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Epigenetic modification in TNFa-gene

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Introduction

Periodontitis is characterized as a chronic inflammatory disease of the periodontal supporting tissue of teeth induced by periodontotpathogens. That's why several factors of the immune response have been proposed as potential markers for the development of this disease.
Cytokines, including the potent proinflammatory TNF-a, may be regarded as markers of the progression and severity of periodontitis as well as indicators of an appropriate response to treatment.
The expression of genes involved in inflammatory processes is influenced among others by genetic and epigenetic modifications. Moreover, many risk factors associated with periodontitis, including bacterial occurrence, smoking, or diabetes are known to induce epigenetic changes.
Epigenetic modification may occur at DNA level (methylation of CpG islands) or at histone level.
Despite TNF-a does not contain a classical CpG island a region of the promoter exhibits CpG rich sequences. Therefore, its presumably that the TNF-a is under epigenetic regulation via DNA methylation.

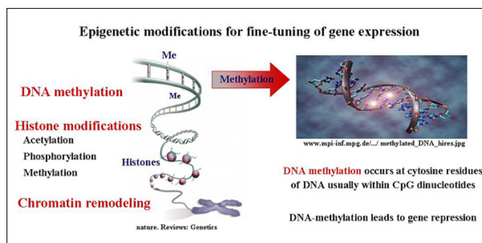


Fig. 1: Epigenetic modifications for fine-tuning of gene expression

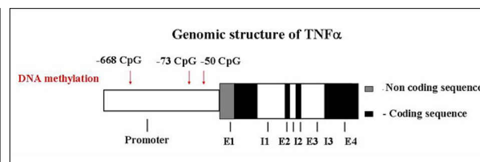


Fig. 2: Genomic structure of TNFa

Objectives

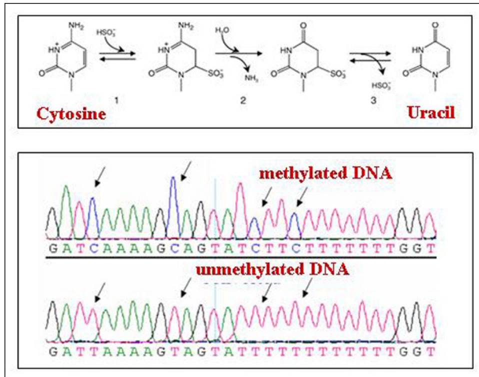
The aim of the present clinical study was to establish methods for evaluating the DNA methylation status of TNF-a. Furthermore, in first experiments the methylation status of CpGs -668, -73, -50 in TNF-a promoter was investigated in gingival biopsies and blood of patients with generalized periodontitis as well as periodontitisfree controls.

Material and Methods

DNA-Isolation
Preparation of genomic DNA from human venous EDTA-blood was carried out using the blood extraction kit (Qiagen).
200µl EDTA-blood and 20 µl protease were mixed in a 1,5 ml tube.
After adding of 200 µl denaturation buffer AL and pulse-vortexing for 15 sec the samples were incubated at 56°C for 10 min.
200 µl of ethanol was added to the samples, vortexed and the samples were applied to a QIAamp Spin Column were the DNA is bound.
After two washing steps (buffer AW1 and AW2) the DNA bound to the column is dried by centrifugation.
200 µl distilled water is added to the samples, incubated at room temperature for 5 min and then centrifuged. The solved DNA is now in the filtrate.
Long-term storage of DNA is possible at -20°C.

DNA-isolation from gingival biopsies
During periodontal surgery gingival biopsies were obtained and were immediately frozen in liquid nitorgen
Preparation of genomic DNA from biopsies were carried out using the QIAamp® DNA Micro Kit (Qiagen).
The frozen tissue sample was transferred into a tube and 180µl buffer ATL was added immediately
After equilibration to room temperature 20µl Proteinase K was added and vortexed.
After overnight incubation at 56°C the same procedure as described above for EDTA-blood samples was carried out.

Bisulfite conversion (EpiTect® Bisulfite Conversion (Qiagen))



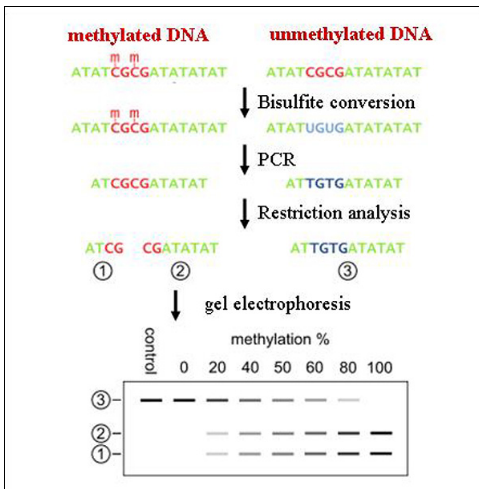
The methylation status of DNA can be evaluated using sodium bisulfite: Incubation of DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged.

-Bisulfite reaction components (20µl)	Thermal cycling conditions		
250ng DNA	Denaturation:	5min	95°C
RNase-free Water	Incubation:	25min	60°C
Bisulfite Mix	Denaturation:	5min	95°C
DNA protection buffer	Incubation:	85min	60°C
	Denaturation:	5min	95°C
	Incubation:	175min	60°C
	Hold:		20°C

Cleanup via binding of converted single-stranded DNA to the membrane of an EpiTect spin column, washing, desulfonation, washing, and elution

Fig. 3a-b: Principle of EpiTect® Bisulfite Conversion (Qiagen)

Combined bisulfite restriction analysis (COBRA)



Amplification
During PCR the originally methylated residues remain cytosines whereas the unmethylated residues become thymine
Primers used do not contain CpG sites → no discrimination of methylation status at this level

PCR components (25µl)		Thermal cycling conditions		
2xMastermix (Promega)		Denaturation:	2min	94°C
10pmol/µl forward primer	30 cycles	Denaturation:	15sec	92°C
10pmol/µl reverse primer	30 cycles	Annealing:	30sec	50°C
Ad water	30 cycles	Extension:	30sec	72°C
		last extension	10min	72°C
		Hold:		10°C

Restriction digest
Application of restriction enzymes (Hinf I, Aci I) which are able to discriminate between the DNA-seqeunce containing the thymine residues or the cytosine residues
10µl PCR-product is incubated with 10U restriction enzyme (New England Biolabs) in the appropriate buffer at 37°C overnight.

Fig. 4a-b: Combined bisulfite restriction analysis (COBRA)

inclusion criteria of the probands

Generalized aggressive periodontitis (AP):
clinical manifestation before 35th year of life
attachment loss in at least 30% of the teeth with a minimum pocket depth of 4 mm
severity of attachment loss was inconsistent to the amount of mineralized plaque
more vertical than horizontal approximal bone loss was visible in the radiographs

Generalized chronic periodontitis (CP):
attachment loss in at least 30% of the teeth with a minimum pocket depth of 4 mm
The amount of the attachment loss was consistent with the presence of mineralized plaque
More horizontal than vertical approximal bone loss was visible in the radiographs.

Periodontitisfree controls

probing depth ≤ 3.5 mm, no gingival recession due to periodontitis
Clinical attachment loss > 3.5mm as a consequence of traumatic tooth brushing, overhanging dental fillings, orthodontic therapy etc. was not considered as a case of periodontitis.

Results

Probands:

Generalized aggressive periodontitis (AP):
n=8
mean age: 42.7+6.3y
male: n=5

Generalized chronic periodontitis (CP):
n=11
mean age: 55.2+7.7y
male: n=6

Periodontitisfree controls:
n=9
mean age: 34.0+12.2y
male: n=6

Validation of COBRA-analysis
Methodological error
2 x 10 DNA samples of the same proband were analysed using bisulfite conversion followed by COBRA
The evaluated methodological error was < 5%

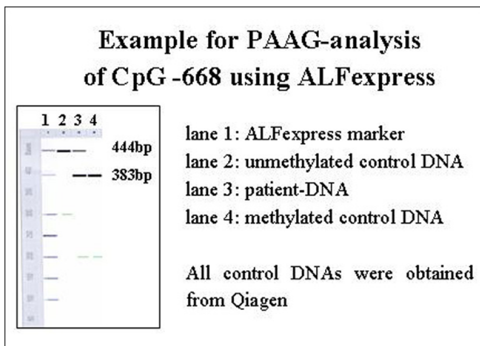


Fig. 5: Example for PAAG-analysis of CpG -668 using ALFexpress

CpG-site	Enzyme	detected fragments in ALF express analysis	
		methylated DNA	unmethylated DNA
-668	Hinf I	383bp	444bp
-73	Aci I	32bp	124bp
-50	Aci I	55bp	124bp

Tab. 1: Restriction enzymes for COBRA

Epigenetic evaluation
In first experiments differences in DNA methylation pattern were investigated at CpG-site -668 of TNF-a promoter.
It could be shown, that in patients suffering from severe periodontitis the methylation level in gingival biopsies was significantly decreased compared to venous blood.
Furthermore, a significant reduction in DNA-methylation was assessed comparing the results of gingival biopsies of the patients with periodontitisfree controls.

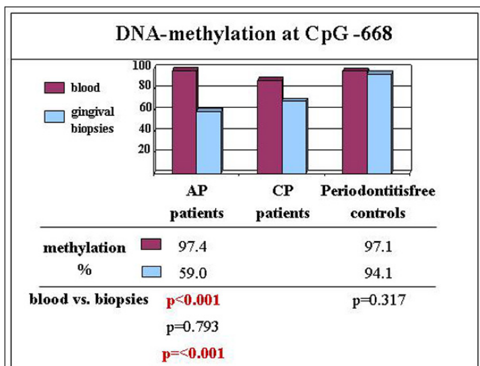


Fig. 6: Epigenetic evaluation: DNA-methylation at CpG -668

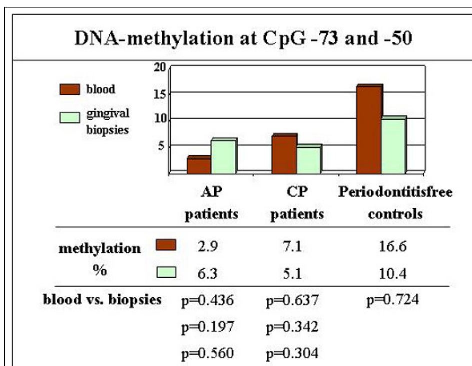


Fig. 7: Epigenetic evaluation: DNA-methylation at CpG -73 and -50

Conclusions

A quantitative method for evaluating DNA-methylation of TNF-a CpG sites -668, -73 an -50 was established and validated using COBRA and detection via ALFexpress (pharmacia biotech).
For the first time a change of epigenetic pattern in TNF-a gene was assessed comparing inflamed gingival tissue and venous blood at CpG site -668. In biopsies of patients with generalized periodontitis (AP as well as CP) a significant reduction in DNA-methylation was obviously comparing to methylation status in blood as well as in biopsies of periodontitisfree controls. The reduction in DNA-methylation could be an indicator for an elevated TNF-a gene expression already described for inflamed gingival tissue.
However, for the CpG sites -73 and -50, which are shown to be regulated in unison, no significant differences could be evaluated. Both CpG sites are little methylated, suggesting a stable TNF-a expression regardless the inflammatory periodontal disease.

This Poster was submitted by Dr. Susanne Schulz.

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Poster Faksimile:

Epigenetic modification in TNFα-gene and periodontal disease

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Objectives:

Epigenetic investigations

DNA Isolation

Results and discussion

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