T, *PIGR*IVS3-156G→T, and *PIGR*1739C→T. The reaction mixtures consisted of 100 ng of genomic DNA and the following set of primers at the respective concentrations: 0.1 μM of *CR2*IVS2-848C→T primers (5'-CTTTCTGTGCAGACCACGTT-3' and 5'-GATCTATGGTAGCCAGTTGG-3'*PIGR*IVS3-156G→T primers (5'-TCAGCCAGGGTAAGGATCC-3' and 5'-TGATGGTCACCGTTCTGCC-3'), or 0.2 μM of *PIGR*1739C→T primers (5'-GGGTCCCGCGATGTCAGCCTAG-3' and 5'-TTCTCCGAGTGGGGAGCCTT-3'). The DNA samples were amplified in the presence of 200 μmol dNTPs, 5 μl of 10X PCR buffer (20 μM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$ and 4U Taq DNA polymerase (Gibco). The PCR condition for *CR2*IVS2-848C→T comprised an initiation denaturation step at 94°C for 4 minutes, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and final extension step at 72°C for 7 minutes. *PIGR*IVS3-156G→T was amplified 40 cycles at an annealing temperature of 60°C for 1 minute, and extension at 72°C for 3 minutes. The conditions for *PIGR*1739C→T was 35 cycles at 60°C annealing temperature for 1 minute and extension at 72°C for 1 minute. Subsequently, RFLP analysis was performed on 20 microliters each of the respective PCR products by subjecting them to the following restriction enzymes (New England Biolabs) at a 10U concentration: *TaqI* for *CR2*IVS2-848C→T, *PvuII* for *PIGR*IVS3-156G→T, and *HgaI* for *PIGR*1739C→T with overnight incubation at 65°C for *TaqI* and at 37°C for both of *PvuII* and *HgaI*. The resulting products were further analyzed by 2% agarose gel electrophoresis.

ARMS of PIGR*1093G→A*

The Amplification Refractory Mutation System (ARMS) [26] was used to detect SNP *PIGR*1093G→A. The primers can be divided into 2 sets, A and B. Set A primers comprised of 1093Fa and 1093R (GCCCCACTGTGGTGAAGGGGGTGGCAGGTG and ACTGGGCCTTAACCCACCCC), whereas 1093Fb (GCCCCACTGTGGTGAAGGGGGTGG CAGGTA) and 1093R were mixed in set B. For each sample, the PCR reaction was performed in duplicate, set A and B, in a total volume of 50 μl containing 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 5 μl of 10X buffer (20 μM Tris-HCl pH 8.4, 50 mM KCl), 4U Taq Gold DNA polymerase (Perkin Elmer) and 0.3 μM of primer set A or set B. PCR conditions in-

cluded initial denaturation at 95°C for 10 minutes followed by 30 cycles of 95°C for 1 minute, 65°C for 1 minute, 72°C for 1 minutes and final extension for 72°C for 7 minutes in a Perkin-Elmer/DNA thermal Cycle 480. Each SNP was investigated by 2% agarose gel electrophoresis for complementarity of the PCR product with the 3' nucleotide of primers set A and/or set B.

Statistical analysis

Both cases and controls were subdivided based on ethnic, sex and age at sampling (i.e., <30, 30–49, 50–69, ≥ 70 years). The association between certain alleles of the *CR2* or *PIGR* and NPC development was estimated by the statcalc from Epi info 2000 program http://www.cdc.gov/epiinfo/ to calculate the odds ratio (OR) and 95% confidence interval (CI), Mantel-Haenszel chi squares and associated p values. Mantel-Haenszel weighted OR, summary chi square and p value were adjusted for the confounding effect of ethnic, and/or age and sex by combining stratified 2 × 2 tables. The effect exerted by genotype was estimated as if autosomal inheritance according to actual number of alleles contributed to a significant OR [27]. Haplotype frequencies for pairs of alleles, as well as Chi square values for allele associations were estimated by the Estimating Haplotype-frequencies (EH) software program [28]. The number of each haplotype from each ethnic were estimated based on the allele frequencies multiplied by the number of cases. The haplotype numbers of all the cases as well as controls were summation of the haplotype numbers of Thai, Chinese and Thai-Chinese groups. The association between each haplotype and NPC development was calculated based on the OR using Epi info 2000 program. The haplotype differences between groups were estimated by $T(x^2/2)$ = In(L, group1)+In(L, group2)-In(L, group1+group2) as previously described [28].

Sequencing

All 11 exons of *PIGR* genomic DNA were amplified from DNA samples of 16 unrelated patients. Each exon was amplified by a pair of primers locating on the following positions in relation with nucleotide number AL359089: 93732C-93751G and 94163A-93782C, 99668C-99687T and 100025A-100044C, 100724C-100743T and 101427C-101446T, 102578A-102597G and 103454C-103473A, 104403C-104422A and 105039T-105058A, 105584C-105604G and 106080G-106099G, 107094C-107113A and 107518G-107537C, 107737G-107756C and 108066C-108085C, 108524A-108543T and 109039G-109059T, and 109935G-109954G) and 110656G-110675C. Subsequently, PCR products were purified and directly sequenced in both directions by dye terminators into cycle-sequencing products.

Authors' contributions

RH and NK gathered case and control samples as well as informations and performed the molecular genotyping experiments and statistical analysis. KS, PS and NV collected patients and clinical data. AJ sequenced the whole gene, reviewed statistical analysis methodology and partly wrote the articles. AM hypothesized, designed the experiments and interpreted the genotyping, statistical, sequencing results and wrote the article.

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References

1. Mutirangura A **Molecular mechanisms of nasopharyngeal carcinoma development.** *Research Advances and Research Updates in Medicine* 2000, **1:**18-27
2. McDermott AL, Dutt SN and Watkinson JC **The aetiology of nasopharyngeal carcinoma.** *Clin Otolaryngol* 2001, **26:**82-92
3. Cheung F, Pang SW, Hioe F, Cheung KN, Lee A and Yau TK **Nasopharyngeal carcinoma in situ: two cases of an emerging diagnostic entity.** *Cancer* 1998, **83:**1069-1073
4. Mutirangura A, Tanunyutthawongese C, Pornthanakasem W, Kerekhanjanarong V, Sriuranpong V, Yenrudi S, Supiyaphun P and Voravud N **Genomic alterations in nasopharyngeal carcinoma: loss of heterozygosity and Epstein-Barr virus infection.** *Br J Cancer* 1997, **76:**770-776
5. Pathmanathan R, Prasad U, Sadler R, Flynn K and Raab-Traub N **Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma.** *N Engl J Med* 1995, **333:**693-698
6. Jeannel D, Hubert A, de Vathaire F, Ellouz R, Camoun M, Ben Salem M, Sancho-Garnier H and de-The G **Diet, living conditions and nasopharyngeal carcinoma in Tunisia – a case-control study.** *Int J Cancer* 1990, **46:**421-425
7. Yu MC, Huang TB and Henderson BE **Diet and nasopharyngeal carcinoma: a case-control study in Guangzhou, China.** *Int J Cancer* 1989, **43:**1077-1082
8. Simons MJ, Wee GB, Chan SH and Shanmugaratnam K **Probable identification of an HL-A second-locus antigen associated with a high risk of nasopharyngeal carcinoma.** *Lancet* 1975, **1:**142-143
9. Ren EC and Chan SH **Human leucocyte antigens and nasopharyngeal carcinoma.** *Clin Sci (Lond)* 1996, **91:**256-258
10. Hildesheim A, Anderson LM, Chen CJ, Cheng YJ, Brinton LA, Daly AK, Reed CD, Chen IH, Caporaso NE, Hsu MM, Chen JY, Idle JR, Hoover RN, Yang CS and Chhabra SK **CYP2E1 genetic polymorphisms and risk of nasopharyngeal carcinoma in Taiwan.** *J Natl Cancer Inst* 1997, **89:**1207-1212
11. Kongruttanachok N, Sukdikul S, Setavarin S, Kerekhjanarong V, Supiyaphun P, Voravud N, Poovorawan Y and Mutirangura A **Cytochrome P450 2E1 polymorphism and nasopharyngeal carcinoma development in Thailand: a correlative study.** *BMC Cancer* 2001, **1:**4
12. Voravud N **Cancer in the Far East.** *In Treatment of Cancer (Edited by: Sikora K and Halman KE) Chapman and Hall Medical: London* 1990, 887-894
13. Fingeroth JD, Weis JJ, Tedder TF, Strominger JL, Biro PA and Fearon DT **Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2.** *Proc Natl Acad Sci U S A* 1984, **81:**4510-4514
14. Chang Y, Tung CH, Huang YT, Lu J, Chen JY and Tsai CH **Requirement for cell-to-cell contact in Epstein-Barr virus infection of**

nasopharyngeal carcinoma cells and keratinocytes. *J Virol* 1999, **73:**8857-8866
15. Imai S, Nishikawa J and Takada K **Cell-to-cell contact as an efficient mode of Epstein-Barr virus infection of diverse human epithelial cells.** *J Virol* 1998, **72:**4371-4378
16. Shao X, He Z, Chen Z and Yao K **Expression of an Epstein-Barr-virus receptor and Epstein-Barr-virus-dependent transformation of human nasopharyngeal epithelial cells.** *Int J Cancer* 1997, **71:**750-755
17. Zhang JR, Mostov KE, Lamm ME, Nanno M, Shimida S, Ohwaki M and Tuomanen E **The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells.** *Cell* 2000, **102:**827-837
18. Norderhaug IN, Johansen FE, Schjerven H and Brandtzaeg P **Regulation of the formation and external transport of secretory immunoglobulins.** *Crit Rev Immunol* 1999, **19:**481-508
19. Gan YJ, Chodosh J, Morgan A and Sixbey JW **Epithelial cell polarization is a determinant in the infectious outcome of immunoglobulin A-mediated entry by Epstein-Barr virus.** *J Virol* 1997, **71:**519-526
20. Reich V, Mostov K and Aroeti B **The basolateral sorting signal of the polymeric immunoglobulin receptor contains two functional domains.** *J Cell Sci* 1996, **109:**2133-2139
21. Lin CT, Lin CR, Tan GK, Chen W, Dee AN and Chan WY **The mechanism of Epstein-Barr virus infection in nasopharyngeal carcinoma cells.** *Am J Pathol* 1997, **150:**1745-1756
22. Chien YC, Chen JY, Liu MY, Yang HI, Hsu MM, Chen CJ and Yang CS **Serologic markers of Epstein-Barr virus infection and nasopharyngeal carcinoma in Taiwanese men.** *N Engl J Med* 2001, **345:**1877-1882
23. Tabor HK, Risch NJ and Myers RM **Candidate-gene approaches for studying complex genetic traits: practical considerations.** *Nature Reviews Genetics* 2002, **3:**391-397
24. Fujisaku A, Harley JB, Frank MB, Gruner BA, Frazier B and Holers VM **Genomic organization and polymorphisms of the human C3d/Epstein-Barr virus receptor.** *J Biol Chem* 1989, **264:**2118-2125
25. Krajci P, Kvale D, Tasken K and Brandtzaeg P **Molecular cloning and exon-intron mapping of the gene encoding human transmembrane secretory component (the poly-Ig receptor).** *Eur J Immunol* 1992, **22:**2309-2315
26. Dracopoli N, Haines J and Korf B **Current protocol in human genetic.** *John & Sons, New york.* 2000,
27. Spurdle A, Hopper J and Dite G **CYP17 Promoter Polymorphism and Breast Cancer in Australian Woman Under Age Forty Years.** *Journal of the National Cancer Institute* 2000, **92:**1674-1681
28. Zhao JH, Curtis D and Sham PC **Model-free analysis and permutation tests for allelic associations.** *Hum Hered* 2000, **50:**133-139
29. Banting G, Brake B, Braghetta P, Luzio JP and Stanley KK **Intracellular targetting signals of polymeric immunoglobulin receptors are highly conserved between species.** *FEBS Lett* 1989, **254:**177-183
30. Hughes G, S F, L-A S, AJ R, HR M and J-C J **Human free secretory component of the polymeric immunoglobulin receptor.** *FEBS Lett* 1997, **410:**443-446