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The role of SNPs in TGF-b1 at codon 10 and 25 and the occurrence of severe periodontitis

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Introduction

Periodontitis is characterized as a chronic inflammatory disease induced by periodontopathogens. TGF-b1 is a pleiotropic cytokine that exerts its effects on bone and connective tissue metabolism which are of great importance in periodontal disease. Functional important polymorphisms are described for TGF-b1:
(L10P) associated with higher circulating levels and secretion of TGF-β1, Yokota et al.2000; Dunning et al., 2003
(R25P) associated with interindividual variation in TGF-b1, Awad et al. 1998

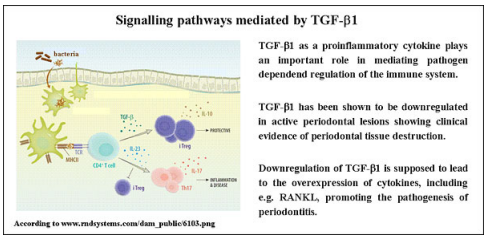


Fig. 1: Signalling pathways mediated by TGF-β1

Aim of the study

In the present study possible associations were investigated between the genetic variants of TGF-b1 and chronic/aggressive periodontitis and ist clinical features, including smoking status, plaque (API) and bleeding indexex (BOP), pocket depth (PD), clinical attachment loss (CAL) and subgingival bacterial colonization.

Material and Methods

Inclusion criteria of probands

Generalized aggressive periodontitis (AP, n=81): clinical manifestation before 35th year of life attachment loss in at least 30% of the teeth with a minimum pocket depth of 4 mm > 3 affected teeth had to be no first molars or incisors 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, n=68): 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 (n=82): 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

Genomic investigations

DNA-isolation from EDTA-blood
Preparation of genomic DNA from human venous EDTA-blood was carried out using the blood extraction kit (Quiagen).
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.

Haplotype specific PCR of TGF-β1
The detection of genotypes and haplotypes of TGF-b1 SNPs (L10P and R25P) was carried out using the CYTOKINE Genotyping array CTS-PCR-SSP Tray kit of the Collaborative Transplant Study, Department of Transplantation Immunology of the University Clinic of Heidelberg.
For every PCR a fragment of 440bp of the human CRP gene was coamplified as a positive control.
The PCRs were performed using sequence specific primers for detection of possible haplotypes prepipetted and lyophilized in thin-walled plastic 96-well PCR trays.
For every PCR 10µl of a Mastermix containing 1U Taq-Polymerase (Invitek), 100ng genomic DNA, 5% glycerol, and PCR reaction buffer was added.
PCR-program (2 min 94°C; 10 cycles: 15 sec 94°C, 1 min 64°C; 20 cycles: 15 sec 94°C, 50 sec 61°C, 30 sec 72°C)
After cycling was completed, the PCR products were loaded onto a 2% agarosegel for electrophoresis.
After electrophoresis, the ethidium bromide stained gel is photographed and interpreted.

Evaluation of periodontopathic bacteria in subgingival pockets

Subgingival sampling
Paper points for collection of subgingival samples were used to bind periodontopathogens of the deepest pocket of each quadrant.

DNA-isolation
Preparation of bacterial DNA was carried out using the QIAamp DNA Mini Kit (Quiagen).
The paper points were incubated with 180 µl ATL-buffer and 20 µl proteinase K and incubated at 70°C for 10 min.
200 µl buffer Al was added and the mixture was incubated at 96°C for 5 min.
The mixture (without paper points) was applied to a QIAamp Spin Column and washed twice with buffer AW1 and AW2.
The DNA was solved in 400 µl AE-buffer and stored at -20°C.

Multiplex-PCR
For specific amplification of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola the micro-Ident® test of HAIN-Diagnostik based on alkaline phosphatase mediated staining reaction was used.
Mastermix provided in the micro-Ident® test (containing buffer, biotynilated primer, DNA for positive control), 2U Taq-polymerase (Eppendorf), and 5 µl of isolated bacgterial DNA were mixed.
PCR was performed (5 min 95°C; 10 cycles: 30 sec 95°C, 2 min 58°C; 20 cycles: 25 sec 95°C, 40 sec 53°C, 40 sec 70°C; 8 min 70°C)
The quality of PCR product was checked by agarosegelelectrophoresis.

Bacteria specific hybridization
20 µl of the PCR product were mixed with 20 µl of the denaturation solution in the well of the tray and incubated at room temperature for 5 min.
1 ml prewarmed (45°C) hybridization buffer was added to the sample and a strip (hybridized with DNA sequences of each bacteria as well as a positive control) was placed in the well of the tray.
The tray was incubated at 45°C for 30 min in a shaking water bath.
1 ml of stringent wash solution was added and incubated at 45°C for 15 min.
The strip was washed once with 1 ml rinse solution for 1 min and 1 ml of conjugate solution was added (room temperature for 30 min).
After washing 1 ml of substrate solution was added.
The occurrence of bacteria was evaluated visually by means of colored bands.
Two positive controls for amplification reaction and for conjugate were included in the test.

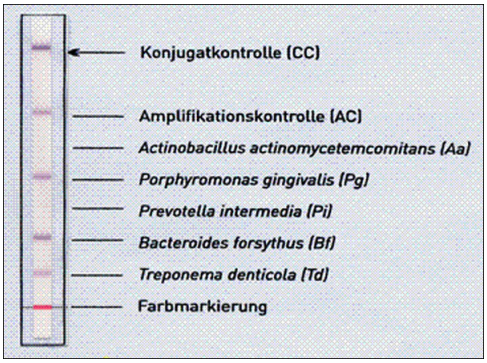


Fig. 2: Bacteria specific hybridization according to HAIN

Results

Clinical and demographic characterization

	Chronic periodontitis (CP)	Aggressive periodontitis (AP)	healthy controls	p values vs. controls	
	n=68	n=81	n=82	CP	AP
Mean age (years)	48.9 ± 9.6	40 ± 9.5	46.6 ± 10.7	n.s.	<0.001
Gender (% female)	64.2	63	53.7	n.s.	n.s.
Smoking (%)	25	34.6	21.2	n.s.	n.s.
Approximal plaque index (%)	61.8 ± 25.5	52.8 ± 28.5	47.3 ± 21.4	<0.001	n.s.
Bleeding on probing (%)	70.3 ± 24.6	78 ± 23.2	46 ± 23.9	<0.001	<0.001
Pocket depth (mm)	5.2 ± 1.2	5.7 ± 1.4	2.6 ± 0.7	<0.001	<0.001
Pocket depth on microbial test site (mm)	6.9 ± 1.6	7.5 ± 1.6	3.1 ± 0.4	<0.001	<0.001
Clinical attachment loss in general (mm)	6 ± 1.5	6.5 ± 1.5	3.0 ± 0.8	<0.001	<0.001
Clinical attachment loss on microbial test site (mm)	7.6 ± 1.9	8.4 ± 1.8	3.3 ± 0.5	<0.001	<0.001

Tab. 1: Clinical and demographic characterization of the patients

No significant differences between the patient groups and the healthy control group could be proven investigating gender, smoking status, and age. Sole exception was the age of the patients suffering from aggressive periodontitis because of the young age of onset of disease. As expected, both patient groups showed significant more severe clinical symptoms compared to the control group.

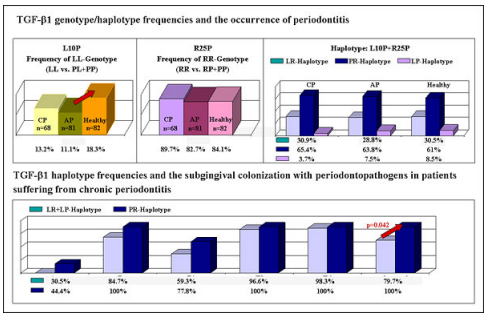
Microbiological assessment

Aggregatibacter actinomycetemcomitans (%)	30.6	47.2	22.2	n.s.	0.001
Porphyromonas gingivalis (%)	86.6	76.5	23.2	<0.001	<0.001
Prevotella intermedia (%)	61.8	61.7	32.9	<0.001	<0.001
Tannerella forsythia (%)	97.1	86.4	68.2	<0.001	0.005
Treponema denticola (%)	98.5	85.2	64.7	<0.001	0.002
Pg, Td, Tf (%)	82.4	71.3	23.5	<0.001	<0.001

Tab. 2: Microbiological assessment

As expected, both patient groups showed distinct and mostly significant increase in the occurrence of periodontopathic bacteria. Interestingly, no significant difference in the subgingival colonization with Aa could be shown for patients suffering from CP.

Genetic evaluation



Genetic evaluation

Conclusions

Hardy-Weinberg criteria were fulfilled for both TGF-b1 SNPs L10P and R25P.
No significant association of the genetic background of TGF-b1 including genotype and haplotype analyses and the occurrence of either chronic or aggressive periodontitis could be proven. A trend for a lower occurrence of the LL-genotype in patients suffering from aggressive periodontitis could be demonstrated.
Among patients with chronic periodontitis bacteria of the red complex (P.g., T.f., T.d.) occurred less frequently in carriers of the PR-haplotype.
However, in binary logistic regression analyses the SNPs L10P and R25P and haplotypes could not be proven as independent risk factors for bacterial colonization considering age, gender, smoking and API as cofounders.

This Poster was submitted by Dr. Susanne Schulz.

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Poster Faksimile:

21. Jahrestagung der Deutschen Gesellschaft für Humangenetik in Hamburg vom 02. - 04.03.2010

The role of SNPs in TGF-β1 at codon 10 and 25 and the occurrence of severe periodontitis

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[3] Institute of Human Genetics and Medical Biology, Martin-Luther-University, Halle-Wittenberg
[4] Department of Operative Dentistry and Periodontology, University Hospital Aachen

Introduction

Periodontitis is characterized as a chronic inflammatory disease induced by periodontopathogens.

TGF-β1 is a pleiotropic cytokine that exerts its effects on bone and connective tissue metabolism which are of great importance in periodontal disease.

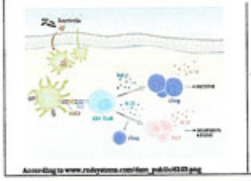
Functional important polymorphisms are described for TGF-β1:

(L10P) associated with higher circulating levels and secretion of TGF-β1 — Yokota et al.2000; Duming et al., 2003

(R25P) associated with interindividual variation in TGF-β1 — Awad et al. 1998

Aim of the study:
In the present study possible associations were investigated between the genetic variants of TGF-β1 and chronic/aggressive periodontitis and bl clinical features, including *smoking status, plaque (API) and bleeding indexes (BOP), pocket depth (PD), clinical attachment loss (CAL) and subgingival bacterial colonization.*

Signalling pathways mediated by TGF-β1



TGF-β1 as a proinflammatory cytokine plays an important role in mediating pathogen dependend regulation of the immune system.

TGF-β1 has been shown to be downregulated in active periodontal lesions showing clinical evidence of periodontal tissue destruction.

Downregulation of TGF-β1 is supposed to lead to the overexpression of cytokines, including e.g. RANKL, promoting the pathogenesis of periodontitis.

Material and Methods

Inclusion criteria of probands

Generalized aggressive periodontitis (AP): n=81
clinical manifestation before 35th year of life
attachment loss in at least 30% of the teeth with a minimum pocket depth of 4 mm
> 3 affected teeth had to be on first molars or incisors
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): n=68
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: n=83
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

Genomic investigations

DNA-Isolation from EDTA-blood
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.
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Long-term storage of DNA is possible at –20°C.

Haplotype specific PCR of TGF-β1
The detection of genotypes and haplotypes of TGF-β1 SNPs (L10P and R25P) was carried out using the CYTOKINE-Genotyping array CTS-PCR-SSP Tray kit of the Collaborative Transplant Study, Department of Transplantation Immunology of the University Clinic of Heidelberg.
For every PCR a fragment of 448bp of the human CD2 gene was exemplified as a positive control.
The PCRs were performed using sequence specific primers for detection of possible haplotypes prepipetted and lyophilized in thin-walled plastic 96-well PCR trays.
For every PCR 10µl of a Mastermix containing 10U Taq-Polymerase (Invitek), 100ng genomic DNA, 5% glycerol, and PCR reaction buffer was added.
PCR-program (2 min 94°C; 10 cycles: 15 sec 94°C, 1 min 64°C; 20 cycles: 15 sec 94°C, 50 sec 61°C, 30 sec 72°C)
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After electrophoresis, the ethidium/bromide stained gel is photographed and interpreted.

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Mastermix provided in the micro-IDent® test (containing buffer, biotinylated primer, DNA for positive control), ZO Taq-polymerase (Eppendorf), and 5 µl of isolated bacterial DNA were mixed.
PCR was performed (3 min 95°C, 10 cycles: 30 sec 95°C, 3 min 58°C, 20 cycles: 25 sec 95°C, 40 sec 53°C, 40 sec 70°C, 8 min 70°C)
The quality of PCR product was checked by agarosegelelectrophoresis.

Bacteria specific hybridization
20 µl of the PCR product were mixed with 20 µl of the denaturation solution in the well of the tray and incubated at room temperature for 5 min.
1 ml prewarmed (45°C) hybridization buffer was added to the sample and a strip (hybridized with DNA sequences of each bacteria as well as a positive control) was placed in the well of the tray.
The tray was incubated at 45°C for 30 min in a shaking water bath.
1 ml of stringent wash solution was added and incubated at 45°C for 15 min.
The strip was washed once with 1 ml rinse solution for 1 min and 1 ml of conjugate solution was added (room temperature for 30 min).
After washing 1 ml of substrate solution was added.
The occurence of bacteria was evaluated visually by means of colored bands.
Two positive controls for amplification reaction and for conjugate were included in the test.

Results and discussion

Clinical characterization of the patient groups

Clinical and demographic characterization

	Chronic periodontitis (CP) n =69	Aggressive periodontitis (AP) n=81	healthy controls n=83	p values vs. controls CP	p values vs. controls AP
Mean age (years)	48.2±9.6	40±9.5	46.6±10.7	n.s.	<0.001
Gender (% female)	64.2	63	53.7	n.s.	n.s.
Smoking (%)	25	34.6	21.2	n.s.	n.s.
Approximal plaque index (%)	61.8±25.5	52.8±28.3	47.3±21.4	<0.001	n.s.
Bleeding on probing (%)	70.3±24.6	70±33.2	46±23.9	<0.001	<0.001
Pocket depth (mm)	3.2±1.3	3.7±1.4	2.4±0.7	<0.001	<0.001
Pocket depth on microbial test site (mm)	4.7±1.6	5.7±1.4	3.1±0.4	<0.001	<0.001
Clinical attachment loss in general (mm)	4±1.5	6.5±1.5	3.0±0.8	<0.001	<0.001
Clinical attachment loss on microbial test site (mm)	7.4±1.9	8.4±1.8	3.3±0.5	<0.001	<0.001

Its significant differences between the patient groups and the healthy control group could be proven investigating gender, smoking status, and age. Sole exception was the age of the patients suffering from aggressive periodontitis because of the young age of onset of disease. As expected, both patient groups showed significant more severe clinical symptoms compared to the control group.

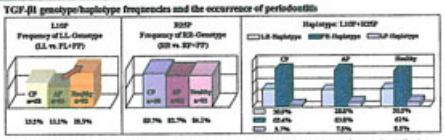
Microbiological assessment

Aggregatibacter actinomycetemcomitans (%)	30.6	47.2	22.2	n.s.	0.006
Porphyromonas gingivalis (%)	84.6	74.5	23.5	<0.001	<0.001
Prevotella intermedia (%)	61.8	61.7	32.9	<0.001	<0.001
Tannerella forsythia (%)	97.1	84.4	68.2	<0.001	0.009
Treponema denticola (%)	98.5	95.2	64.7	<0.001	0.002
Pg, Td, If (%)	82.4	71.3	23.5	<0.001	<0.001

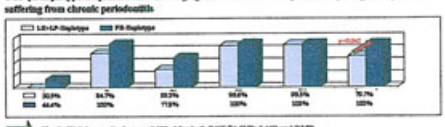
As expected, both patient groups showed distinct and mostly significant increase in the occurrence of periodontopathic bacteria. Interestingly, no significant difference in the subgingival colonization with Aa could be shown for patients suffering from CP.

Genetic evaluation

TGF-β1 genotype/haplotype frequencies and the occurrence of periodontitis



TGF-β1 haplotype frequencies and the subgingival colonization with periodontopathogens in patients suffering from chronic periodontitis



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No significant association of the genetic background of TGF-β1 including genotype and haplotype analyses and the occurrence of either chronic or aggressive periodontitis could be proven. A trend for a lower occurrence of the LL-genotype in patients suffering from aggressive periodontitis could be demonstrated.
Among patients with chronic periodontitis bacteria of the red complex (*Pg, Tf, Td*) occurred less frequently in carriers of the PR-haplotype.
However, in binary logistic regression analyses the SNPs L10P and R25P and haplotypes could not be proven as independent risk factors for bacterial colonization considering age, gender, smoking and API as cofounders.