Applied Medical Informatics
Original Research Vol. 27, No. 3/2010, pp: 43-52

The Analysis of Genetic Polymorphism. The Relationship between Interleukin – 4 Polymorphisms and Intraepithelial Cervical Neoplasia

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Received: 24 August 2010 /Accepted: 28 September / Published online: 30 September 2010

Abstract

Objectives: Interleukin 4 plays a critical role in T helper 2 responses to HPV infection and angiogenesis. The present study aim to study the association between the IL4 promoter polymorphism – 590 C>T, respectively VNTR intron 2 polymorphism and cervical intraepithelial neoplasia. *Material and method*: We have realized a prospective case controls study that included 128 cases of intraepithelial neoplasia positive for HPV HR testing and 111 controls negative for intraepithelial lesion and also negative for HPV HR. Clinical examination was performed on each patient; blood and cervical sample were obtained. Cervical probes were analyzed regarding cytology and HPV HR testing. From peripheral blood DNA sample was obtain followed by genotype analysis for IL4 -590 C>T using PCR RFLP, respectively IL4 70 bp VNTR determined by PCR. *Results*: The absolute frequency of genotypes for IL4 -590 C>T was T/T-5, C/T-42, C/C-81 in the cases group respectively T/T-2, C/T-32, C/C-77 in the control group. The chi-square test had a value of 0.983 (p=0.321) while considering the presence of a minimum one single variant allele as a risk factor for cervical cancer, respectively 0.926 (p=0.336) for homozygous variant genotype. Odds ratio was 0.761 (95%CI [0.443-1.306]) while considering C/T+T/T respectively 2R/3R, 2R/2R as a risk factor, and 0.451 (95%CI 95% [0.086-2.374]) - TT respectively 2R/2R as a risk factor. *Conclusion*: No linear statistical significant association has been found between IL4 polymorphism and cervical neoplasia (p = 0.322).

Keywords: Genetic polymorphism; Restriction fragment length polymorphism (RFLP); Polymerase chain reaction (PCR); Interleukin-4 (IL4); Cervical intraepithelial neoplasia (CIN); Human papiloma virus (HPV) infection; Human papiloma virus high risk (HPV-HR); Negative for intraepithelial lesion (NILM) .

Introduction

The search of MEDLINE database after „genetic polymorphism" has identified 154125 articles published between September 1949 and September 2010 [1]. Genetic polymorphisms represent genetic variations encountered in the genome with a frequency of more than 1% of the population [2]. Both coding and noncoding regions can be affected; regions situated inside or outside genes structure [2]. Two types of genetics polymorphisms have been described: Single Nucleotide Polymorphism (SNP) - the allelic variants differ in a single nucleotide at a specific position, respectively simple sequence length polymorphisms (SSLP) - the allelic variants differ in the number of tenderly repeated short nucleotide sequences in noncoding DNA. The later situation has two distinct categories: short tandem repeat (STR) – repetition of a short number of base pairs (two up to four) for three up to ten times, respectively variable number of tandem repeats (VNTR) when larger sequnces are repeated (20-200 bp) [2].

HPV represents a necessary but not sufficient condition for cervical intraepithelial neoplasia [4]. Nowadays important progresses were made regarding the cervical carcinogenesis, but the predictor factor for developing cervical neoplasia in HPV infected women are still evaluated. The HPV infection plays a crucial role in oncogenesis; a shift from T helper 1 to T helper 2 immune response has been described. The genetic individual susceptibility of immune response signaling molecules could be the answer [5].

Interleukin 4 is secreted by T helper 2 cells [6]. Important roles have been identified for IL4 in the context of the immune response: it stimulates the developing of Th2 lymphocytes by acting upon the undifferentiated T cells after exposure to antigens, it induces the shift from Ig M and Ig G towards Ig E production by plasmocytes, and it determined the secretion of the whole Th2 cytokine spectrum [7]. In the meanwhile has an inhibitory effect upon interferon - γ (IFN- γ) secretion and it inhibits the differentiation of Th1 lymphocytes. Supplementary to the elevated level of IL-4 in HPV infected patients this particular interleukin plays a role in the process of angiogenesis and therefore tumorigenesis [9].

The present study aim to analyze the relation between two genetic polymorphisms (a SNP located at -590 in the promoter region and a 70 bp repeate polymorphism) of interleukin 4 in patients with intraepithelial neoplasia HPV HR (High Risk Human Papiloma Virus) compared to healthy controls.

Material and Method

Cases Selection

This study was designed as a longitudinal prospective case control study, realized on a Romanian population between 1st February 2008 and 30th June 2010. We have included 128 cases, patients diagnosed with intraepithelial neoplasia, positive at HPV HR testing (Atypical squamous cells- cannot exclude HSIL - ASC-H: 7, Atypical Squamous Cells of Undetermined Significance – ASCUS: 4, low-grade squamous intraepithelial lesion – LSIL: 36, high-grade squamous intraepithelial lesion – HSIL: 70, in situ carcinoma- CIS: 11), respectively 111 controls negatives for intraepithelial lesion and also negative at HPV HR testing.

The patients were recruited from the mass of patients that were hospitalized or only consulted on an ambulatory base during this period at First Clinic of Obstetrics and Gynecology Cluj-Napoca, Romania willing to participate at this study. Written consent was obtained for each patient.

Biological Probes Collection

For each patient a gynecological examination was performed. Immediately after the correct positioning of the speculum cytology was obtained using a special device (Cytoprep®) equipped with the brush immediately immersed in a special solution Cytofast®. The use of this technique has

allowed the concomitant realization of cervical cytology and HPV probe from the same sample obtained at a single visit. Cervical cytology results were formulated according to Bethesda nomenclature [10]. Colposcopic examination was performed for each patient. After the gynecological examination a blood sample was obtained using a 2ml blood collection tube. The blood samples were immediately store at 4-8°C.

The Analysis of Cervical Samples

The cervical cytology was examined by a cytologist after a preliminary Papanicolau staining in the laboratory of the above mentioned hospital. HPV HR testing was performed using an immunohistochemistry-based kit Viroactiv® (Virofem®).

DNA-Extraction. Genetic Analysis

The genomic DNA was extracted from peripheral leukocytes contained in 300 µl of whole blood using a commercial kit (Wizzard Genomic DNA Purification Kit, Promega®) according to producer instructions. Once extracted the DNA was stored in a freezer at -20°C.

The VNTR 70 bp repeate polymorphism of intron 2 was determined using a genetic molecular technique, polymerase chain reaction – PCR after a protocol described by Wallney and Cookson and later by Pontes CC [11, 12]. Briefly the PCR reaction was realized in 25 µl, total volume containing: 12,5 µl 2xPCR mix - Taq DNA polymerase 0,05 U/µl, MgCl2 4 mM, dNTPmix 0,4mM each (Fermentas MBI, Lithuania®), 1 µl BSA solution (Bovine Serum Albumin, Fermentas MBI, Lithuania®) 2 mg/ml, 1µL from each primer (8 pM), forward and reverse (Eurogentec, Belgium®) (the sequence of the primers is presented in table 1) µL 75-100 ng genomic DNA. Free nuclease water was added in order to reach the total volume.

Table 1. Primers sequences - 70bp intron 2 polymorphism after Walley et al. [11]

IL-4 70 bp Fwd	5'-TAG GCT GAA AGG GGG AAA GC-3'
IL-4 70 bp Rev	5'-CTG TTC ACC TCA ACT GCT CC-3'

The PCR reactions were performed using a thermocycler (Mastercycler Gradient, Eppendorf®); the conditions of the amplification are described in table 2.

Table 2. PCR protocol –70bp intron 2 polymorphism [11, 12]

No.	Step	Temperature	Time
1.	Initial denaturation	95°C	15 min
2.	Denaturation	94°C	60 sec
3.	Annealing	55°C	60 sec
4.	Elongation	72°C	60 sec
Repeat steps 2 to 4 for 40 times			
5.	Final elongation	72°C	10 min

The amplification was followed by electrophoresis using a 2% MetaPhor® Agarose. The variant allele has a length of 183 bp while the common allele 253 bp. After ethidium bromides staining the amplification products were visualized in UV Transiluminator (Figure 1).

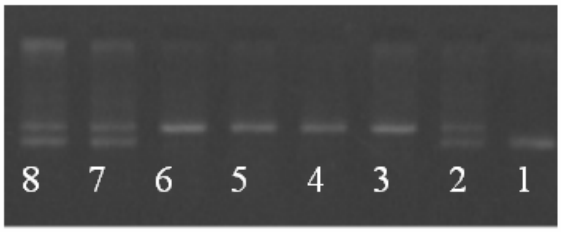

Figure 1. 2% MetaPhor® Agarose gel –70 bp intron 2 polymorphism - 8 patients: 1 – homozygous for variant allele(2R/ 2R); 2, 7, 8 heterozygous genotype (2R/3R), 3,4,5,6 – common homozygous for common allele (3R,/3R)

In order to perform the genotyping of IL4 -590C>T promoter polymorphism a PCR RFLP technique (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) was realized following a protocol described by Wallney and Cookson [11] and later by Pontes CC [12]. The same PCR mix was used for PCR using specific primers (see Table 3). The conditions of the amplification are presented in Table 4.

Table 3. The sequence of the primers - PCR protocol IL4 -590 C>T after Pontes CC [12]

IL-4 Fwd	5'-ACT AGG CCT CAC CTG ATA CG- 3'
IL-4 Rev	5'-GTT GTA ATG CAG TCC TCC TG- 3'

Table 4. The amplification conditions - IL4 -590 C>T after Pontes CC [12]

No.	Step	Temperature	Time
1.	Initial denaturation	95°C	15 min
2.	Denaturation	94°C	60 sec
3.	Annealing	57°C	60 sec
4.	Elongation	72°C	60 sec
Repeat step 2 - 4 - 40 times			
5.	Final elongation	72°C	10 min

The amplification was followed by enzymatic digestion using 3 UI FaqI® (Fermentas) and 1x Buffer overnight at 37°C. The presence of IL4 -590 C>T the cleavage site is abolished resulting a 252 bp product. For exemplification an agarose gel fragment examined in the UV light is presented (Figure 2).

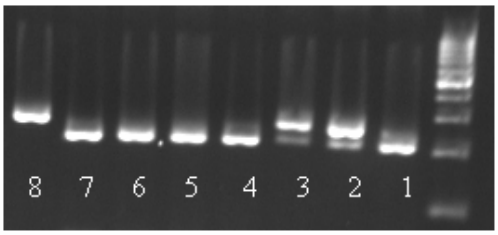

Figure 2. 2% MetaPhor® Agarose gel – IL4 -590 C>T: DNA ladder; CC 1, 4, 5, 6, 7; CT 2, 3; TT-8

Data Analysis

The quantitative continuous variables were express using centrality and dispersion parameters. The qualitative variables were expressed as percents accompanied by 95% confidence intervals. The mean and the standard deviation were used for summarizing continuous quantitative variables. Percentage expression was used for summarizing qualitative variables [13, 14].

Genotypes distribution comparison between cases and controls was realized using chi-square test. The differences were considered significant if p-value was lower than 0.05.

Z score was used for comparing two proportions; the level of significance was 5%. CatROM program was used in order to calculate the statistical parameters associated to the 2x2 contingency table for the investigation of genetic polymorphisms as risk factor for cervical cancer [15].

Tests: Kolmogorov-Smirnov [16] and Shapiro-Wilks [17] were used for normality testing for all experimental quantitative data at a level of significance of 5%.

Data were analyzed and the results were summarized using specific software: SPSS 16.0 and Microsoft Excel 2007.

Results

Studied Group Analysis

One hundred and twenty eight patients have fulfilled the inclusion criterias for cases while one hundred and eleven patients were included in the control group. The distribution of cases regarding the cytological diagnosis is illustrated in Figure 3.

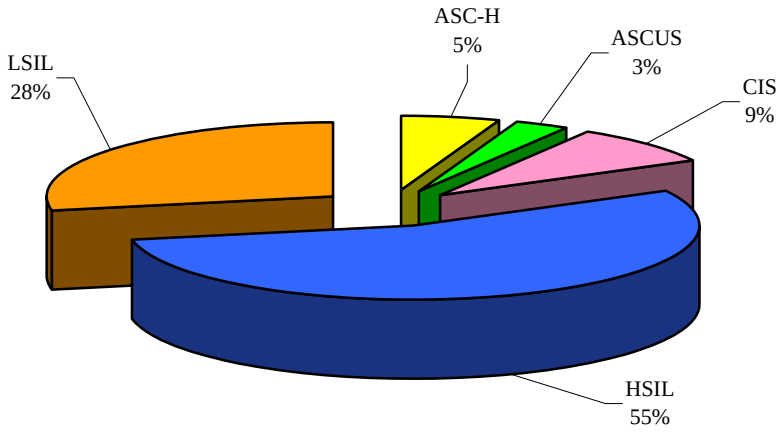

Figure 3. Cases distribution after cytological diagnosis

Z test was applied on the cases group for comparing two proportions; the results are presented in Table 5.

The statistical descriptive parameters associated with age variable are presented in Table 6.

The age variable had not a normal distribution (Kolmogorov-Smirnov test, Shapiro-Wilks test) at a significance level of 5% ($p < 0.04$); therefore the use of parametrical tests for comparing age average for cases and controls was not allowed.

Age comparison for cases group was realized using Kruskal-Wallis, respectively medians testing (Table 7). Kruskal-Wallis test had a value of 10.127, with an associated p-value of 0.038. Because approximately 40% of the expected values are lower than 5 the obtained results are only orientative.

Mann-Whithney test was applied to the cases group in order to identify the differences for age variable divided by cytological diagnosis into five classes; results are presented in Table 8.

Table 5. A comparative analysis of frequency of different cytological classes

	ASCUS (3%)	CIS (9%)	HSIL (55%)	LSIL (28%)
ASC-H (5%)	0.4150	0.2109	< 0.0001	< 0.0001
ASCUS (3%)		0.0443	< 0.0001	< 0.0001
CIS (9%)			< 0.0001	0.0001
HSIL (55%)				< 0.0001

Table 6. Statistic parameters for age variable

	AVR [95% CI]	Median	Module	StDev	Min	Max
Case group (n = 128)	37.91 [36.25-39-57]	37	41	9.48	18	61
ASC-H (n=7)	42.57 [33.91-51.23]	39	multimodal	9.36	33	58
ASCUS (n=4)	36.00 [13.46-58.54]	30	multimodal	14.17	27	57
CIS (n=11)	39.91 [33.93-45.88]	40	36	8.89	28	61
HSIL (n=70)	39.26 [36.99-41.52]	39.5	41	9.5	18	61
LSIL (n=26)	33.97 [31.18-36.76]	33	41	8.25	23	50
Control group (n=111)	35.68 [33.71-37.66]	32	32	10.49	21	69

95%CI = 95% confidence interval; StDev = standard deviation

Table 7. Age median comparison in the case group

	Group of cases				
Cytological diagnosis	ASCUS	ASC-H	CIS	HSIL	LSIL
> Median	1	4	6	38	11
<= Median	3	3	5	32	25

n = 128; median = 37; $\chi^2 = 6.72$ (df = 4); p = 0.152

Table 8. Mann-Whithney test for cases group (results)

	ASC-H	ASCUS	CIS	HSIL	LSIL
ASC-H		0.131	0.190	0.321	1.000
ASCUS			0.683	0.473	0.026
CIS				0.926	0.068
HSIL					0.008

Combined Analysis of The Two Genetic Polymorphism (IL4 -590 C>T; IL4 70 bp repeated polymorphism of intron2)

The frequencies of genotypes apparition for IL4 -590C>T polymorphism and 70 bp repeate polymorphism of intron 2 are shown in Table 9 and respectively 10.

Spearman correlation matrix was calculated in order to perform concordance analysis between the two IL4 polymorphisms (Table 11). Cronbach's concordance coefficient had a value of 0.440 ($p = 5.9 \cdot 10^{-6}$).

Table 9. IL4 -590C>T genotyping

	Cases		Controls		p
	Absolute frequency	% [95%CI]	Absolute frequency	% [95%CI]	
T/T	5	3.9 [1.57-8.59]	2	1.8 [0.08-6.30]	0.3724
C/T	42	32.8 [25.00-41.10]	32	28.8 [20.73-37.83]	0.5061
C/C	81	63.3 [54.69-71.87]	77	69.4 [60.36-77.47]	0.3305
Total	128	100	111	100	
Allele frequency					
C	204	79.68	186	83.78	
T	52	20.31	36	16.21	
Total	256	100	222	100	

Table 10. IL4 70 bp, intron 2 - genotyping

	Cases		Controls		p
	Absolute frequency	% [95%CI]	Absolute frequency	% [95%CI]	
2R/2R	5	3.9 [1.57-8.59]	2	1.8 [0.08-6.30]	0.3724
3R/2R	42	32.8 [25.00-41.10]	32	28.8 [20.73-37.83]	0.5061
3R/3R	81	63.3 [54.69-71.87]	77	69.4 [60.36-77.47]	0.3305
Total	128	100	111	100	
Allele frequency					
3R	204	79.68	186	83.78	
2R	52	20.31	36	16.21	
Total	256	100	222	100	

Table 11. Spearman correlation matrix for cases (n-128) and controls (111)

		IL4 70 bp
Cases		
IL4-590 C>T	Correlation Coefficient	1.000(**)
	Sig. (2-tailed)	.
	N	128
Controls		
IL4-590 C>T	Correlation Coefficient	1.000(**)
	Sig. (2-tailed)	.
	N	111

While trying to evaluate the relation between polymorphism presence and cervical intraepithelial neoplasia we have utilized two hypotheses:

1. First hypothesis – the presence at least one variant allele was considered a risk factor (hetero- or homozygous status)
2. Second hypothesis – the presence of homozygous variant genotype was considered risk factor

The results are shown in Table 12. Taking into consideration the perfect concordance between the two polymorphisms the statistical calculated parameters are presented in only one table for both polymorphisms.

Table 12. The risk evaluation – IL4 polymorfisms – CIN - OR

	Value	95% Confidence Interval	
		Lower	Upper
First hypothesis (CT+TT respectively 2R/3R+ 2R/2R considered a risk factor)			
OR	0.761	0.443	1.306
Second hypothesis (TT respectively 2R/2R considered a risk factor)			
OR	0.451	0.086	2.374

For testing the relation between polymorphism presences a risk factor for cervical intraepithelial neoplasia chi-square test was also used. The results are presented in Table 13.

Table 13. Chi-square test

		Cases	Controls	Total
Genotype	CC (3R/3R)	81	77	158
	CT &TT (2R/3R&2R/2R)	47	34	81
Total		128	111	239

$\chi^2 = 0.983$; df = 1; p = 0.321

		Cases	Controls	Total
Genotype	CC &CT (3R/3R/2R/3R)	123	109	232
	TT (2R/2R)	5	2	7
Total		128	111	239

Fisher's Exact Test; p = 0.455

Discussion

Previous studies have shown an association between these polymorphisms, autoimmune diseases and several neoplasias (colorectal cancer [18], oral cancer [19], renal cancer [20], and gastric cancer [21]). The link can be explained by the implication of these molecules in the systemic response to infection and carcinogenesis, but the intimate mechanisms are far from being elucidated. The presence different genotypes at -590 C>T in IL4 promoter region is associated with an increased IL4 activity in vivo caused by the facilitated binding of particular transcription activating factors (NFAT-1 - nuclear factor of activated T cells) [22]. A raise in the seric level of immunoglobulin (Ig) E has described in asthmatic patients [23] with IL4 -590 TT genotype but also in parasitic infections (malaria [24], brucellosis [25], and leishmaniasis [26]). Others associations of this polymorphism were with autoimmune disorders (lupus [27], rheumatoid arthritis [28]), lichen planus [29]), and other inflammatory disorders (periodontal disease [30]). It is well known that IL-4 produces a shift of the immune response towards Th2 in the context of HPV infection [31], but also in others disorders where the association with this polymorphism was proved (Sjogren syndrome [32] or with other infection (HIV [33]) . All these data represents a solid argument for the search of a link between IL-4 -590 and IL 4 intron 2 70bp VNTR on one hand and cervical intraepithelial neoplasia on the other hand.

In the present study HSIL was the most frequent class of cervical intraepithelial neoplasia encountered; theses can be explained as a consequence of the modality of selecting patients. The majority of patients were recruited from hospital admitted patients; in the meanwhile the patients with less severe CIN (LSIL, ASCUS, and ASC-H) are monitored usually in ambulatory. Actually in everyday practice the incidence of LSIL is the highest [34].

Regarding patients age a statistical significant difference was found between the rages associated with the five classes for cases group ($p = 0.038$). The medians of ages for the five pathologies were not significantly different for the median of the whole group. The average of age range was statistically significant higher (Mann-Whitney test = 5799.5, $p = 0.014$) for cases (130.19, $n = 128$) compared to controls (108.25, $n = 111$).

Cronbach's coefficient value (0.440 ($p = 5.9 \cdot 10^{-6}$) has showed a perfect concordance between IL4 -590C>T polymorphism and IL4 70 bp VNTR intron 2.

The conclusion of this study is that there is no significant linear association between the two above mentioned IL4 polymorphisms and cervical intraepithelial neoplasia ($p = 0.322$). Odds ratio values of 0.761 while considering the presence of a single variant allele as a risk factor, respectively 0.451 while only the homozygous variant genotype was taken into consideration suggests a possible protector role for a variant allele towards cervical intraepithelial neoplasia.

The relation between interleukin 4 genetic polymorphism and HPV infection remains an unsolved issue, supplementary study on a larger number of patients being necessary. It is important to take into consideration the interrelation between cytokines, chemokines and other paracrine factors that might influent each other in a cascade type reaction.

On the other hand the genetic complexity of immune system signal molecules would necessitate a vast project in order to obtain the exact sequence of nucleotide of interleukins (using sequencing) followed by their integrated analysis. A project of such amplitude on a large population is difficult to perform and requires an important amount of both human and financial resources.

Conclusions

1. A linkage disequilibrium was found between IL4 -590 C>T and IL4 intron 2, 70 bp repeated VNTR.
2. No statistical significant differences were found between the genotypes between the two groups (cases – controls.
3. The presence of a variant allele (T at -590 respectively 2R) seems to be a protective for cervical intraepithelial neoplasia.

Conflict of Interest

The author(s) declare that they have no conflict of interest.

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