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ROLE OF MANNOSE-BINDING LECTIN (*MBL2*) GENOTYPING IN PREDICTING THE RISK OF RECURRENT OTITIS MEDIA (rOM)

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1. INTRODUCTION

Otitis media (OM) is the most common childhood disease. Almost all children experience at least one episode of otitis media with effusion (OME) or acute otitis (OMA) during childhood, and a substantial subgroup is confronted with recurrent episodes. If not treated adequately, OM may lead to temporary or permanent hearing loss. It is well known that bacteria and viruses cause OMA, and may play a role in OME. Bacteria such as *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae* account for about of 85% of OMA cases, with viruses making up the remaining 15%. The chronic and recurrent forms are thought to be superimposed on an underlying middle ear ventilation problem, sometimes expressed as OME. Although the complete pathophysiological picture still remains to be fully elucidated, bacteria and viruses are certainly thought to play an important role in both chronic and recurrent otitis forms. The treatment for acute otitis media is antibiotics, usually for 7 to 10 days. In the case of rOM one may elect for surgery (adenotomy or tympanotomy with placement of grommets) in order to prevent future remissions.

It has been widely accepted that environmental and infectious factors trigger the development of OM in general. However, there is significant evidence from epidemiologic, anatomic, physiologic, and immunologic studies that susceptibility to recurrent episodes of OM is also partially genetically determined[2,3], although unlikely to be due to a single major gene[12]. The specific

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genes conferring susceptibility to OM are largely unknown, but ongoing genome-wide linkage studies may provide new insights into susceptibility and the pathogenesis of OM in the near future[4,3]. One such approach seeks to understand the genetic links between innate immunity and OM.

The innate immune system represents the first line of host defense against infectious agents, permitting an immediate response by a constellation of sensing and effector mechanisms (antimicrobial peptides, phagocytic cells, and complement pathways) that recognize, contain, and usually remove pathogens swiftly. It relies on a pool of germline-encoded pattern-recognition receptors (PRRs) that can bind to highly conserved structures known as pathogen-associated molecular patterns (PAMPs), which are commonly present across large groups of invasive microorganisms. The PRRs may be divided into three classes: signalling, endocytic, and secreted. The latter class functions as opsonins by binding to microbial and viral cell walls and activating the complement system. The most important receptor is mannose-binding lectin (MBL), which is a soluble protein, synthesized mainly by the hepatocytes. MBL not only binds to microbial carbohydrates but also to phospholipids, nucleic acids, and nonglycrosylated proteins, properties that may be relevant to the clearance of apoptotic cells and avoidance of autoimmunity.

Upon binding of MBL to the surface of microorganisms, MBL undergoes a conformational change leading to activation of two MBL-associated serine proteases (MASPs). In a next step, the complement factor C4 is activated by the MBL/MASP complex, resulting in formation of the C3 convertase, the key reaction leading to terminal complement pathway initiation.

During the past decade, a steadily increasing number of clinical studies aimed at elucidating the role of MBL in health and disease has been published[11,16]. From these data, it became apparent that MBL deficiency is selectively associated with an increased risk for infections and autoimmune conditions, and influences the severity and course of several diseases. Initially, quantification of MBL in serum or plasma using ELISA technologies were used. However, as it becomes more apparent that the serum levels of MBL are influenced by genetic polymorphisms in the gene encoding MBL, i.e., the *MBL2* gene, genotying seems to be the method of choice due the simplicity of the test.

From the literature, there are strong suggestions that innate immunity may play a role in the development of rOM. In view of the importance of bacterial or viral agents in OM, the gene encoding mannose-binding lectin (*MBL2*) was chosen as a first focus of attention for the following reasons:

- MBL is a key component of innate immunity as a molecular sensor for bacteria or viruses[10]. It activates the complement cascade via the lectin pathway using the MASP proteins as co-activators.
- Association studies have shown that individuals with diminished MBL activity show a higher susceptibility to sepsis or recurrent infection[17,11].

- It has been shown that the MBL protein is present in both nasopharyngeal secretions and middle ear effusions, suggesting a defensive role for this protein in the middle ear[6].
- The incidence of OM is highest in young children where the adaptive immune system has not yet fully developed, and where infections are recognized mainly by the receptors of the innate immunity system.
- From earlier studies, a possible role of MBL in the development of OM has been suggested, although contradictory results have been reported[6,15,7,13].

An exploratory pilot study was therefore conducted to investigate the occurrence of MBL2 gene polymorphisms in rOM cases.

2. *MBL2* GENE AND POLYMORPHISMS

The functional human *MBL2* gene is located on chromosome 10q11.2-q21 and consists of four exons. Six key variations (Figure 1) affecting expression and functionality of MBL have been described. The polymorphisms are localized in the promotor and 5′ untranslated region [–550G>C, –221G>C, and +4C>T], and affect expression of the *MBL2* gene. In exon 1, encoding the collagen-like region necessary for correct multimer formation, three polymorphisms [R52C, G54D, and G57E] influencing the functionality of the protein have been identified (Table 1).

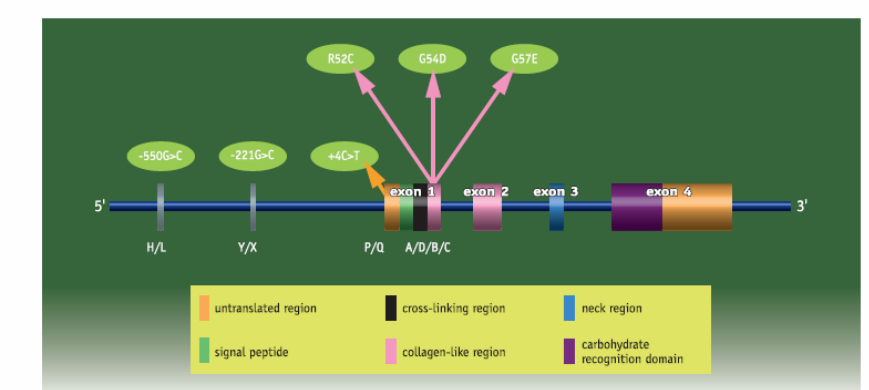

Figure. 1. Structure and organization of the *MBL2* gene. The localization of the principal structural and promoter polymorphisms with respect to the four domains of the MBL molecule is indicated.

Table 1. Overview of the 6 Polymorphisms in the *MBL2* Gene Detected by the INNO–LiPA MBL2 Kit

Common name	Abbreviation commonly used in the literature	Nomenclature according to HGVS	Minor allele frequency n=172
–550G>C	H/L	–619C>G	0.41
–221G>C	Y/X	–290C>G	0.19
+4C>T	P/Q	–66C>T	0.23
R52C - CGT>TGT	A/D	154C>T	0.06
G54D - GGC>GAC	A/B	161G>A	0.14
G57E - GGA>GAA	A/C	170G>A	0.03

The nomenclature for the *MBL2* polymorphisms is generally accepted by the scientific community[8,9]. To avoid confusion, a conversion table is given showing the nomenclature using the guidelines as recommended by the Human Genome Variation Society (HGVS)[5].

The characterization of the promotor polymorphisms led to identification of four commonly found haplotypes: LXP, LYP, LYQ, and HYP. Since each of the three exon 1 mutations is in strong linkage disequilibrium with a different promotor haplotype, seven common haplotypes have been described: HYPA, LYPA, LXPA, LYQA, HYPD, LYPB, and LYQC. Two very rare haplotypes, HXPA[14] and LYPD[1], have also been reported in the literature. An abbreviated nomenclature, taking into account only one promotor polymorphism (X/Y) and the presence of an exon 1 polymorphism (normal exon 1 sequence is expressed as A), are written as YAYA, YAYO, YAXA, YOYO, YOXA, and XAXA.

3. MATERIALS AND METHODS

3.1. Patients and Controls

Seventeen patients who presented at an ear, nose, and throat (ENT) clinic with OM and showing clinical evidence of recurrent or persistent disease took part in the study. Recurrence/persistence was judged based on the patient's history.

All patients were under 10 years of age at entry, and all were scored as having "severe" rOM according to the clinician's judgement of the recurrence/persistence rate. The score "severe" was given in case of 3 successive

ENT visits with positive otitis findings (OME or OMA) or in the case of a history of three or more otitis episodes with antibiotic treatment during the preceding year. As a control population, 172 healthy volunteers were included. Informed consent was obtained from all control subjects and patients.

3.2. MBL2 Genotyping

Genomic DNA was isolated from buccal cells from children with recurrent OM or leukocytes from healthy volunteers using standard procedures. Genotyping was caried out using the INNO-LiPA *MBL2* assay (Innogenetics, Belgium). In total, 17 patient and 172 control samples were tested; for all samplesboth haplotypes could be identified.

The INNO-LiPA MBL2 assay is a multi-parameter assay allowing simultaneous detection of the 6 relevant DNA variants in the *MBL2* gene using reverse hybridization technology (Figure 2). The test is easy to perform and takes about two hours starting from amplified product.

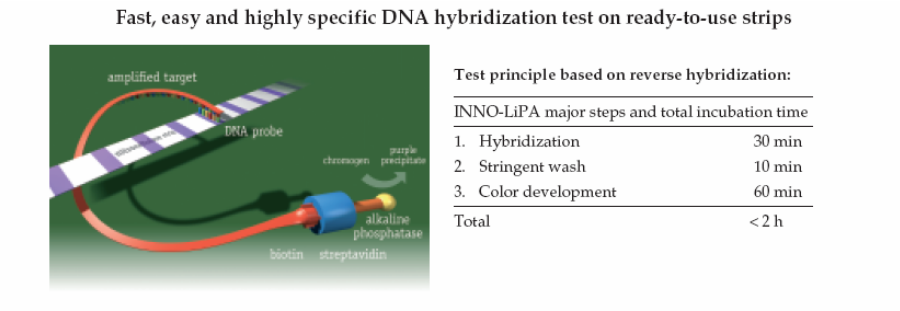

Figure 2. The INNO-LiPA principle.

Specific probes designed to hybridize with their complementary sequences are coated as parallel lines on a nitrocellulose membrane. The hybridized probes can be visualized as purple lines on the strip (Figure 3). Interpretation of the results can be performed using the LiRAS® for LiPA MBL software or manually using a typing table.

4. RESULTS

An overrepresentation of the *MBL2-G54D* variant (B-allele) [OR 2.9 (95% CI 1.35–6.44)] was found in the rOM patients compared to a healthy control population (Figure 4A). When the combined genotypes of rOM patients and healthy controls were compared, the genotype comprising one exon-1 polymorphism in combination with the minor allele of the promotor polymorphism in position

–221G>C was found to be significantly overexpressed in the patient group [OR 14.35 (95% CI 4.3-47.6)] (Figure 4B).

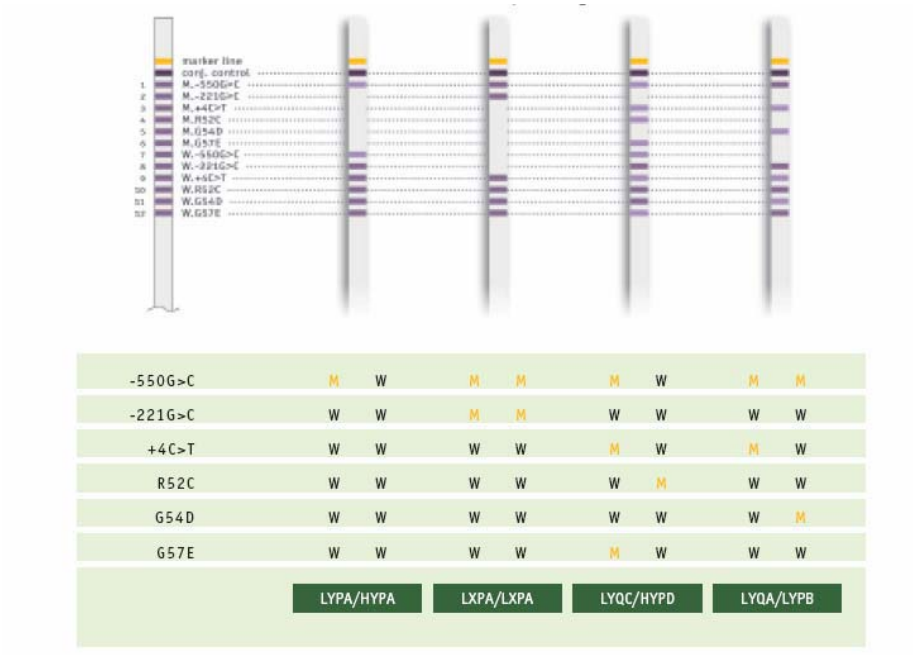

Figure 3. The INNO-LiPA MBL2 assay is a validated, CE-marked multiparameter assay allowing simultaneous genotyping of 6 DNA variants in the human *MBL2* gene using reverse hybridization technology. Specific probes designed to hybridize with their complementary sequences are coated as parallel lines on a nitrocellulose membrane. The hybridized probes can be visualized as purple lines on the strip. Interpretation of the results can be performed using the LiRAS™ for LiPA *MBL* software, or manually using a typing table. The test is easy to perform and takes about two hours starting from amplified product.

5. DISCUSSION

OM and OM-related upper respiratory problems are encountered with great frequency in pediatric and ENT practices. Most children experience at least one episode of OM during their first years of life. Many different types of OM have been defined, mainly on clinical grounds rather than based on pathophysiological criteria. The most clear-cut form is OMA. This results from a microbial infection of the middle ear, leading to a purulent collection in the middle ear cavity. The spontaneous course of this infection is almost always uncomplicated, with healing occurring either spontaneously or after antibiotic treatment. Some

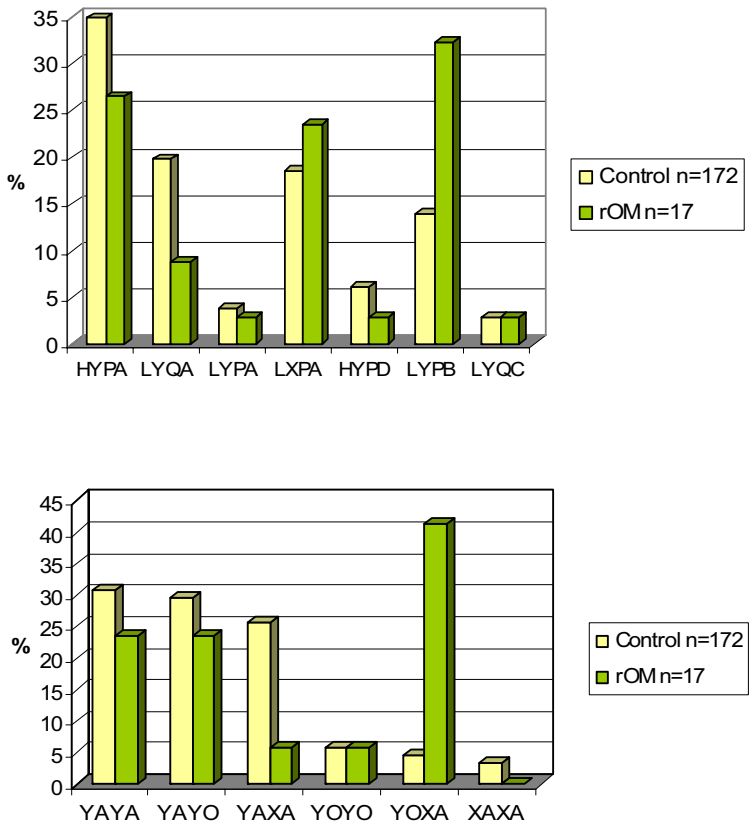

Figure 4. Haplotyping results and comparison of haplotype frequency. (a) Haplotyping results of 17 OM patients and 172 healthy subjects using the INNO–LiPA MBL2 kit. A significant overrepresentation of the LYPB genotype in the rOM group was detected [OR 2.9 (95% CI 1.35–6.44)]. (b) Comparison of the frequency of the combined haplotypes using the two-letter code expression showed a significant overrepresentation of the YOXA genotype in the patient group [OR 14.35 (95% CI 4.3–47.6)].

children, however, develop chronic or recurrent forms of otitis and an underlying chronic middle ear ventilation problem (often controversially called "eustachian tube dysfunction") is believed to be a basic factor in both forms of OM. In addition, microbial factors have been found to contribute to OM pathogenicity, as have genetic factors, in view of the familial occurrence that is often seen. These chronic or recurrent forms cause important morbidity with multiple episodes of pain, fullness, hearing loss, fever, behavioral problems, language developmental delays, etc., resulting in significant drug consumption and surgical interventions.

A remarkable feature of all forms of otitis media is its age-related occurrence. Under the age of 6–7 years, many children seem to be very prone to OM, whereas its incidence dramatically drops beyond this age. Since the adaptive immune system in this population is not yet fully matured, innate immunity thereby bears the burden of protecting the child against upper respiratory infections. This could explain the age-specific proneness of this kind of infections. A deficient or inadequate level of innate immunity in a subpopulation of these children would undermine this sole protective shield, and could be associated with more severe forms of upper respiratory infections, such as rOM.

Given the importance of innate immunity and, more particularly, complement pathway activation as the first line of defense in young children, significantly decreased MBL functionality in serum may be a clinically relevant factor for susceptibility to upper airway infection in this age group. In general, the majority of individuals in the general population with low MBL levels do not suffer from diseases directly related to decreased MBL functionality. However, hypersensitivity to infection becomes particularly apparent when constitutively low MBL levels, due to the presence of one or two mutations affecting the expression rate or functionality of MBL, occur in the context of coexisting primary (e.g., C4-null alleles) or secondary immune deficits (e.g., SLE).

The involvement of MBL in otitis media has been studied previously, but inconsistent results were obtained. This may be due to several reasons, such as low numbers of patients included, differing inclusion/exclusion criteria, alternative approaches to measurement of MBL (serum levels vs. genotyping), etc. A possible association between recurrence of otitis media and low concentrations of MBL in plasma and upper airway secretions was investigated in 89 children with several presentations of OM[6]. Their results did not support the assumption that low MBL levels alone predispose to recurrence of otitis media in Caucasian children. Subsequently, the authors of [15] investigated 51 children between 6 and 48 months with OMA. They observed a defective opsonization in the majority of patients, and indirectly concluded that an MBL deficiency might be involved.[7] Homoe et al. (1999) studied a group of Greenlandic children, although no association with low MBL levels was found. Straetemans et al.[13] recently showed a role of serum MBL levels together with the FcγRIIa–R/R131 polymorphism in the pathogenesis of recurrent OM. Our results, performed on a limited group of children with severe rOM, found that *MBL2* genotyping may be useful for patient stratification, especially in identifying children at high risk for severe recurrent otitis media. These results confirm the findings that innate immunity, especially MBL and complement activation, could well play an important role in first-line defense in the middle ear. The presence of the *MBL2*–G54D variant or the combination of an exon 1 variant on one allele and the C-allele of the *MBL2*–221G>C variant on the second allele, is associated with susceptibility to recurrent otitis media.

The overexpression of genotypes leading to diminished production of MBL in our study group provides a possible causative explanation for the phenotypic

trait of rOM. This seems to be a particularly promising line for further research. If confirmed, these results may lead to the development of new molecular diagnostic tests enabling the clinician to identify children at risk for OM and thereby help in preventing the possible consequences of the disease, such as hearing loss and developmental problems. Furthermore, these data might be used in the future for the development of more focused treatments. At present, no specific therapy is available, but it might become so in the future in the form of MBL supplementation therapy. Alternatively, the subgroup of children with rOM and deficient MBL may become candidates for new forms of personalized therapies (such as more aggressive antibiotic therapy), coupled with more intensive follow-up.

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