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Research Article

The Involvement of Interleukin-10 Promoter Genetic Polymorphism in Epstein-Barr Virus-Associated Nasopharyngeal Carcinoma from North Africa

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Abstract

Objectives: Interleukin-10 (IL10) is a pro-inflammatory cytokine that plays a pivotal role in inflammatory diseases as well as in the pathogenesis of diverse tumors, including nasopharyngeal carcinoma (NPC). The present study was designed to evaluate the importance of the functional promoter polymorphisms of IL10 (-1082 A>G and -592 A>C) in the development of NPC.

Methods: A total of 384 patients with NPC were recruited into this study, together with 375 matched control subjects. Polymorphisms within the promoter region of IL10 gene were analysed using PCR-RFLP method.

Results: We report a lack of association between IL10 polymorphisms and NPC in the overall population (P>0.05). However, the 1082 A>G polymorphism was significantly associated with the susceptibility to NPC among young patients (age ≤30 years). The GG genotype was found to be associated with a significantly higher risk of NPC as compared with the AA genotype among young patients (OR=2.534; 95% CI, 1.189–5.398, P=0.016).

Conclusion: The results of the present study indicate an association between IL10 -1082 GG genotype and NPC among young North African subjects. This difference in IL10 polymorphism association with different ages at the onset of NPC suggests that the younger and older onset patients are genetically different and may involve different mechanisms.

Keywords: Nasopharyngeal Carcinoma, IL10, North African population

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The Epstein-Barr virus (EBV)–associated nasopharyngeal carcinoma (NPC), a rare neoplasm in most parts of the world, occurs with high frequencies in China and South East Asia with an age-adjusted incidence varying between 5 to 40 cases per 100 000 per year.[1] High rates approaching those observed in southern China are also seen among In-

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uit and other natives of the Arctic region.[2] In North Africa, where this malignancy occurs at intermediate risk, the age incidence curve of NPC shows a bimodal distribution with a significant age peak in the teens.[3,4] Family clustering and migrant studies suggested the involvement of genetic and environmental factors in the aetiology of this tumor which is believed to have EBV as a necessary aetiological agent.[1]

Biologic mechanisms leading to the development of NPC are not clearly understood, but the role of cytokines in cancer immunity and carcinogenesis has been well established.[5,6]

Interleukin-10 (IL10) is a multifunctional cytokine produced by a variety of immune cells with both immunosuppressive and anti-angiogenic function. It seems to play a dual, controversial role in carcinogenesis, as a tumor promoting and tumor inhibiting factor.[7–9] Raised levels of serum and peri-tumoral IL10 production have been reported in many malignancies, which have been interpreted in support of a role of IL10 in tumor escape from the immune response. However, gene transfection studies in a number of malignancies argue more convincingly for an anti-tumor function of IL 10, possibly via inhibition of pathways of angiogenesis.[10]

The IL10 gene (OMIM:124092) is located on chromosome 1 at q31-32, contains five exons and four introns that encode a 178-amino-acid protein.[11,12] Alterations in IL10 expression have been linked to polymorphisms in the promoter region that span at least 5 kb upstream of the transcription start point and known to contain at least 27 polymorphic sites.[13] These polymorphisms include functional single nucleotide polymorphisms (SNPs) located in the 50-flanking promoter region at –1082 (G to A substitution) and –519 (C to A substitution) have been reported to regulate IL10 gene transcription and expression and the secretion of IL10 consequentially.[14–17] These alterations, result in abnormal cell proliferation and cancer development.[18,19] IL10 –1082 GG and –592 CC genotypes has been shown to be associated with higher IL10 production.[15] The homozygous AA allele at IL10 -1082, which influences the transcription of IL10 mRNA and the expression of IL10, leading to a lower IL10 expression, has been associated with several cancers, including advanced cutaneous malignant melanoma, gastric cancer,[20] breast cancer[21] and prostate cancer,[22] while the higher producing genotype (GG) has been linked to cervical cancer.[23] There are also indications that IL10 has a regulatory role in EBV–induced infections.[24–27] Some studies further reported that the haplotypes defined by SNPs in the IL10 gene promoter, may be associated with different levels of IL10 production.[19,28,29]

Numerous molecular epidemiological studies have investigated the association between IL10 gene polymorphisms and cancer risk, such as lung cancer,[30] breast cancers,[31] digestive cancer,[32] cervical cancer,[32] and NPC.[33–35] However, the results were not consistent.

On the basis of the role of IL10 in the regulation of the immune response and EBV-associated tumor development and to evaluate whether IL10 gene functional promoter polymorphisms are associated with NPC in our North African population, we considered it worthwhile to investigate the association between genetic polymorphisms in IL10 genes and NPC. The present study reports the association analysis of IL10 -1082 and -592 polymorphisms in a large-scale sample of NPC.

Methods

Study Subjects

The general design of the International Agency for Research on Cancer (IARC) international study of NPC has been described in detail elsewhere.[36,37] The present study consisted of 384 NPC cases and 369 unrelated controls recruited from 5 hospitals in Morocco, Algeria, and Tunisia. Inclusion criteria for both cases and controls stipulated that all four grandparents of each subject were of Moroccan, Algerian or Tunisian origin in order to avoid all risk of admixture bias. Controls were frequently matched by center, age, sex and household type (urban/rural) during childhood. Individuals with other diseases sharing risk factors with NPC were carefully excluded (i.e., ear, nose, and throat conditions; alcohol- and tobacco-related diseases; or potential HLA-related disorders such as autoimmune diseases). For both cases, subjects less than 15 years old were also excluded. At time of the blood sampling, information on demographic features and clinical details were collected through personal interview and written informed consents were obtained from each subject. The study was also approved by the ethics committee review board of the IARC.

IL10 Polymorphisms Genotyping

The IL10 -1082 A>G (rs1800896) and -592 A>C (rs1800872) genotypes were determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay as previously described.[38] Genomic DNA was extracted from peripheral blood leukocytes using the proteinase K digestion and phenol–chloroform extraction or using the Gentra Puregene Blood Kit and then amplified using forward primer 5'-CCTAGGTCACAGTGACGTGG-3' and reverse primer 5'-GGTGAGCACTACCTGACTAGC-3' for -592 A/C SNP and forward primer 5'-CTCGCTGCAACCCAACTGGC-3' and reverse primer 5'-TCTTACCTATCCCTACTTCC-3' for -1082 A/G SNP. The PCRs were performed with a 25 µL reaction

mixture containing 100 ng of genomic DNA, 0.25 µmol/L of each primer, 200 µmol/L of each dNTP, 1.5U of Taq DNA polymerase (Promega), 10× PCR buffer supplied by Invitrogen Corp (10 mmol/l Tris-HCl, pH 8.8, 50 mmol/l KCl), and 2.0 mmol/L MgCl2. The PCR profile consisted of an initial melting step of 5 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 45 seconds at 58°C for -1082A>G and 60°C for -592 A>C; 55 s at 72°C; and a final elongation at 72°C for 10 min. The restriction endonucleases MnlI and RsaI were used to distinguish the IL10 gene -1082A/G and -592A/C polymorphisms, respectively (Agarose gel electrophoresis images are provided as Supplementary figure 1). We randomly selected 20 % of DNA samples to be plated in duplicate for quality control checks. The concordance rate for duplicate genotyping was 100%.

Statistical Analysis

The statistical analyses were performed using SPSS 25.0 software. the chi-square (χ^2) goodness of fit test was used to examine Hardy-Weinberg equilibrium (HWE) in controls. Student's t test or the χ^2 test was used to analyze the genotype frequency differences between the NPC patients and the healthy controls. Logistic regression adjusted by age, sex and country of origin was used to test for associations between SNPs and NPC. The linkage disequilibrium (LD) between the polymorphisms was quantified using Haploview 4.2 software. The haplotypes and their frequencies were estimated using the R statistical programming language version 3.5.1. The sample size and the statistical power was calculated using "Power and Sample Size" program. Bonferroni corrections were applied to correct for multiple comparisons, and the threshold for statistical significance was set at p<(0.05/n), where n is the number of independent tests.

Results

Demographic Characteristics

Comparisons of distributions of demographic characteristics between cases and controls are shown in Table 1. There was no significant difference between cases and controls regarding gender, age groups or countries. The mean age at onset of the cases was 40.80 years old, comparable to the mean age of the controls (41.06 years old; p=0.64).

Sample Size Analysis

Based on the frequency of the risk allele of the SNPs -592A>C (MAF = 28.66%) and -1082 A>G (MAF = 34.13%), the number of subjects required for our study to detect a hypothesized odds ratio of 2.0 with a probability (statistical power) of 0.80, were 143 and 135, respectively.

Table 1. Characteristics of the study population

Characteristics	Cases N (%)	Controls N (%)	p*
Whole sample	384	375	
Gender			
Male	295 (67.04)	298 (68.50)	
Female	145 (32.95)	137 (31.49)	0.644
Age mean (years)	40.80	41.06	0.992
Age			
≤30	136 (30.90)	136 (31.26)	
>30	304 (69.09)	299 (68.73)	0.910
Country			
Tunisia	213 (48.40)	177 (40.68)	
Morocco	204 (46.36)	175 (40.22)	
Algeria	23 (5.22)	83 (19.08)	

N= number of subjects; * P-value of chi^2 comparing cases and controls.

Genotypic and Allelic Distributions of IL10 Polymorphisms

Table 2 shows the genotypic and allelic frequencies of the IL10 -1082 A>G and IL10 -592 A>C polymorphisms in the NPC cases and control groups. The two studied polymorphisms were conformed to be in HWE in genotypes distribution of the control groups (p>0.05 for both). There were no significant differences in the genotype and allele frequencies between the case and control groups for both SNPs.

LD and Haplotype Analysis

The results indicate that LD existed between the IL10 -1082 A>G and IL10 -592 A>C polymorphisms (r^2= 0.21). As IL 10 haplotypes in the promoter gene were found to be associated with differential secretion of IL10 cytokine,[19,28,29] we tested the association between the frequencies of the haplotypes and NPC risk (Table 3) despite the low DL observed. No significant association between the haplotypes and NPC risk was observed.

Genotypic Distributions of IL10 Polymorphisms According to Sex

As NPC is more frequent among men than women,[39] we performed analysis with the stratification of the samples by sex. The results showed that there was no significant difference in IL10 -1082 A>G and IL10 -592 A>C polymorphisms between men and women (Supplementary Table 1).

Genotypic Distributions of IL10 Polymorphisms According to Age

Since cases from the first age incidence peak (≤30) are believed to have a strongest genetic susceptibility to NPC in our North African population,[39,40] the selected IL 10 geno-

Table 2. Genotype and allele frequencies of IL10 gene polymorphisms in NPC Cases and Controls

Polymorphism	Cases N (%)	Controls N (%)	OR (CI 95%)^	p*	OR (CI 95%)‡	p*
IL10 -592 A>C						
Genotype						
CC	215 (55.98)	195 (52.0)	1.00	-	1.00	-
CA	141 (36.71)	145 (38.66)	0.882 (0.652 – 1.193)	0.415	0.841 (0.616 – 1.148)	0.275
AA	28 (7.29)	35 (9.33)	0.726 (0.426 – 1.237)	0.237	0.707 (0.405 – 1.233)	0.222
Allele						
A	571 (74.34)	535 (71.33)	1.00	-	1.00	-
C	197 (25.66)	215 (28.66)	0.858 (0.68 – 1.083)	0.204	0.848 (0.674 – 1.066)	0.159
IL10 -1082 A>G						
Genotype						
AA	182 (47.39)	169 (45.06)	1.00	-	1.00	-
AG	138 (35.93)	156 (41.60)	0.821 (0.602 – 1.120)	0.214	0.830 (0.603 – 1.143)	0.253
GG	64 (16.66)	50 (13.33)	1.189 (0.777 – 1.818)	0.426	1.172 (0.757 – 1.815)	0.476
Allele						
A	502 (65.36)	494 (65.86)	1.00	-	1.00	-
G	266 (34.63)	256 (34.13)	1.022 (0.836 – 1.243)	0.847	1.009 (0.823 – 1.236)	0.934

N= number of subjects; ^Unadjusted Odds ratios and confidence interval for case-control comparison; ‡Odds ratios and confidence interval adjusted on age, sex and country; *Statistically significant threshold was set at p<0.025 (0.05/n, n=2) determined by Bonferroni correction.

Table 3. Estimation of haplotype frequencies and haplotype association with NPC

Haplotype -1082 A>G – -592 A>C	Frequency	OR (CI 95%)^	p*
C A	0.457	1.00	-
A A	0.198	0.85 (0.62 – 1.16)	0.294
A G	0.073	0.93 (0.60 – 1.45)	0.750
C G	0.270	0.98 (0.75 – 1.28)	0.895

^Unadjusted Odds ratios and confidence interval for case-control comparison; *Statistically significant threshold was set at p<0.01 (0.05/n, n=3) determined by Bonferroni correction.

types on NPC risk were evaluated according to age (Table 4). Our data showed a significant difference in the genotype frequency of the IL10 -1082 A>G polymorphism between young (≤30 years old) and older patients (over 30 years old). The -1082 GG was found to be associated with a significantly increased risk of NPC as compared with the -1082 AA genotype (OR=2.534; 95% CI, 1.189 – 5.398, p=0.016). This association remains significant even after employing the Bonferroni correction.

SNP-SNP Interaction Models for IL10 Polymorphisms

To perform data mining regarding the SNP-SNP interactions, all possible pair combinations between all IL10 -1082 A>G and IL10 -592 A>C genotypes were analyzed. However, no significant association was observed (Table 5).

Discussion

Despite the importance of IL10 in NPC pathogenesis,[41–43] the literature concerning the role of the IL10 polymorphism in relation to NPC is small. On these grounds, the present study was designed to evaluate the importance of the functional promoter polymorphisms of IL10 (-1082 A>G and –592 A>C), a key immune-regulatory-related gene in the development and disease onset of NPC. The IL10 -1082 A>G and –592 A>C SNPs have been associated with increased production of IL10, and thus influence the expression and function of protein,[18,44,45] which can contribute in the variability of susceptibility between individuals to a disease and its severity.

In the current study, the frequencies of the IL10 -1082 G and -592 C alleles among healthy controls were 0.387 and 0.635 respectively, and these were similar to those observed in healthy Caucasians (0.380 and 0.710 respectively). These frequencies were significantly higher than those of healthy Chinese (0.114 and 0.333 respectively) (hapmap.org). These corroborate previous results showing a remarkable difference between Caucasian and Asian population in IL10 gene promoter polymorphisms, with G alleles at -1082 loci being very rare in Asians populations.[46]

In agreement with a study conducted in Tunisia,[47] our results did not show any significant difference in allele and genotype frequencies between IL10-promoter polymorphisms –1082 A>G and –592 A>C and NPC risk in North Africa among overall patients. Similarly, a study in Portugal

Table 4. Genotype distribution of IL10 gene polymorphisms according to age in NPC cases and controls

Polymorphism	Cases N (%)	Controls N (%)	OR (CI 95%)^	p*	OR (CI 95%)‡	p*
IL10 -592 A>C						
≤ 30						
CC	71 (55.90)	67 (53.17)	1.00	-	1.00	-
AC	48 (37.79)	48 (38.09)	0.944 (0.560 – 1.589)	0.827	1.075 (0.614 – 1.882)	0.799
AA	8 (6.29)	11 (8.73)	0.686 (0.260 – 1.810)	0.445	1.000 (0.335 – 2.987)	1.000
>30						
CC	144 (56.03)	128 (51.40)	1.00	-	1.00	-
AC	93 (36.18)	97 (38.95)	0.852 (0.588 – 1.235)	0.398	0.767 (0.524 – 1.123)	0.173
AA	20 (7.78)	24 (9.63)	0.741 (0.391 – 1.404)	0.356	0.618 (0.318 – 1.199)	0.155
IL10 -1082 A>G						
≤ 30						
AA	51 (40.15)	60 (47.61)	1.00	-	1.00	-
GA	48 (37.79)	53 (42.06)	1.065(0.621–1.829)	0.818	1.208 (0.688 – 2.120)	0.511
GG	28 (22.04)	13 (10.31)	2.534(1.189 – 5.398)	0.016	2.485 (1.143 – 5.404)	0.022
>30						
AA	131 (50.97)	109 (43.77)	1.00	-	1.00	-
GA	90 (35.01)	103 (41.36)	0.727 (0.497 – 1.063)	0.100	0.693 (0.468 – 1.024)	0.066
GG	36 (14.00)	37 (14.85)	0.810 (0.479 – 1.368)	0.430	0.812 (0.473 – 1.393)	0.449

N= number of subjects; ^Unadjusted Odds ratios and confidence interval for case-control comparison; ‡Odds ratios and confidence interval adjusted on age, sex and country; *Statistically significant threshold was set at P<0.025 (0.05/n, n=2) determined by Bonferroni correction.

Table 5. The two-way interaction of IL10 polymorphisms in the risk of NPC.

Genotypes -592 A>C – -1082 A>G	Cases N (%)	Controls N (%)	OR (CI 95%)^	p*
CC – AA	69 (21.03)	69 (21.90)	1.00	-
CC – GA	73 (22.25)	68 (21.58)	1.074 (0.671 – 1.717)	0.767
CC – GG	34 (10.36)	26 (8.25)	1.308 (0.711 – 2,407)	0.388
CA – AA	76 (23.17)	55 (17.46)	1.382 (0.854 – 2.236)	0.187
CA – GA	38 (11.58)	54 (17.14)	0.704 (0.413 – 1.199)	0.195
CA – GG	15 (4.57)	14 (4.44)	1.071 (0.481 – 2.387)	0.866
AA – AA	10 (3.04)	17 (5.39)	0.588 (0.252 – 1.375)	0.218
AA – GA	7 (2.13)	11 (3.49)	0.636 (0.233 – 1.738)	0.375
GG – GG	6 (1.82)	1 (0.31)	6 (0.704 – 51.157)	0.065

N= number of subjects; ^Unadjusted Odds ratios and confidence interval for case-control comparison; ‡Odds ratios and confidence interval adjusted on age, sex and country; *Statistically significant threshold was set at p<0.0006 (0.05/n, n=8) determined by Bonferroni correction.

did not identify any association of the –1082 A>G polymorphism with NPC, while another European study showed a borderline statistically significant increase in the frequency of IL10 –1082G allele in NPC patients from Italy.[48,49] In two studies conducted on NPC patients from Asia (China and Taiwan), the –1082 AG (an intermediate IL10 expression) or GG (a high IL10 expression) genotypes were found to be associated with a significantly increased risk of NPC as compared with the –1082 AA genotypes.[34,35] Interestingly, our finding is corroborated by the results of a meta-analysis conducted by Ma et al.,[33] reporting that the –1082 A>G polymorphism may be associated with a greater risk of NPC in Asians but not in non-Asians in stratified analysis by geographic location.

The difference observed in the IL10 promoter polymorphism between NPC patients from different population might be related to the differences observed in IL10 allelic frequencies in the different populations, and/or different genetic risk factors in the different ethnic populations.

The bimodality is an inherent feature of NPC age-incidence curve in North Africa. As cases from the first age peak (≤30) are believed to have a strongest genetic sus-

ceptibility, we tested association between NPC risk and IL10 polymorphisms in two subgroups, ≤30 and >30 year of age. A significantly higher frequency of the –1082 GG genotype was found in the group of young patients (under 30 years of age) as compared to the main age group (above 30 years of age). Because the GG genotype was reported to confer a higher IL10 phenotype, this result suggests that NPC probably arises in young individuals in a scenario of higher levels of basal IL10 synthesis. However, the only study conducted in a North African country on the IL10 promoter polymorphism in NPC patients (Tunisia) did not find such association, maybe the sample size was not large enough to detect a difference between the two age groups.[47]

Our results show a genetic predisposition to NPC in the IL10-1082 A>G promoter polymorphism, relevant only in young patients. This difference in IL10 polymorphism association with different ages at onset suggests that there is heterogeneity within the NPC population. This heterogeneity may be even more pronounced in moderate incidence areas for NPC (i.e., North Africa) where the age incidence curves usually show two peaks, one at adolescence and one at later-onset age.

We can argue that NPC in young North African patients represents a different pathogenic entity, as proposed originally by de-Vathaire et al. 1988,[50] which is further supported by other studies revealing significant different biological and clinical characteristics of NPC tumors of young NPC patients as compared to elderly ones. Interestingly, Ben Nasr et al. showed that a higher level of EBV latent membrane protein-1 (LMP-1) expression occurs in the juvenile form of NPC patients.[51] It is also noteworthy that LMP-1 induces the production of hIL10 in infected B cells, that acts as autocrine factor and modulate the immune response.[52] According to these observations, we can speculate that among young NPC patients carrying G allele (high IL- 10 production), the level of IL10 is abnormally high causing reduced immune response, suggesting that in both age groups malignant cells in NPC tend to use distinct pathways to escape the immune system and complete malignant transformation. However, further studies are required to decipher whether such an effect may be linked to the observed increased risk of NPC in the young age population.

The limitations of the present study need to be addressed, although we had the power to find the associations reported previously. Because of the relatively small sample size, this study may not be powerful enough to test the interaction among genes with low frequencies.

Conclusion

In conclusion, although the molecular explanation for the relationships between the -1082 A>G and the age of patients' warrants further studies, our results indicate that each age peak may possesses its own particular oncogenic mechanisms. In fact, we illustrate that the strength of a genetic effect can vary by age, causing « age-varying » associations. In future studies, it will be important to investigate in both age groups of NPC the status of proteins which could be expected to be expressed with a rather heterogeneous pattern.

Disclosures

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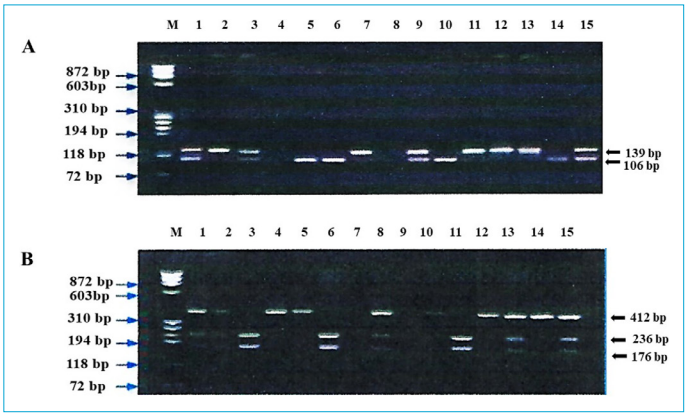
Supplementary Table 1. Genotype distribution of IL10 gene polymorphisms according to sex in NPC cases and controls

Polymorphism	Cases N (%)	Controls N (%)	OR (CI 95%)^	p*	OR (CI 95%)†	p*
IL10 -592 A>C						
Male						
CC	142 (56.12)	134 (53.17)	1.00	-	1.00	-
AC	92 (36.36)	96 (38.09)	0.904 (0.624 – 1.310)	0.595	0.872 (0.593 – 1.282)	0.486
AA	19 (7.50)	22 (8.73)	0.815 (0.422 – 1.573)	0.542	0.733 (0.384 – 1.556)	0.470
Female						
CC	73 (55.72)	61 (49.59)	1.00	-	1.00	-
AC	49 (37.40)	49 (39.83)	0.836 (0.496 – 1.408)	0.500	0.748 (0.437 – 1.282)	0.291
AA	9 (6.87)	13 (10.56)	0.579 (0.232 – 1.445)	0.238	0.577 (0.224 – 1.486)	0.255
IL10 -1082 A>G						
Male						
AA	124 (49.40)	116 (44.61)	1.00	-	1.00	-
GA	90 (35.85)	113 (43.46)	0.745 (0.512 – 1.084)	0.124	0.735 (0.499 – 1.082)	0.119
GG	37 (14.74)	31 (11.92)	1.117 (0.651 – 1.916)	0.689	1.050 (0.602 – 1.830)	0.864
Female						
AA	58 (43.60)	53 (46.08)	1.00	-	1.00	-
GA	48 (36.09)	43 (37.39)	1.020 (0.586 – 1.777)	0.944	1.066 (0.599 – 1.896)	0.828
GG	27 (20.30)	19 (16.52)	1.299 (0.648 – 2.602)	0.461	1.416 (0.691 – 2.902)	0.342

N= number of subjects; ^Unadjusted Odds ratios and confidence interval for case-control comparison; ‡Odds ratios and confidence interval adjusted on age, sex and country; *Statistically significant threshold was set at P<0.025 (0.05/n, n=2) determined by Bonferroni correction.



Supplementary Figure 1. The restriction fragment length polymorphism analysis to determine the genotype. **(a)** For IL10 -1082 A>G polymorphism, the AA genotype shows one 139-bp band (2, 7, 11, 12 and 13); the GA genotype shows two bands at 139-bp and 106-bp (1, 3, 9 and 15); and the GG genotype shows one 106-bp band (5, 6, 10 and 14). **(b)** For -592 A>C polymorphism, the AA genotype shows two bands at 236-bp and 176-bp (3, 6 and 11); the AC genotype shows three bands at 412-bp, 232-bp and 176-bp (1, 8, 13 and 15); the CC genotype shows one 412-bp band (2, 4, 5, 10, 12 and 14).