

## EXTENDED REPORT

## Lactoferrin Glu561Asp facilitates secondary amyloidosis in the cornea

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*Br J Ophthalmol* 2005;89:684-688. doi: 10.1136/bjo.2004.056804

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Accepted for publication  
5 October 2004

**Aim:** To elucidate the pathogenic mechanism of amyloid formation in corneal amyloidosis with trichiasis.

**Methods:** Ophthalmological examination was performed in nine patients to determine secondary corneal amyloidosis with trichiasis. Congo red staining and immunohistochemistry using anti-human lactoferrin antibody were used for biopsied corneal samples. For genetic analyses, single strand conformation polymorphism (SSCP), direct DNA sequence analysis, and polymerase chain reaction (PCR) induced mutation restriction analysis (IMRA) were employed to detect lactoferrin gene polymorphism.

**Results:** All patients had had trichiasis at least for 1 year, and all amyloid-like deposits were found in one eye with trichiasis. Ophthalmological examination revealed that eight patients showed gelatinous type of amyloid deposition and one showed lattice type of amyloid deposition. Studies of biopsied corneal samples with Congo red stain revealed positive staining just under the corneal epithelial cells. Immunoreactivity of anti-human lactoferrin antibodies was recognised in all tissues with positive Congo red staining. Lactoferrin gene analysis revealed that seven patients were heterozygotic and two were homozygotic for lactoferrin Glu561Asp. The frequency of the polymorphism in the patients was significantly different from that in 56 healthy control subjects.

**Conclusion:** Lactoferrin Glu561Asp is a key polymorphism related to facilitating amyloid formation in corneal amyloidosis with trichiasis.

Amyloidosis is a disorder of protein metabolism in which normally soluble autologous proteins are deposited in tissues as abnormal insoluble fibrils, which cause structural and functional disruptions.<sup>1, 2</sup> Thus far, 24 different precursor proteins of amyloid fibrils have been identified in systemic and localised amyloidoses.<sup>3</sup> Of these precursors, the mutated proteins, with conformations different from those of wild type proteins, often become amyloidogenic proteins.<sup>4-6</sup>

Ocular tissue is one type of tissue in which several different types of amyloid precursor proteins deposit as amyloid fibrils.<sup>7-8</sup> Most of the precursors that occur in ocular tissues in systemic amyloidosis have been identified.<sup>9-14</sup> Secondary corneal amyloidosis has been reported to occur in cases of keratoconus, trachoma, phlyctenular keratitis, bullous keratopathy, interstitial keratitis, syphilis, and trichiasis. Although amyloid deposits, with possible co-localisation of several proteins, have been observed,<sup>14, 15</sup> the precursor protein of the amyloid remained to be unidentified. We previously reported that the precursor protein in corneal amyloidosis associated with trichiasis is lactoferrin.<sup>16</sup> Although all of the patients in the previous study were heterozygotes for lactoferrin Glu561Asp gene, no statistical significance was observed when compared with that of the polymorphism in healthy control subjects. Although trichiasis itself is not so uncommon, the incidence of corneal amyloidosis is quite low.<sup>10, 17</sup> Some additional factor or factors, such as polymorphism in lactoferrin gene, may thus be involved in corneal amyloidosis with trichiasis.

Because we recently encountered an additional six patients with corneal amyloidosis with trichiasis, we examined the relation between the lactoferrin gene polymorphism and corneal amyloidosis.

## PATIENTS AND METHODS

## Patients

The patients' profiles are presented in table 1. Neurological examination and blood analysis indicated no signs of

systemic amyloidosis, and no amyloid deposition was found by Congo red staining of a biopsy specimen of the gastric and duodenal mucosae. After informed consent, the corneal regions of patient 1-5, and 8 and the cilia of patient 9 were excised and subjected to histological examination. The study was approved by the ethics committee of Graduate School of Medical Sciences, Kumamoto University.

## Materials

Polyclonal anti-human lactoferrin antibody and other antibodies, such as polyclonal anti-human transthyretin, anti-human kappa, lambda light chain, anti-human lysozyme antibodies, and monoclonal anti-human AA and anti-human keratin antibodies were purchased from Sigma Chemical Co (St Louis, MO, USA) and Dako Corp (Carpinteria, CA, USA), respectively. Chemicals used in histochemical and biochemical studies were of analytical grade.

## Congo red staining

For all specimens, formalin fixed, paraffin embedded sections were stained with haematoxylin and eosin and Congo red and were examined under polarised light for the presence of green birefringence.

## Immunohistochemical analysis of biopsy specimens

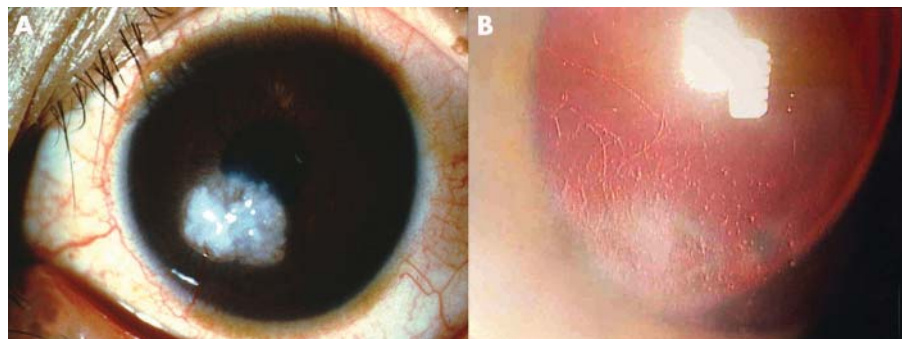
Specimens were fixed in 4% buffered paraformaldehyde. Paraffin embedded biopsy samples were serially cut at 4 µm. To detect the immunoreactivity for antibodies in amyloid deposits, the ABC method was used (Dako, Glostrup, Denmark) according to the manufacturer's instructions.

**Abbreviations:** IMRA, induced mutation restriction analysis; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism

**Table 1** Patient profile

Patient No	Age (year)	Sex	Lesion	Duration of disorder (years)	Histopathology	Genotype
1	30	M	Right downward	3	+	Hetero
2	41	F	Right downward	16	+	Hetero
3	67	F	Right downward	10	+	Hetero
4	17	F	Right downward	4-5	+	Hetero
5	62	F	Right downward	3	+	Homo
6	12	F	Right downward	1<	np	Hetero
7	78	F	Right downward	10	np	Hetero
8	17	F	Left downward	17	+	Hetero
9	85	M	Right downward	1-2	np	Homo

Hetero, heterozygotic for the lactoferrin Glu561Asp gene; Homo, homozygotic for the lactoferrin Glu561Asp gene. np: not performed. 1<: more than 1 year.



**Figure 1** Amyloid deposits in the cornea of patients 1 (A) and 9 (B).

**DNA isolation**

In patients 1 and 4-9 in table 1, total genomic DNA was isolated from peripheral blood cells as described previously.<sup>18</sup> In patients 2 and 3, total genomic DNA was isolated from paraffin sections by using the Depat kit (Takara Co, Shiga, Japan).

**Single strand conformation polymorphism (SSCP) analysis**

SSCP analysis was performed according to the method of Orita *et al.*<sup>19</sup> The polymerase chain reaction (PCR) primer sets used based on previous reports<sup>20</sup> (GenBank Database accession no U95626) was described in table 2.

**Direct DNA sequence analysis**

The PCR products (5 ng) of exons 2, 9, 10, and 15 from the patient and the control subjects were analysed, using 5' and

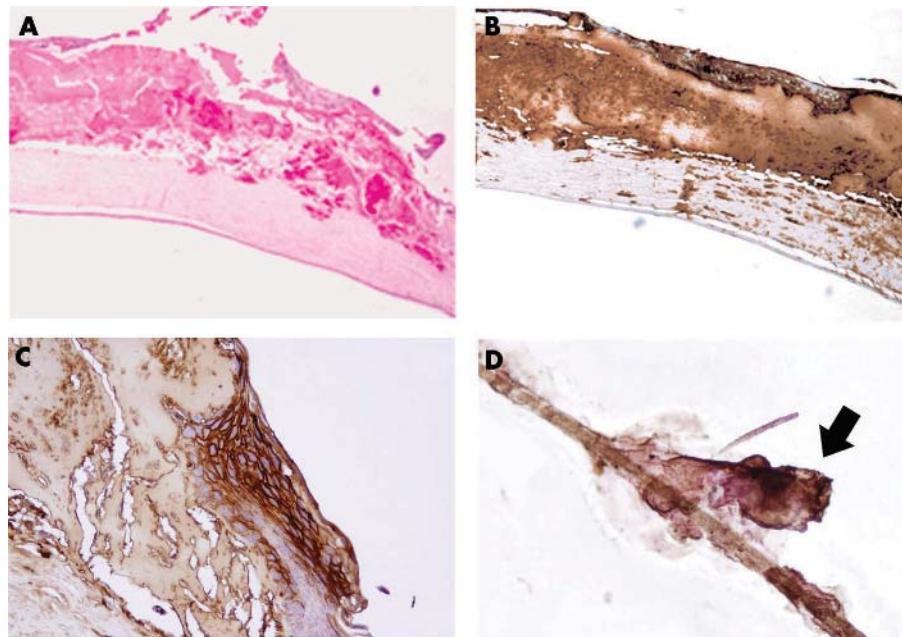
3' primers, by Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham, Uppsala, Sweden).

**PCR induced mutation restriction analysis (IMRA)**

To confirm the polymorphism Ala11Thr, PCR was performed with the exon 2 primer set, and then PCR products were digested with *Apa*I. To confirm the other polymorphism (Glu561Asp), we prepared the Glu561Asp PCR-IMRA primer (5'-GTCTGCCAGCTTCA AATCCTTAGC CCAGAC-3'), which annealed immediately 3' to the polymorphism and contained mismatch bases (GA instead of a normal TG at the 2', 3' position from the 3' end) that created a unique *Aat*II restriction site, only when the lactoferrin gene had a C at position 3 of codon 561. After PCR amplification using the Glu561Asp PCR-IMRA primer and exon 15 outer primer (5'-GAAGTCTCTTCTGTTCCTCACA-3'), PCR products were digested with *Aat*II. After informed consent was obtained,

**Table 2** Primer sets used in this study

Exon	Upstream primer	Downstream primer
1	5'-CCAGCCGAGTTTCTCAAGTC-3'	5'-CCCCAGGCACCTGCACTCAC-3'
2	5'-CCTTGGCCCTCTCTCCCAG-3'	5'-AACACCCGGCATTGACTCAC-3'
3	5'-TCTGGCCTCTTTACTTTCAG-3'	5'-GGGTCCCCAGGCAGAACTAC-3'
4	5'-TTCTGTCTGCCCTTTGCGAG-3'	5'-TATGCCCCAGCCATCTTAC-3'
5	5'-CACTTCTCTGTGTTAACAG-3'	5'-AAGGGGACAGGGTCACTCAC-3'
6	5'-GATGGTTTCTTTTCACAG-3'	5'-GCTCATTACCCTGCTCTTAC-3'
7	5'-CCACCTCACCTCCCTGCAG-3'	5'-AAGTGGGGAGGACCGTGGGT-3'
8	5'-TCCCTATTACCATTGACAC-3'	5'-AAGTAGAAGACCACACCAGG-3'
9	5'-GCAAAGCTCAGGTTGCCAG-3'	5'-ATGCCAGGCCCTAGTCTT-3'
10	5'-AGCCTCACTGTGGTCTGGA-3'	5'-ACTCCATGACCCAGAGGGA-3'
11	5'-AGAGTTTGTGGCTTCTCACT-3'	5'-CCAGCAACAAGAACTTATCC-3'
12	5'-CCTGGAGGTTAAGACTTGT-3'	5'-CCACAGCACAATATGCCTAC-3'
13	5'-TGGACTCAGGTTGAAGAGC-3'	5'-GCTGAGGGATGAGGTAAGTC-3'
14	5'-GAAAGCCCCACTAGTTTCTC-3'	5'-CCAAAGACTCTGCTTTGAAG-3'
15	5'-CGTGGATGATGCCACCTTCT-3'	5'-GCCACACAGCTAAGAAAGC-3'
16	5'-CTTAGCTACTACTGTCTGC-3'	5'-CTTACCCTATGGTGGTTTCT-3'
17	5'-GTTTCTGAATCTCTTGCTCT-3'	5'-AGAGCAGGGAATTGTAAGCA-3'



**Figure 2** Histochemical analysis for the excised cornea, (A) and (B) Congo red staining and an anti-human lactoferrin antibody staining of the sample from patient 8. (C) An anti-human lactoferrin antibody staining of the corneal epithelium of patient 8. (D) Immunohistochemistry using an anti-human lactoferrin antibody for cilia in patient 9. The arrow indicates the positive stained mass stuck on the cilia. Magnification  $\times 100$ .

56 healthy Japanese volunteers were also examined in the same way to determine the frequency of these polymorphisms.

#### Statistical analysis

Frequency of lactoferrin Glu561Asp gene was compared in control subjects and the patients using Fisher's exact test. p Values less than 0.05 were considered significant.

## RESULTS

#### Macroscopic examination

Eight patients showed gelatinous type of amyloid deposition (fig 1A), and one showed lattice type of amyloid deposition (fig 1B). All patients had had trichiasis at least for 1 year (table 1), and all amyloid-like deposits were found in one eye with trichiasis.

#### Histochemical analyses of biopsied corneal samples

Congo red staining for biopsied corneal samples in patient 1–5 and 8 revealed positive staining just under the corneal epithelial cells that extended into the stroma (fig 2A). No vascularisation was observed. Immunoreactivity of anti-human lactoferrin antibodies was recognised in all tissues with positive Congo red staining (fig 2B). Notably, extra-cellular space of the corneal epithelial cells was clearly stained with an anti-lactoferrin antibody (fig 2C). Other antibodies showed no immunoreactivity for the amyloid

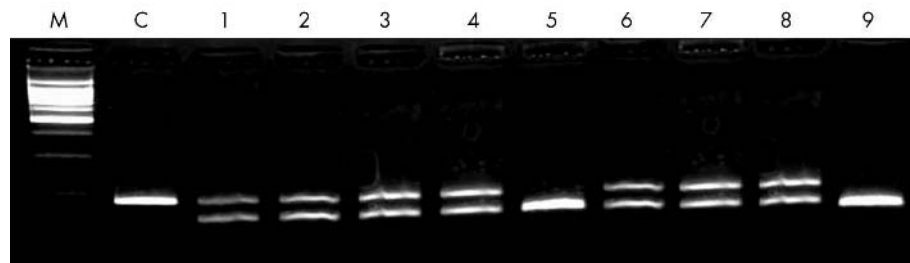
deposits. Specificity controls were obtained by preincubating the anti-human lactoferrin antibody with lactoferrin (1–10  $\mu\text{g/ml}$ ).<sup>21</sup>

Although we could not perform a biopsy on patient 9 for ethical reasons, a lactoferrin positive material stuck on the excised cilia (fig 2D).

#### Analyses of the lactoferrin gene

SSCP analyses for DNA from a 30 year old male patient (No 1) revealed abnormally migrating bands in exons 2, 9, 10, and 15. Direct sequencing using exons 2, 9, 10, and 15 PCR products from the patients' DNA was performed to identify these polymorphisms. In exons 9 and 10, two polymorphisms were detected (data not shown): a GTC (Val) to GTT (Val) substitution in codon 346 of exon 9 and a GGA (Gly) to GGG (Gly) substitution in codon 398 of exon 10. In exons 2 and 15, two polymorphisms were detected: amino acid substitutions at codon 11 from GCC (Ala) to ACC (Thr) and at codon 561 from GAG (Glu) to GAC (Asp) (data not shown). Since the conversion of amino acid was observed in lactoferrin Ala11Thr and Glu561Asp, PCR-IMRA was performed in other patients to examine these polymorphisms.

Analysis via PCR-IMRA revealed that seven of nine patients were heterozygotic and two homozygotic for lactoferrin Glu561Asp (fig 3). Although one patient was heterozygotic for lactoferrin Ala11Thr, other patients showed no such polymorphism. We also used genetic analysis to



**Figure 3** Detection of the lactoferrin Glu561Asp gene by means of PCR-IMRA, M represents the 100 bp DNA size marker; C represents the data from control subject; Numbers 1–9 correspond the patient numbers given in table 1. Analyses for lactoferrin Glu561Asp were performed via PCR-IMRA as described.<sup>16</sup> When allele for lactoferrin Glu561Asp only exists, the digestion bands (158 bp and 28 bp) are observed.

**Table 3** Frequency of the lactoferrin genotype in healthy volunteers

	Lactoferrin Glu561Asp	Number of subjects	Frequency of genotype (%)
No polymorphism	-/-	31	56.3
Heterozygote	+/-	18	32.7
Homozygote	+/+	7	12.7

determine the frequency of the lactoferrin Glu561Asp gene in 56 healthy Japanese volunteers. Comparison of this frequency in healthy volunteers with the frequency in patients revealed significant polymorphism in the patients (Fisher's exact test = 0.0119,  $p < 0.01$ ) (table 3).

## DISCUSSION

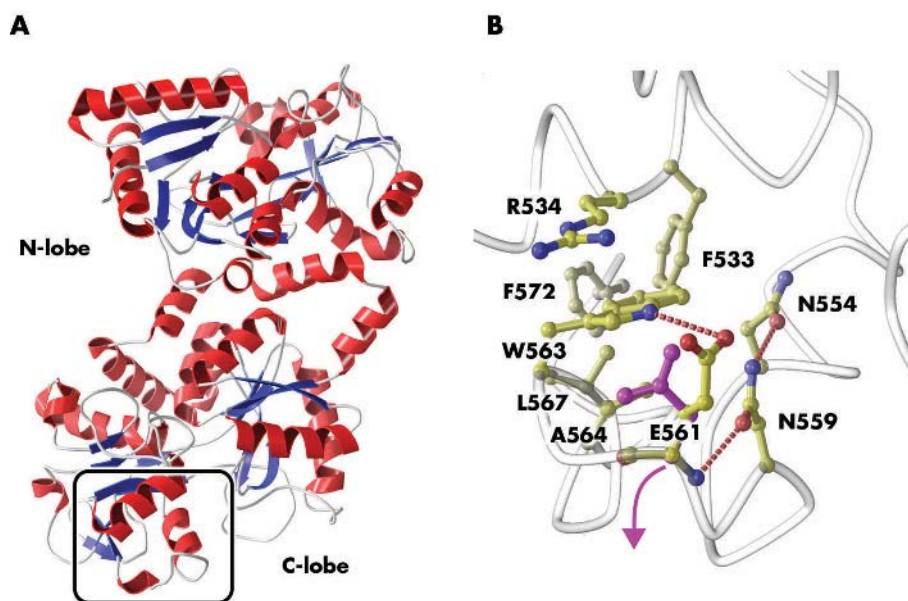
We suggested in this study that lactoferrin Glu561Asp facilitates amyloid formation in corneal amyloidosis with trichiasis. We speculate that lactoferrin from tears should be a source of amyloid formation in the cornea of patients for the following four reasons. Firstly, lactoferrin is the major component of tears. Secondly, anti-human lactoferrin antibody reactive mass stuck to cilia (fig 2D). Thirdly, lactoferrin was clearly detected in the intracellular space of the corneal epithelium. And fourthly, lactoferrin was observed in amyloid deposits in all samples examined (fig 2). Although we cannot deny the possibility that continuous stimulation by trichiasis may induce corneal epithelial cells and stromal fibroblasts to secrete the amyloidogenic protein, because these cells have the potential producing amyloid protein in certain situations,<sup>22</sup> we conclude that mutated lactoferrin derived from tears might infiltrate into the site of amyloid deposition through the extracellular space of the epithelial cells. We also performed the lactoferrin gene analysis and histochemistry of three samples of corneal amyloidosis with keratoconus. Although anti-lactoferrin antibody showed a positive reaction, no polymorphism in the lactoferrin gene was detected (data not shown). In this type of amyloidosis, the parenchyma already has abnormal structures that may

show an affinity with native lactoferrin. But, in corneal amyloidosis with trichiasis, the polymorphism may be an indispensable factor because the parenchyma, the targeted lesion of amyloid deposition, has normal structures.

We carefully checked the pattern of anti-lactoferrin antibody reactivity of lactoferrin Glu561Asp gene in the heterozygotic and homozygotic patients. However, no obvious difference was detected. This may be because the degree of the amyloidotic changes in the corneal may be too immature to be compared. Accumulation of such cases for comparison is needed.

We previously reported that lactoferrin forms an amyloid mass both in *in vitro* examination and in the cornea.<sup>16</sup> However, the relation between lactoferrin gene polymorphisms and the amyloid formation mechanism remained to be elucidated because statistical differences in the frequency of the polymorphism between the patients and control subjects was not clear.

To investigate whether the mutated form of lactoferrin has amyloidogenic ability, a possible conformational change of the protein was simulated. According to the PDB code ([www.rcsb.org/](http://www.rcsb.org/)), the amino acid at position 561 in lactoferrin locates in a loop region at the bottom of the C-lobe (fig 4A). This loop region has relatively high B factors, which indicates this region's flexibility.<sup>23</sup> Oxygen of the Glu561 side chain forms a weak hydrogen bond with the side chain's nitrogen atom of Trp563 at a distance of 3.32 Å (fig 4B). The polymorphism of Glu561Asp in lactoferrin seems to have no hydrogen bond or has a weaker interaction with Trp563. This might enhance flexibility of this loop region, and then



**Figure 4** Structural feature of the human lactoferrin Glu561. (A) A ribbon diagram of human lactoferrin. The region depicted in close up (B) is indicated by a rectangle. (B) The main chain backbone is shown in grey. The residues, which involve the charged or hydrophobic interaction network in this region, are shown as a ball and stick model. The Asp mutated from Glu561 is shown in purple.

expose the hydrophobic patch. Consequently, the mutant lactoferrin may form amyloid fibrils via this exposed hydrophobic patch.

Clinically, our nine patients showed the two different types of amyloidosis. It is well known that hereditary corneal amyloidosis has been classified into two types, gelatinous and lattice,<sup>24</sup> with a pathogenesis related to mutated M1S1<sup>25 26</sup> and TGFBI<sup>27</sup> genes, respectively. Majima *et al* speculated that amyloid deposition in the corneal stroma resulted in a lattice pattern and deposition in the epithelium resulted in a gelatinous pattern (personal communication). From our patients' clinical observations, we derive the following: in gelatinous-type secondary corneal amyloidosis, lactoferrin aggregates into the epithelial layer through the extracellular space of the epithelium where cilia repeatedly touch. In contrast, in lattice-type secondary corneal amyloidosis, epithelial erosion with destruction of Bowman's membrane might enable lactoferrin to integrate into the stroma and form amyloid deposition.

From our study, we conclude that secondary corneal amyloidosis with trichiasis is predominantly induced by both trichiasis and lactoferrin Glu561Asp polymorphism.

## ACKNOWLEDGEMENTS

This work was supported by grants from the Amyloidosis Research Committee, the Pathogenesis, Therapy of Hereditary Neuropathy Research Committee, the Surveys and Research on Specific Disease, the Ministry of Health and Welfare of Japan, Charitable Trust Clinical Pathology Research Foundation of Japan, and Research for the Future Program Grant, and Grants in Aid for Scientific Research (B) 15390275 and (B) 20253742 from the Ministry of Education, Science, Sports and Culture of Japan. The authors thank Hiroko Katsura, and Miyo Okajima for technical assistance.

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Competing interests: The authors have no proprietary, financial, or commercial interests in any of the companies or products mentioned in this paper.

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*Br J Ophthalmol* 2005 89: 684-688  
doi: 10.1136/bjo.2004.056804

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