

ASSOCIATION OF INTERLEUKIN-1 POLYMORPHISMS WITH PERIODONTAL DISEASE IN THE MENTALLY RETARDED

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ABSTRACT

Genetic test for a composite Interleukin-1 (IL-1) genotype is being marketed to predict risk for progression of periodontitis.

Aim: To elucidate the effect of genetic variance of inflammatory mediators expression and the influence of microbial expression, as risk factors for periodontitis – inflammation around teeth – among Down's syndrome (DS) individuals, as well as to characterize a new procedure to perform this concept through isolation of DNA from dried blood spots collected on a specific filter paper.

Materials & Methods: Twenty normal volunteers and thirty Down's syndrome (DS) children (comparable of age range, 15-20 years old) represented the sample of this study. Their periodontal status was estimated through periodontal examination (full mouth clinical attachment loss measurement, probing depths, plaque index scores, and bleeding on probing). Isolation and detection of certain oral pathogens; *A.actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* was performed. Genotype for bi-allelic IL-1A+4845, IL-1B+3954 gene polymorphisms using blood was detected by PCR based methods.

Results: The study clarified that the distribution of IL-1 α and IL-1 β composite genotype among the normal and Down's syndrome individuals were 30% and 5% & 23.3% and 13.3%, respectively. The total IL-1 (IL-1 α & IL-1 β) were 35% in the normal and 36.7% in the DS individuals. Results showed significant difference between the total composite IL-1 genotype of allele 2 carriage of IL-1A (+4845) & IL-1B (+3953) of DS and normal individuals. There were significant differences between the two groups (DS and normal) only as regards to colonization of Gram (-ve) facultative rods (*A.actinomycetemcomitans*) and nearly all microorganisms showed a higher percentage within DS more than normal (*A. actinomycetemcoitans* were 30% & 5%, respectively, *P. gingivalis*; 23.3% and 10% respectively and *P.intermedia* was not detected within normal and detected in 6.7% of DS individuals, In addition, DNA was stable and could be captured on FTA cards when tested by PCR and matched with results detected through ordinary procedure.

Conclusion: The composite IL-1 genotype is associated with the severity of periodontitis and further

studies have to be conducted to confirm transmission disequilibrium testing with parental DNA to detect excess transmission of the disease associated with genotypes to the affected off-spring. The use of FTA cards were recommended as a new surveillance tool for molecular techniques. It constitutes a significant improvement in the collection of samples (especially with children and handicapped individuals), as well as ease in sample transport.

1. INTRODUCTION

The etiopathogenesis of the periodontal disease is poorly understood, as it is a multifactorial disease, and individual differences in initiation and progression of the disease are dramatic.

The pro-inflammatory cytokine interleukin-1 (IL-1) is a key regulator of the host's responses to microbial infection and a major modulator of extracellular matrix catabolism and bone desorption. It has been reported that variations in the IL-1 gene cluster on chromosome 2 are associated with increased susceptibility to severe periodontitis (Kyoko, et. al., 2000 and Mc Devitt, et. al., 2002). Therefore, a genetic test was being marketed to predict the risk for periodontal disease progression (Higashi, 2002).

The reason for different inflammatory response among some people more than others in responding to the same stimulus may be speculated as occurring due to the dys-regulated production of IL-1, which over-rides the feedback mechanisms that normally master the dose of inflammation to a level sufficient to fight microbial invasion without long-lasting damage to the tissues involved (Agerbaek, et. al., 2006).

Down's syndrome (DS) individuals often develop severe early-onset marginal periodontitis in early adulthood; however, there is little information available on the microbiology of DS periodontitis (Amano, et. al., 2001). Those individuals (DS) differed in their oral condition, compared with the healthy population and showed more severe periodontal disease which may be related to their immune deficiency (Zaldivar-Chiapa, et. al., 2005). Dried blood samples on filter paper (DBS) represented easily handled, stored, and shipped resources of analysis. These have been used worldwide for the neonatal screening of congenital disorders (Guthrie, 1992). Recently, FTA (Flinders Technology Associates) card was introduced to detect bacterial DNA or viral RNA from different biological samples, such as blood, saliva, tissues (Burgoyne,

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1996). These cards are cotton-based cellulose papers impregnated with anionic detergent and buffer that provide chelating and free radical-trapping properties. FTA cards contain reagents designed to adapt the storage, transport and integrity of samples to kill or inhibit saprophytes during drying or bouts of high humidity (Burgoyne, 1996). Moreover, it contains lyophilized chemicals that lyse many types of bacteria and viruses. Most cell types are lysed on contact with FTA, including white blood cells (Devost and Choy, 2000) and bacteria (Lampel, et. al., 2000). Viruses are also inactivated, leaving the nucleic acids suitable for molecular identification (Katz, 2002).

The aim of this study was elucidating the effect of genetic variance of inflammatory mediator expressions and the influence of microbial expression, as a risk factors for periodontitis among DS individuals. Also, it aimed to characterize a new procedure to perform this concept through isolation of DNA from dried blood spots collected on specific filter paper.

2. MATERIALS AND METHODS

2.1 Subject and Samples

A. Subjects

- i. Thirty DS children of age range from 15-18 years were selected from "Mentally Retarded School-Dokki area. (Each case was subjected to full clinical anthropometric evaluation, to assess their growth and development).
- ii. Twenty normal healthy volunteers of age range from 18-20 years.

B. Samples

- i. *Blood samples:*
Five millilitre of venous blood from all individuals.
Aliquots of sample (50µl) were obtained by finger prick from the same individuals, blotted onto the filter paper (Whatmann FTA cards) (1 cm in diameter) and allowed to air-dry at 20 to 25°C till use.
- ii. *Plaque samples:*
Using paper-point, plaque samples were collected from each case and transferred in a tube full of sterile reduced transport media (Thioglycolate broth).

All cases were also examined carefully for oral and

dental evaluation as follows:

C. Dental examinations

- i. Periodontal examination; probing pocket depth, bleeding on probing, clinical attachment loss and gingival recession (A North Carolina probe was used in the examination).
- ii. Clinical attachment levels both measured with a manual probe on six locations around each tooth.
- iii. Recorded gingival inflammation using gingival index according to Löe and Silness (1963); the teeth and gingiva were dried and adequate illumination, a plain mouth mirror and number 3 periodontal probes, were used. The probe was used to press on the gingiva to determine the degree of firmness. The probe was used to run along the soft tissue-wall near the entrance of the gingival sulcus, to evaluate bleeding.

D. Scoring gingival index criteria depended on the following investigation:

- | | | |
|---|---|---|
| 0 | = | Normal gingiva |
| 1 | = | Mild inflammation—slight change in color, slight edema. No bleeding on probing. |
| 2 | = | Soft debris, covering more than one third but not more than two thirds of the exposed tooth surface |
| 3 | = | Soft debris, covering more than two thirds of the exposed tooth surface. |

Debris covering the surface area was estimated by running the side of the tip of an explorer across the teeth surface.

E. Identifying the routine professional tooth-cleaning procedures

F. Family history

Bleeding disorders, cardiovascular disease or diabetes mellitus, ethnic origins of their parents.

2.2 Analysis of Interleukin (IL-1 α and IL-1 β) Genetic Polymorphism:

A. DNA extraction:

- i. *Using whole blood:*
QIA amp DNA Mini-kit. QIA En, Ltd, UK (Genomic DNA purification kit) was used for

Table - 1: Total anaerobic count (CFU/ml) in relation to gingival index of individuals tested

Total anaerobic count (CFU/ml)	Normal individuals			Down Syndrome individuals (DS)	
	Good GI	Fair GI	Bad GI	Bad GI	Fair GI
103	0/9 (0%)	0/7 (0%)	1/4 (25%)	3/25 (12%)	0/5 (0%)
104	0/9 (0%)	1/7 (14.3%)	1/4 (25%)	9/25 (36%)	1/5 (20%)
105	0/9 (0%)	1/7 (14.3%)	2/4 (50%)	13/25 (52%)	2/5 (40%)

extraction of DNA from the whole blood, as instructed.

ii. *Using filter paper:*

Each dried sample was transferred to micro-centrifuge tube containing lysis buffer which is shaken for 5 minutes at room temperature, vortex for 15 sec and centrifuged for 30 sec at 12,000g. This wash step was repeated three times. The pellet was resuspended in TE buffer vortex, incubated for 10 min with periodical vortex, and is set to centrifuging for 2 min. After drying, pellets were ready to be subjected to PCR reaction .

3. ANALYSIS OF POLYMORPHISMS IN GENES OF THE IL-1A&B

The bi-allelic polymorphisms at position -889 within the promoter region of the IL-1A gene McDowell, et. al., (1995) and at position +3954 (Taq I RFLP) within exon 5 of the IL-1B gene Bioque, et. al., (1995), were determined according to previously described methods.

3.1 Microbiological Analysis

The following Media were used, as instructed:

- Trypticase soy agar (ETSA), Trypticase soy agar supplemented with sucrose, crystal violet and polymixin B sulfate (HLE) and Tryptic soy serum-bacitracin-vancomycin agar (TSBV) were

prepared as per instruction.

- Samples were diluted in ten-fold step, with repeat homogenization on vortex mixer at a maximal setting for 10 seconds between successive dilutions. Aliquots of 0.1 ml of the dilution were spread on freshly prepared enriched tryptic soy agar (ESTA), (HLR) and (TSBV). ESTA plates were anaerobically incubated for seven days, HLR and TSBV agar plates were incubated in 5% CO₂ air incubator for five days at 37°C. After the incubation periods, one ESTA plate suitable for counting; CFU/ml were calculated considering the respective dilution factor. The relative proportion of all different colony morph types were determined. Representative colonies of these types were sub-cultured on ESTA and incubated for four to seven days. Characterization and identification were performed based on colony and cellular morphology. Grams stain reaction, phase contrast microscopy for registration of motility, biochemical reactions, agar fermentation pattern, and fluorescence in long wave ultra violet light.

Confirmation was done place by using API (fermentative 29 biochemical standardized enzymatic reactions, which depend on the biochemical properties of the tested anaerobic microorganisms).

Individuals examined were further classified, according to their Gingival-index (GI) score, into good, fair and bad GI score groups.

Table - 2: Percentage of certain bacterial species isolated from plaque samples of Down syndrome individuals compared with normal individuals

Bacterial species	% of different types in normal individuals	% of different types in DS individuals	Z.score	P
Gram (-) facultative rods <i>A. actinomycetemcoitans</i>	(1/20) 5%	30% (9/30)	1.96	0.02
Gram (-) anaerobes <i>Porphyromonas gingivalis</i>	(2/20)10%	23.3% (7/30)	1.3	0.09
Gram (-)anaerobes <i>Provetella intermedia</i>	(0/20) 0%	6.7% (2/30)	-	-

4. RESULTS

Table-1 demonstrates the total anaerobic count among all examined individuals. It was noticed that the highest CFU/ml (105) and (104) was counted only among bad and fair gps of both normal and DS individuals. The lowest CFU/ml (103) was found only among the bad GI score gps.

There were significant differences between the two groups (DS and normal) only as regards colonization of Gram (-ve) facultative rods (*A. actinomycetemcomitans*). Nearly all microorganisms showed a higher percentage within DS more than normal (*A. actinomycetemcomitans* were 30% & 5%, respectively, *P.gingivalis*; 23.3% and 10%, respectively, and *P.intermedia* was not detected within normal group and was detected in 6.7% of DS individuals (Table-2).

Table-3 reveals that only 11 out of the 30 subjects (36.7%) carried the composite IL-1 genotypes consisting of both IL-1 α and IL-1 β . There were significant differences between the total carrier of allele 2 from fair and bad GI scores groups (20% with fair GI score and 40% with bad GI score). IL-1 α polymorphism was carried by a more percentage than IL-1 β 23.3% (7/30) and 13.3 % (4/30) of all subjects tested, respectively.

Table-4 revealed that 7 out of the 20 individuals (35%) carried the composite IL-1.

Genotypes consisted of both IL-1 α and IL-1 β . There were significant difference between the total carrier of allele 2 from fair and bad GI scores groups compared to those with good GI score group (11.1% of them were with good GI score 42.8% with fair GI score & 75% with bad GI score). IL-1 α polymorphism was carried by a more percentage than IL-1 β (30 % (6/20) and 5%

(1/20) of all subjects tested, respectively.

Table-5 showed a significant difference of the total composite IL-1 genotype of allele 2 carriage of IL-1A(+4845) and IL-1B (+3953) of DS compared to normal individuals DNA was stable and could be captured on FTA cards when tested by PCR and matched with results detected through ordinary procedure.

5. DISCUSSION

Periodontitis is a multifactorial chronic inflammatory disease. However, it is difficult to ascertain the role of the different factors involved in its pathogenesis. Our microbiological study focused on a number of microbial species (*A. Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia*), which were proposed to be a useful tool for the identification of susceptible individuals (Slots and Listgarten, 1988; Maiden et. al., 1990; and Haffajee, et. al., 1991). Substantial data supported the current concept that specific bacteria were essential for initiation and progression of chronic periodontitis (Page, et. al., 1997). The rates of progression and disease severity were determined by host modifiers, such as smoking, diabetes, and genetic influences.

Schroeder and Listgarten (1997) suggested that the continuous presence of such large numbers of bacteria probably accounts for varied host defense mechanisms against bacterial invasion and growth that could be found in the gingival tissues. Reports by Marsh and Martin (1999) supported our results that nearly all micro-organisms show a higher percentage within DS as compared with normal (*A. actinomycetemcomitans*) percentages were 30 & 5 respectively, *P.gingivalis*; 23.3 and 10 respectively and *P.intermedia* was not detected in normal cases but

Table - 3: Distribution of composite IL-1 genotype of allele 2 carriage of IL-1A (+4845) & IL-1B (+3953) among examined Egyptian Down Syndrome children divided according to their GI score

Groups according to GI score	IL-1 genotype of allele 2 carriage of-IL-1A(+4845)and IL-1B(+3953		Total carriers of allele 2
	IL-1 α	IL-1 β	
Fair (GI)gp	1/5 (20%)	0/5 (0%)	1/5 (20%)
Bad (GI)gp	6/25 (24%)	4/25(16%)	10/25 (40%)
Chi square between fair GI and bad GI gp			0.004
Total studied sample	7/30 (23.3%)	4/30(13.3%)	11/30 (36.7%)

Table - 4: Distribution of composite IL-1 genotype of allele 2 carriage of IL-1A (+4845) & IL-1B (+3953) among Examined Normal Egyptian individuals divided according to their GI score

Groups according to GI score	IL-1 genotype of allele 2 carriage of-IL-1A(+4845)and IL-1B(+3953)		Total carriers of allele 2	Chi square between goodGI score group and other GI score gps
	IL-1 α	IL-1 β		
Good (GI) gp	1 /9 (11.1%)	0/9 (0%)	1/9 (11.1%)	-
Fair (GI) gp	3/7 (42.8%)	0/7 (0%)	3/7 (42.8%)	0.002
Bad (GI) gp	2/4 (50%)	1/4 (25%)	3/4 (75%)	0.0066
Total studied sample	6/20 (30%)	1/20 (5 %)	7/20 (35%)	

was detected in 6.7% of DS. In addition, Sakellari, et. al., (2005) noted that DS individuals displayed more severe periodontal destruction earlier, and a heavier colonization with periodontal pathogens compared with age-matched healthy individuals.

Prevalence of the total IL-1 (IL-1 α and IL-1 β) polymorphism in subjects of our results of both normal and DS individuals with bad GI score (75% and 40%) almost double those with fair GI (42.8% and 20%) respectively, which indicated that there were evidence of a linkage between severity of periodontal disease and IL-1 gene expression. These results coincided with the findings reported by Di Giovine, et. al., (1996), Salvi, et. al., (1998), Socransky, et. al., (2000), Mary, et. al., (2001) and Laine, et. al., (2002). Genes which encode inflammatory cytokines were subjected to polymorphisms in their regulatory regions that may affect both the level and ratio of cytokines produced in response to exogenous stimuli. These variant alleles were observed in a large percent of the population and are often associated with increased or decreased susceptibility or severity (modifiers) to infectious, immune or inflammatory diseases (Yucesoy, et. al., (2003). Our results correlated the severity of periodontitis to presence of carriers of allele 2 genotype in the IL-1A and IL-1B genes. Our data agreed with Kornman, et. al., (1997) who reported the same correlation and explained this finding as genetic mechanism by which some individuals, if challenged by bacterial accumulations, might have more vigorous immune-inflammatory response leading to more severe periodontitis. Moreover, Kornman (2006) added that monocytes from individuals homozygous

for the IL-1 B +3953 allele 2 produce four-fold more IL-1 β and heterozygous cells produce approximately two-fold more IL-1 β from individuals homozygous for allele-1.

The complex interactions that occur between host-response mechanisms and oral pathogens in periodontal disease have made elucidation of genetic factors in disease susceptibility more difficult (Hassell, et. al., 1995).

Our results showed that, within the sample of this study IL-1 α and IL-1 β polymorphisms were carried by 30%, 23.3% and 5%, 13.3 % among the normal and DS individuals, respectively, i.e., IL-1 α polymorphism was more frequent in our sample than IL-1 β . This finding was in accordance to finding of Gary, et. al., (2000) that IL-1 β polymorphism was much rare with only 3.3% (10/300) in their study on Chinese population. In contrast, findings of Walker, et. al., (2000) suggested that IL-1 β polymorphism was the most prevalent allele in a study of 37 individuals in the general African-American population in Western North Carolina .

There was a concept mentioned that the prevalence of IL-1 genotype positive subjects differ according to ethnic populations. It was found to be 26% in a Hispanic Mexican population (Caffesse, et. al., 2002). A higher percentage (38.9%) was observed by (Mary, et. al., 2001) in European heritage.

The present study recommends the use of FTA cards, as it establishes a new surveillance tool for molecular

Table - 5:Chi square for comparison of the distribution of composite IL-1 genotype of allele-2 carriage of IL-1A (+4845) & IL-1B (+3953) among all examined individuals

Total carriers of allele-2 among DS individuals	Total carriers of allele 2 among normal individuals	Chi square
7/20	11/30	0.00005

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techniques. In agreement with our recommendation, the studies of Abe, et. al., (1998) and Abe and Konomi (1998) confirm similar results on analysis of frozen serum compared with plasma dried on the filter paper related to the same samples. Moscoso, et. al., (2005) and Elizabeth, et. al., (2006) documented that molecular characterization is feasible in stored samples on FTA under unfavourable environmental conditions (41°C) for at least 15 days. Generally, many scientists preferred this method, due to safety of storage and shipment to laboratory in settings where these issues are problematic. Furthermore, this method is practically valuable for small-volume samples, large population-based studies, in case of problems with cold storage and transportation.

In conclusion, the composite IL-1 genotype is associated with the severity of periodontitis and further studies have to be conducted to confirm transmission disequilibrium testing with parental DNA, to detect excess transmission of the disease-associated genotypes to affected off-spring. The use of FTA cards are recommended as a new surveillance tool for molecular techniques. It constitutes a significant improvement in the collection of samples (especially with children and handicapped individuals), as well as ease in sample transportation.

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