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The role of the c.-374T>A SNP in the promoter region of RAGE-gene in aggressive periodontitis

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Introduction

Periodontitis is characterized as a chronic inflammatory disease induced by periodontopathogens.
The receptor of advanced glycation end products (RAGE) is mediating inflammatory processes e.g. it is involved in the occurrence of severe periodontitis via activation of transcription factor NF-kB. Its expression is influenced by the c.-374T>A-SNP, located in the promoter region of this gene.

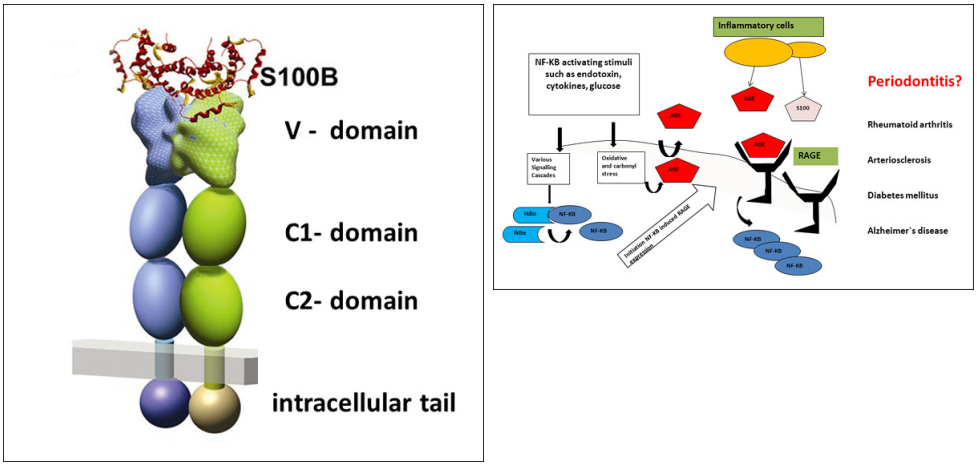


Fig. 1a-b: Signalling pathways mediated by RAGE

Aim of the study

In the present study possible associations were investigated between this SNP and the occurrence of aggressive periodontitis and its clinical features including smoking status, plaque (API) and bleeding indexes (BOP), pocket depth (PD), clinical attachment loss (CAL) and subgingival bacterial colonization (Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola).

Material and Methods

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.

Periodontitis-free controls n=85:
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.

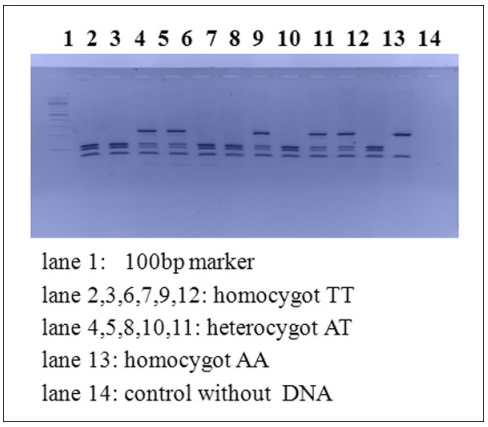


Fig. 2: RFLP of c.-374T>A SNP

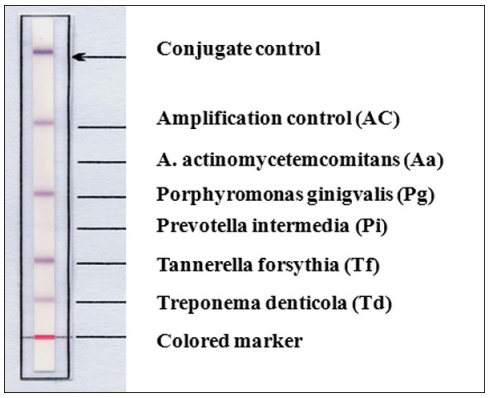


Fig. 3: Bacteria specific hybridization

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.

Specific PCR and RFLP of c.-374T>A SNP
The detection of genotypes c.-374A>T SNP was carried out using a Forward primer 5'-3', RAGE-5: tca gag ccc ccg atc cta ttt, and a Reverse Primer 5'-3', RAGE-6: ggg ggc agt tct ctc ctc.
For every PCR 25µl of a Mastermix containing 12,5µl Mix (Promega);0,5µl primer RAGE-5; 0,5µl primer RAGE-6; 10,5µl water and 1µl genomic DNA was added.
PCR-program (2 min 94°C; 40sec 92°C, 15 cycles: 40sec 54°C, 1 min 72°C; 1sec delay; 40sec 92°C; 25 cycles: 40sec 49°C, 1min 72°C, 1sec delay; 5min 72°C; hold 4°C)
For digestion of the PCR products, to every PCR-mix 2,5µl 10* buffer (NEB) and 4U Tsp5091 (0,4µl, NEB) were added, for digestion the mixture was incubated for 8h at 65°C.
The DNA fragments 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.

Results

Clinical characterization of the patient groups
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.

	Aggressive periodontitis (AP)	**Chronic periodontitis (CP)**	**Periodontitis-free controls**
	n=81	**n=68**	**n=85**
Mean age (years)	40.4±9.8*	48.9±9.6	46.7±10.8
Gender (% female)	63	63.2	54.1
Smoking (%)	34.6	25.0	21.2
Approximal plaque index (%)	52.2±28.9	61.8±25.5*	47.3±21.4
Bleeding on probing (%)	78.9±22.6*	70.3±26.6*	46.0±23.9
Pocket depth (mm)	5.7±1.4*	5.2±1.2*	2.6±0.7
Pocket depth on microbial test site (mm)	7.5±1.5*	6.9±1.6*	3.1±0.4

Clinical attachment loss in general (mm)	6.5±1.5*	6.0±1.5*	3.0±0.8
Clinical attachment loss on microbial test site (mm)	8.4±1.8*	8.4±1.9*	3.3±0.5

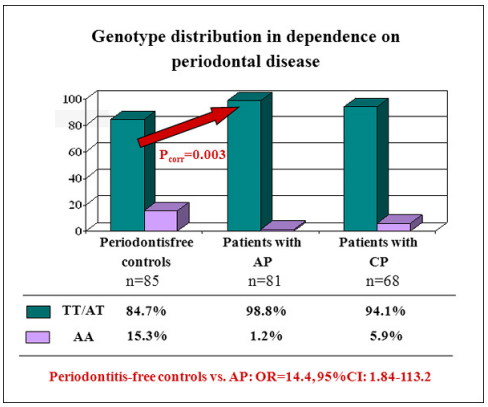
Tab. 1: Clinical characterization of the patient group – I
* $p < 0.05$ vs. periodontitis-free controls

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.

Aggregatibacter actinomycetemcomitans (%)	40.7	32.4	18.8
Porphyromonas gingivalis (%)	76.5	86.6	23.5
Prevotella intermedia (%)	61.7	61.8	32.9
Tannerella forsythia (%)	86.4	97.1	68.2
Treponema denticola (%)	85.2	98.5	64.7
Pg, Td, Tf (%)	70.4	82.4	23.5

Tab. 2: Clinical characterization of the patient group – II: Microbiological assessment

Genetic evaluation
Since the T-allele was associated with decreased promoter activity the genotypes carrying the T-allele were evaluated as "risk genotypes" (TT+AT) according to a dominant genetic model.
Significant differences in genotype as well as allele distribution could be proven between the periodontitis-free control group and the group of AP patients. In binary logistic regression analysis the T-allele could be proven as an independent risk factor for aggressive periodontitis.
The same tendency (n.s.) was proven for genotype and allele distribution in patients suffering from chronic periodontitis.



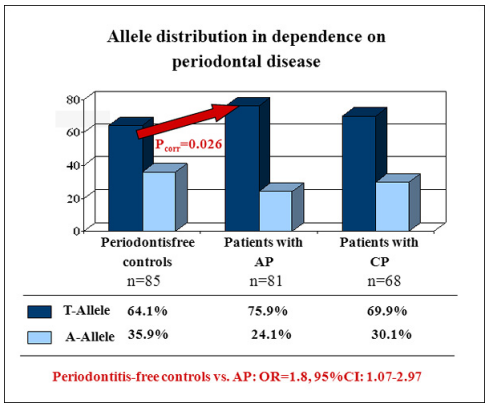


Fig. 4a-b: Genetic evaluation

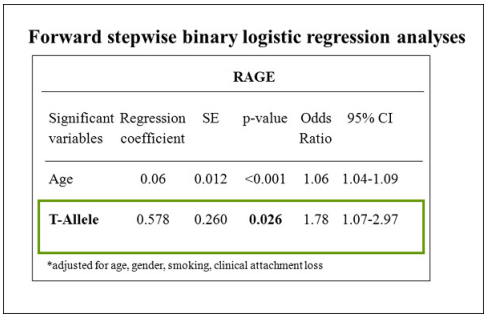


Fig. 5: Multivariate evaluation

Conclusions

The results emphasize the role of the T-allele of RAGE SNP c.-372T>A as a putative risk indicator for aggressive periodontitis in this German Cohort irrespective of further periodontal risk factors.
This finding could be possibly based on an impaired immune response due to the decraesed promoter activity associated with the T-allele.

This Poster was submitted by Diana Stosberg.

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The role of the c.-374T>A SNP in the promoter region of RAGE-gene in aggressive periodontitis

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[1] University School of Dental Medicine, Department of Operative Dentistry and Periodontology, Martin-Luther-University Halle-Wittenberg
[2] private dental department Halle/Saale
[3] Department of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital Aachen (RWTH)
[4] Institute of Human Genetics and Medical Biology, Martin-Luther-University, Halle-Wittenberg

Introduction

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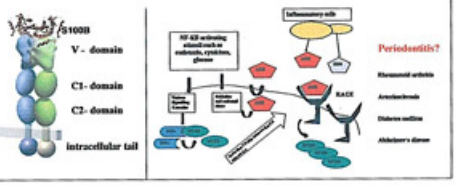
The receptor of advanced glycation end products (RAGE) is mediating inflammatory processes e.g. it is involved in the occurrence of severe periodontitis via activation of transcription factor NF-kB. Its expression is influenced by the c.-374T>A-SNP, located in the promoter region of this gene.

Aim of the study:

In the present study possible associations were investigated between this SNP and the occurrence of aggressive periodontitis and its clinical features including *smoking status, plaque (API) and bleeding indexes (BOP), pocket depth (PD), clinical attachment loss (CAL) and subgingival bacterial colonization (Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola).*

Signalling pathways mediated by RAGE



Material and Methods

Inclusion criteria of probands

Generalized aggressive periodontitis (AP): clinical manifestation before 35th year of life
n=81 attachment loss on 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
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more vertical than horizontal appraximal bone loss was visible in the radiographs

Generalized chronic periodontitis (CP): attachment loss on at least 30% of the teeth with a minimum pocket depth of 4 mm
n=68 The amount of the attachment loss was consistent with the presence of mineralized plaque
More horizontal than vertical appraximal bone loss was visible in the radiographs

Periodontitis-free controls: probing depth ≤ 3 5 mm, no gingival recession due to periodontitis
n=85 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 (Qiaagen)
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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

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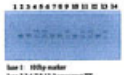
The detection of genotypes c.-374A/T SNP was carried out using a Forward primer 5'-3', RAGE-5 tcc gag ccc cag ac cta tE, and a Reverse Primer 5'-3', RAGE-4 agg ggt agt tct ctc ctc ttc
For every PCR 25µl of a Mastermix containing: 12,5µl Mix (Promega)0,5µl primer RAGE-5, 0,5µl primer RAGE-6, 10,5µl water and 1µl genomic DNA was added
PCR-program (2 min 94°C, 40sec 92°C, 15 cycles: 40sec 94°C, 1 min 72°C, 1sec delay, 40sec 92°C, 25 cycles: 40sec 63°C, 1min 72°C, 1sec delay, 5min 72°C, hold 4°C)
For digestion of the PCR products, to every PCR mix 2,5µl 10* buffer (NEB) and 4U Tsp509I (0,4µl, NEB) were added, for digestion the mixture was incubated for 0h at 65°C
The DNA fragments were loaded onto a 2% agarosegel for electrophoresis. After electrophoresis, the ethidium bromide stained gel is photographed and interpreted.



lane 1 100bp marker
lane 2,3,4,7,8,12 homozygot TT
lane 4,5,8,10,11 heterozygot AT
lane 13 homozygot AA
lane 14 control without DNA

Evaluation of periodontopathic bacteria in subgingival pockets

Subgingival sampling

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

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Preparation of bacterial DNA was carried out using the QIAamp DNA Mini Kit (Qiaagen)
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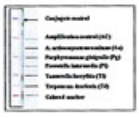
The quality of PCR product was checked by agarosegelelectrophoresis

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.



Results and discussion

Clinical characterization of the patient groups

Clinical and demographic characterization

	Aggressive periodontitis (AP) n=81	Chronic periodontitis (CP) n=68	Periodontitis-free controls n=85
mean age (years)	40,4±9,9*	49,3±9,6	44,7±10,9
Gender (% female)	63,0	63,2	54,1
Smoking (%)	34,6	23,0	21,2
Approximal plaque index (%)	52,2±28,9	61 0±25 5*	47,3±23,4
Bleeding on probing (%)	58,9±22,6*	39,3±26,6*	46,0±23,9
Pocket depth (mm)	5,7±1,4*	5,2±1,2*	2 4±0 3
Pocket depth on microbial test site (mm)	5,3±1,3*	4,9±1,6*	3 1±0 4
Clinical attachment loss on general (mm)	4,5±1,3*	4,0±1,3*	3 0±0 8
Clinical attachment loss on microbial test site (mm)	8,4±1,8*	8 4±1 9*	3 3±0 3

*p<0,05 vs. periodontitis-free controls

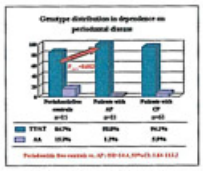
⇒ **No significant differences between the patient groups and the healthy control group could be proven investigating gender, smoking status, and age. Sole exception was the age of the patients suffering from aggressive periodontitis became of the young age of onset of disease. As expected, both patient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

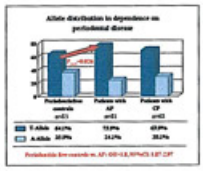
Microbiological assessment

Aggregatibacter actinomycetemcomitans (%)	40,7	32,4	18,8
Porphyromonas gingivalis (%)	74,3	86,8	23 5
Prevotella intermedia (%)	61,7	61,8	32 9
Tannerella forsythia (%)	86,4	97,1	68 2
Treponema denticola (%)	85,2	98,5	64,7
Pg, Tf, Td (%)	70,4	82,4	23 5

⇒ **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





Forward stepwise binary logistic regression analyses

RAGE					
Significant variables	Regression coefficient	SE	p-value	Odds Ratio	95%-CI
Age	0.06	0.012	<0.001	1.06	1.04-1.09
T-Allele	0.578	0.260	0.026	1.78	1.07-2.97

*adjusted for age, gender, smoking, clinical attachment loss

Since the T-allele was associated with decreased promoter activity the genotypes carrying the T-allele were evaluated as "risk genotypes" (TT+AT) according to a dominent genetic model.
Significant differences in genotype as well as allele distribution could be proven between the periodontitis-free control group and the group of AP patients. In binary logistic regression analysis the T-allele could be proven as an independent risk factor for aggressive periodontitis.
The same tendency (n.s.) was proven for genotype and allele distribution in patients suffering from chronic periodontitis.

The results emphasize the role of the T-allele of RAGE SNP c.-374T>A as a putative risk indicator for aggressive periodontitis in this German Cohort irrespective of further periodontal risk factors.
This finding could be possibly based on an impaired immune response due to the decreased promoter activity associated with the T-allele.