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Genetics of Otosclerosis

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Objectives: Otosclerosis is a major cause of acquired hearing loss in adult life affecting exclusively the human temporal bone. Until recently, the etiopathogenesis of otosclerosis was still a matter of debate. Genetic research, however, has evolved enormously the last years and unveiled important clues regarding the cause of otosclerosis. The objective of this article is to review the genetics of otosclerosis with special attention for the links to the bone homeostasis of the otic capsule.
Data Sources: A detailed literature study was performed focusing on the recent genetic findings in otosclerosis and the special bone turnover of the otic capsule. A PubMed search and own research data were used to bring the relevant information for this review together.
Conclusion: Unlike all other bones in the human skeleton, the otic capsule undergoes very little remodeling after development, possibly due to local inner ear factors. Otosclerosis is a process of pathologic increased bone turnover in the otic capsule, which in most cases leads to stapes fixation, resulting in a conductive hearing loss. Although environmental factors such as estrogens, fluoride, and viral infection have been implicated, it is clear that genetic factors play a significant role in the manifestation of otosclerosis. From a genetic viewpoint, otosclerosis is considered to be a complex disease with rare autosomal dominant forms caused by a single gene. Already, 7 monogenic loci have been published, but none of the genes involved have been identified. For the complex form of otosclerosis, caused by an interaction between genetic and environmental factors, the first susceptibility genes were identified by case-control association studies. All 3 replicated genes, *TGFB1*, *BMP2*, and *BMP4*, are a part of the transforming growth factor–β1 pathway. Data from both genetic association studies and gene expression analysis of otosclerotic bone showed that the TGF-β1 pathway is most likely an important factor in the pathogenesis of otosclerosis. **Key Words:** Bone morphogenetic proteins 2 and 4—Genetics—Optic capsule—Otosclerosis—Transforming growth factor-β1.

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CLINICAL CHARACTERISTICS

Otosclerosis is the most common cause of hearing impairment in the white population. In 1740, Valsalva was the first to make a link between hearing loss and stapes fixation (1). In otosclerosis, it is important to distinguish clinical and histologic otosclerosis, which was emphasized for the first time in 1944 by Guild (2). "Histologic otosclerosis" is characterized by a disease process without any clinical symptoms, which can only be discovered by postmortem temporal bone investigation or by high-resolution computed tomographic scanning. A large study of 236 temporal bones of European origin showed a prevalence of 2.5% with no differences between sexes (3,4). A similar prevalence of 2.56% was found in the Japanese population (5). However, "clinical otosclerosis" refers to the presence of otosclerosis at a site where it causes hearing impairment. The prevalence varies in different ethnicities: it is rare in African blacks, Orientals, and South American Indians, and more frequent in populations of European origin, where the prevalence is 0.3 to 0.4% (2–4,6–8). Another characteristic is that otosclerosis is more frequent in women compared with men, with a ratio of 1.4:1 to 2:1 (9,10). Although histologic otosclerosis is as common in the Japanese population as in European populations, the otosclerotic foci are less frequent around the oval window, which can explain the low incidence of clinical otosclerosis (11). To date, however, it is still not known whether the bone remodeling in clinical and histologic otosclerosis is caused by the same trigger.

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Clinical otosclerosis is characterized by a progressive conductive hearing loss that is bilateral in 85% of the cases. In 10% of the patients, a sensorineural component arises (10,12–14). Based on histologic findings (15–18), clinical otosclerosis can be divided into 3 categories. The first category is the classic otosclerosis, which manifests as a conductive hearing loss due to stapes fixation. The second category is characterized by stapes fixation and cochlear involvement resulting in a mixed hearing loss, and the third category manifests as a pure sensorineural hearing loss because of cochlear damage without stapes fixation. True cochlear otosclerosis, however, is an issue that has been widely debated. The most confusing elements in this story are the overestimated bone conduction thresholds in otosclerosis. The causing factor is the Carhart effect, first described by Carhart in 1950. This is a well-known audiologic artifact that arises due to the stapes fixation. Because of this phenomenon, bone conduction thresholds are not a true indicator for the inner ear function (19). Today, sensorineural hearing loss that cannot be correlated to the patient's age has become an accepted feature of otosclerosis (10).

The most frequent symptom associated with otosclerosis is tinnitus. In a study of Mazzoli et al. (20), 45% of the patients reported tinnitus. Gristwood and Venables (21) even reported a prevalence of 65%. Vestibular symptoms such as imbalance and vertigo are less frequent compared with tinnitus. Approximately 10% of the otosclerosis patients report vestibular problems (20).

Microsurgical interventions such as stapedectomy and stapedotomy can restore the conductive component of the hearing loss caused by the fixation of the stapes but cannot correct the sensorineural component or other symptoms. In some rare cases of severe sensorineural involvement, a cochlear implantation may be a good therapeutic option (22).

The disease starts typically between the ages of 20 and 40 years, and up to 90% of the cases are younger than 50 years at the time of diagnosis (9,14). However, a recent study showed that the average age of patients is increasing. Possible explanations given by the authors included general improved health awareness, the use of low-dose contraception, changing socioeconomic factors, and measles vaccination strategies (23).

THE OTIC CAPSULE

Embryonic Development

Already 100 years ago, otologists were aware of the unique bony development of the otic capsule. It consists of an inner endosteal layer, an intermediate endochondral layer, and an outer periosteal layer. The otic capsule arises in fetal development through endochondral ossification, a bone formation process in which a cartilage model is first made, which is then replaced by bone. During this process, cartilaginous remnants are often not removed when the lacunae of degenerating cartilage cells are being replaced by primary bone. These remnants are called "globuli interossei" and are located in the intermediate endochondral layer (24–27).

Chondrogenesis of the Otic Capsule

The otic capsule initially appears as a condensation of periotic mesenchyme around the developing otocyst. It is in response to growth factors secreted by the otocyst epithelium that sites of cellular condensation are formed. Epithelial-mesenchymal interactions are therefore essential in the embryonic development of the otic capsule. Transforming growth factor–β1 (TGF-β1) has a very important role in this entire process of otic capsule chondrogenesis (28). In early stages, the otic epithelium produces TGF-β1 to stimulate the chondrogenesis and to promote growth, and in a later stage, TGF-β1 will selectively inhibit this process to allow perilymphatic space formation and capsular modeling (28,29). However, TGF-β1 alone is not sufficient, and other growth factors such as fibroblast growth factors 2 and 3 (FGF-2 and FGF-3) are needed during the first stages of the chondrogenesis (30). Bone morphogenetic proteins 2 and 4 (BMP-2 and BMP-4), members of the TGF-β superfamily, also stimulate the chondrogenesis and promote growth. However, in later stages, they do not evoke an inhibitory response in the periotic mesenchyme (31). The synergistic interaction between these growth factors, and especially between TGF-β1 and FGF-2, is considered the most important factor during otic capsule formation because it can evoke a full chondrogenic response (30).

Characteristics of the Otic Capsule

Bone is a dynamic tissue that is continuously remodeled by the balanced process of bone resorption by osteoclasts and bone formation by osteoblasts. This process of bone remodeling is responsible for the continuous turnover and renewal of the skeleton, which is important to establish and maintain the architectural features of the skeleton during growth and in response to altered functional demands. Although the otic capsule is the hardest bone of our entire skeleton, it shows very little bone turnover. The overall capsular bone turnover rate was found to be 2.1% per year compared with 10% per year for the rest of the skeleton. In the otic capsule, bone remodeling shows a gradual pattern from almost no turnover near the perilymphatic spaces (0.13%/yr) to normal rates toward the periphery. The inhibition of bone turnover is more pronounced around the cochlea and vestibule than around the semicircular canals (24,32).

Recently, researchers found intrinsic factors produced by the cochlea to be responsible for this specific bone turnover inhibition of the labyrinthine bone. Bone remodeling and the balance between bone formation and resorption in the general skeleton is regulated by various hormonal and biochemical factors. A coupling mechanism between osteoblasts and osteoclast plays the major role in this process and is established by several molecules, including osteoprotegerin (OPG), receptor activator of nuclear factor–κB (RANK), RANK ligand (RANKL), and TGF-β1 (Fig. 1). Several recent studies suggest

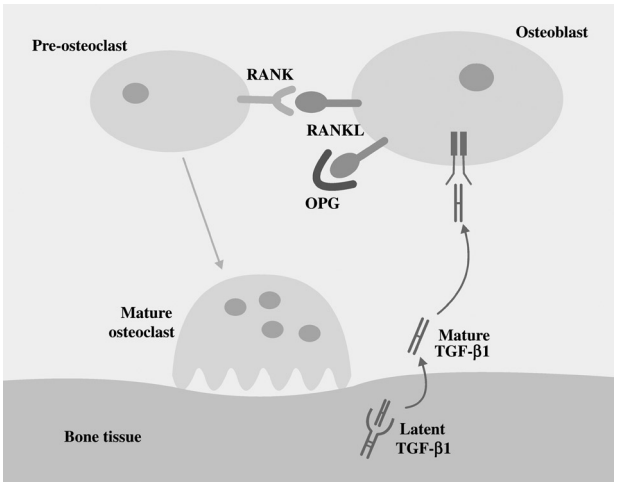

FIG. 1. Schematic representation of the coupling mechanism between osteoblasts and osteoclasts. Receptor activator of nuclear factor–κB (NF-κB) ligand, produced by osteoblasts, binds to its receptor RANK on the osteoclast and activates the NF-κB signaling pathway. This stimulates differentiation and activation of osteoclasts, which results in the promotion of bone resorption. During bone resorption, TGF-β1 that is stored in the bone matrix is released and activated. Active TGF-β1 stimulates osteoblasts, resulting in a decreased expression of RANKL and increased expression of OPG by osteoblasts. Osteoprotegerin is a decoy receptor of RANKL and inhibits RANK/RANKL interaction. Consequently, this leads to an inhibition of osteoclast differentiation and activation because NF-κB signaling will be suppressed. Eventually, this will lead to a decreased bone resorption that results in a decreased amount of active TGF-β1. This makes TGF-β1 a coupling factor between bone resorption and formation (33).

that OPG is the intrinsic factor that inhibits bone remodeling to maintain normal auditory function. Zehnder et al. (34) were the first to report a very high OPG to RANK (mRNA) ratio in the cochlea and a high OPG concentration in the perilymph of mice. Their data suggested that OPG is produced in high concentrations in the spiral ligament of the cochlea and diffuses into the perilymph and the surrounding otic capsule, the latter by way of an extensive system of interconnected canaliculi in the bone of the otic capsule (24). A study by Kanzaki et al. (35,36) showed that OPG plays a crucial role in hearing by protecting auditory ossicles and the otic capsule from osteoclastic bone resorption. In *Opg* knockout mice, which lack OPG, the junction between stapes and otic capsule was fixed because the ligament was replaced by bone tissue. Zehnder et al. (37) also investigated *Opg* knockout mice and demonstrated a progressive and abnormal remodeling process of the otic capsule that was not observed in controls mice. In addition, this active remodeling process showed many similarities to otosclerosis, including sharply defined areas of bone resorption and deposition. The study also demonstrated a progressive hearing loss in *Opg* knockouts. However, there were also clear differences between *Opg* knockouts and clinical otosclerosis. Most importantly, active remodeling in *Opg* knockouts is seen throughout the entire skeleton, including the incus and malleus. Moreover, Zehnder et al. could not find histologic evidence of stapes fixation in these *Opg* knockout mice, which is in contradiction with the study by Kanzaki et al. Another study showed that an increased expression of tumor necrosis factor–α inhibits the protective function of OPG (38). All these studies point to OPG as the most important inhibiting factor of the bone turnover in the otic capsule.

THE PATHOPHYSIOLOGY OF OTOSCLEROSIS

Disease Progression

Otosclerosis is a disease process that is characterized by an increased rate of bone remodeling in the otic capsule. This process takes place in a region of the temporal bone that normally has minimal bone turnover in adult life and increases the turnover to the rate that is found in the rest of the skeleton. In otosclerosis, foci of abnormal bone deposition are particularly frequent around the oval and round window and in the otic capsule close to the cochlea, 3 places where the inhibition of bone remodeling is most prominent (32,39). Otosclerosis only occurs in the temporal bone (40).

The progression of otosclerosis can be divided into 4 stages. In the first stage, the resorptive or active inflammatory phase, the endochondral bone of the otic capsule is resorbed by osteoclasts. This is initiated by an unknown pathologic stimulus and affects certain anatomic sites such as the fissula ante fenestrum and the globuli interossei near the oval window. The bone is then replaced with a highly vascular cellular and fibrous tissue. Subsequently, new bone is formed. This second phase is characterized by the production of a dysplastic, immature basophilic bone and the filling of the vascular spaces with connective tissue and the synthesis of collagen fibrils. The third phase is the remodeling phase in which the basophilic bone is remodeled and becomes a less vascular and more mature acidophilic bone with a laminated matrix. In the fourth and last phase, the mature or otosclerotic phase, mineralization of the dysplastic bone results in a new dense compact bone with a characteristic woven pattern (41–45).

Stapes fixation starts with the calcification of the annular ligament. In this process, the otosclerotic lesion of the oval window fuses with the stapedial footplate. The stapes subsequently becomes fixed by this lesion. The process extends across the ligament onto the footplate until there is no remnant of the original annular ligament (46).

Environmental Factors

In the past, a variety of theories have been postulated to explain the development of otosclerotic foci. Concerning the environmental factors, there is still a lot of controversy. Many studies suggest that endocrine factors such as estrogen or oral contraception could be responsible for the fact that women are more frequently affected than men (47). However, the influence of oral contraception could not be confirmed in a large study (48). Although it is

accepted that otosclerosis can manifest itself during or shortly after pregnancy, there seems to be no correlation with the severity of hearing loss (49). One of the factors that may explain the perceived association with pregnancy is that women sometimes see important life events in relation to their pregnancy (49).

Another factor is the role of sodium fluoride (NaF) in the prevention of otosclerosis. Causse et al. (50,51) thought that moderate doses of NaF would inhibit proteolytic enzymes and therefore decrease disease progression. This hypothesis was supported by epidemiologic studies that showed that otosclerosis was associated with areas of low-fluoride content in the drinking water (52). Although several treatment protocols have been suggested during the last years, there are also studies that contradict this hypothesis (53,54). However, more recently, Grayeli et al. (55) provided a possible molecular explanation for the effect of NaF in otosclerosis. Cells from otosclerotic stapes in cell culture show an abnormal high sulfatation of bone matrix glycosaminoglycans (GAG) (56–58). The diastrophic dysplasia sulfate transporter (DTDST) participates in the GAG sulfatation. It is thought that an increased DTDST activity alters the osteoblastic response to circulating growth factors. In bone cells derived from the stapes and the external auditory canal of otosclerosis patients, the activity of DTDST is increased, and this increase is correlated with the sensorineural hearing loss. Moreover, DTDST activity is specifically inhibited by NaF, which may, for this reason, help to preserve hearing (55). Another molecule with possible therapeutic effects is dexamethasone, which also specifically inhibits the increased DTDST activity in otosclerotic cells, an effect mediated by the inhibition of autocrine/paracrine interleukin 6 secretion (59).

A lot of research has been performed the past 20 years to unravel the possible role of measles virus (MeV) in the pathogenesis of otosclerosis. In 1986, McKenna et al. (60) reported for the first time the presence of filamentous structures resembling paramyxoviral nucleocapsids in osteoblast-like cells of 2 otospongiotic tissue specimens. Later, immunohistochemical investigations using monoclonal and polyclonal antibodies identified MeV proteins (61–63), although this could not be confirmed with the same technique by Roald et al. (64). Measles virus RNA in otosclerotic tissue was detected with reverse-transcriptase–polymerase chain reaction a few years later (65–68). However, a study by Grayeli et al. in 2000 (69) could not confirm these results. Other studies showed a higher percentage of anti-MeV immunoglobulin G in the perilymph and serum of otosclerosis patients (70,71). The reactivity of these antibodies against MeV, however, seemed to be lower in patients (72). A recent report suggests that the incidence of otosclerosis has decreased over the last years since the introduction of measles vaccinations in the early 1970s (73). However, most vaccination recipients are still too young to have developed otosclerosis, and therefore, it is too early to draw definite conclusions. Another aspect is that only humans and primates are hosts of the MeV because of their complementary cell surface structures (CD46 and CD150) (74,75), and that MeV shows a certain organotropism to the otic capsule (66,76). If MeV is involved, this could explain why, to date, there is still no good animal model for otosclerosis. A very recent study by Karosi et al. (77) showed the existence of 4 novel splice variants of the MeV receptor CD46 only present in otosclerotic stapes footplates. It is possible that MeV plays a role in the pathogenesis of otosclerosis, but on the other hand, it is equally possible that the presence of MeV in otosclerotic bone is an epiphenomenon and not a causative agent (66). However, genetic predisposition has to be assumed in both cases.

Disorders With Otosclerosis-Like Lesions and Symptoms

It is known that the otic capsule is an exception among human bones. Except for pathologic conditions, osteoclasts and osteoblasts are not seen in the otic capsule after the endochondral ossification. It retains its fetal structure into adult life, and it responds to pathologic stimuli differently than other bones. In some of these pathologic conditions, besides otosclerosis itself, otosclerosis or otosclerosis-like lesions of the footplate and the otic capsule have been observed. Most of these diseases are inherited bone disorders such as osteogenesis imperfecta (OI), osteopetrosis, Paget disease, osteoporosis, and Camurati-Engelmann disease (CED).

Osteogenesis imperfecta is typically characterized by multiple bone fractures, often resulting from only minimal trauma. It affects 1 in 15,000 to 20,000 individuals. In approximately 50% of the families, hearing loss of conductive or mixed type is present. The hearing loss starts in the late teens and may gradually lead to profound deafness. Tinnitus and vertigo may also occur (78). There is a large similarity between the morphology of the stapedial lesion in OI and otosclerosis. However, OI lesions involve all 3 layers of the otic capsule, whereas it only affects the endochondral layer in otosclerosis (79). The lesions in OI have a greater degree of structural disorganization and larger resorption spaces (43), and the endochondral layer has less "globuli interossei" (80). In 1998, McKenna et al. (81) suggested a common underlying genetic mechanism for otosclerosis and OI. This will be discussed later in the article.

Paget disease is a metabolic bone disease characterized by excessive bone resorption and formation due to activated osteoclasts. It has a prevalence of approximately 3% varying between geographic regions (82). Paget disease can affect 1 or multiple bones of the systemic skeleton, including the temporal bone (42). Like otosclerosis, it is a late-onset disease, occurring in persons older than 40 years. A possible viral pathogenesis has been reported (82). The bony lesions that occur in the otic capsule of Paget patients may seem similar to otosclerosis, but they have more large multinucleated osteoclasts (83), and the pagetoid bone has a typical moth-eaten appearance eroding the otic capsule from the periphery (80). The hearing loss in most of these patients is not due to ossicular lesions.

Osteopetrosis is a bone dysplasia characterized by failure of resorption of cartilage in primitive bone, resulting in an increased density of bone throughout the entire body, including the temporal bone. It has an incidence in the general population of 1 in 250,000 (84). Microscopic examination of the otic capsule in osteopetrosis patients showed thickening of the endosteal and periosteal layer and a greater number of globuli interossei in the endochondral layer compared with otosclerosis (80). Hearing is often compromised by compression of the acoustic nerves.

Osteoporosis is a disease characterized by reduced bone mineral density and disrupted bone microarchitecture. This leads to an increased incidence of fractures. Osteoporosis has a prevalence of approximately 30–40% among postmenopausal women older than 50 years. Fracture rates increase rapidly with age, and women have a more than twofold increased incidence compared with men. A study of 100 women with otosclerosis and 100 women with presbycusis from Massachusetts showed that osteoporosis is more frequent in female otosclerosis patients, which could reflect a possible shared genetic pathogenesis between otosclerosis and osteoporosis (85).

Otosclerosis and otosclerotic lesions have also been observed in CED or progressive diaphyseal dysplasia (86). Camurati-Engelmann disease is a very rare sclerosing bone dysplasia characterized by a rapid bone turnover. Camurati-Engelmann disease is caused by mutations in TGF-β1 (87). Hearing loss has been reported in approximately 18% of the patients. Sensorineural hearing loss is due to auditory nerve damage after narrowing of the internal auditory canals. Some CED patients show a conductive hearing loss that is often due to narrowing of tympanic cavities and fixation or adhesion of the ossicles to the middle ear wall (88). Some of these CED patients clearly show otosclerosis, a diagnosis confirmed during stapes surgery by the presence of stapes fixation, and some researchers even suggest that otosclerosis could be a part of the CED phenotype (86).

Although congenital stapes fixation is rare, it has been reported in families with certain genetic diseases. These include X-linked stapes fixation with perilymphatic gusher (DFN3; *POU3F4*) (89), stapes ankylosis, broad thumbs and toes syndrome (90), proximal symphalangism (91,92), and facio-audio-symphalangism (93). The last 3 syndromes are caused by mutations in the *Noggin* gene. Noggin is a secreted protein that binds and inactivates BMPs and plays a role in bone remodeling and maturation (94). Liu et al. (95) showed that noggin treatment of periotic mesenchyme cell cultures, derived from mice otocysts, results in a dose-dependent suppression of the otic capsule chondrogenesis by inhibiting the actions of BMP-4.

THE GENETICS OF OTOSCLEROSIS

Toynbee (96) was one of the first to report in 1861 a familial pattern of hearing loss that probably represents otosclerosis. Fifteen years later, another family was documented by Magnus (97). In 1966, Fowler (98) conducted a twin study and found concordance for clinical otosclerosis in nearly all 40 pairs of monozygotic twins, which supported the early hypotheses that otosclerosis has an important genetic basis.

The first genetic studies aimed at defining a mode of inheritance. In 1922, Albrecht (99) was the first to conclude that otosclerosis could be inherited as an autosomal dominant disease in certain families. Larsson (100) supported this hypothesis in 1960 and found that in most autosomal dominant families, the penetrance is incomplete and lies between 25 and 40%. In the late 1960s, on the basis of a detailed genetic study in larger otosclerosis families, Morrison and Bundy (101,102) also concluded that otosclerosis is an autosomal dominant disease with 40% penetrance. This finding was confirmed by other studies in 1975 and 1984 (103,104). Although mathematical calculations showed that other modes of inheritance are highly unlikely, studies dated between 1960 and 1975 could not completely rule out the possibility (100,104,105). Baurer and Stein (106) postulated in 1925 a digenic recessive mode of inheritance in their study. However, this was criticized in 1967 by Morrison (101) for inclusion of relatives with deafness of other causes.

Although a strong familial background exists, 40 to 50% of all clinical cases have been reported to be sporadic (9,104,107,108). Morrison and Bundey (102) explained most of these sporadic cases by reduced penetrance but also pointed toward possible new mutations and other modes of inheritance besides autosomal dominant. Gordon (9) suggested complex inheritance to explain the sporadic cases in 1989. Complex or multifactorial diseases are caused by an interaction of several environmental and genetic factors. When we look at all these data today, a complex genetic cause for otosclerosis is by far the most likely explanation for most cases. Clearly, autosomal dominant forms also exist, often with reduced penetrance. This is not different from many other frequent diseases for which both complex genetic and monogenic forms exist. Complex genetic diseases and monogenic diseases need a different research strategy to identify the genes involved.

Monogenic Forms of Otosclerosis

To search for genes involved in monogenic diseases, positional cloning is the best strategy, and linkage analysis is a key factor. Linkage analysis is a technique to identify the chromosomal location of the disease causing mutation by means of genetic markers in a family with a monogenic disease. A locus is linked when the analyzed markers are segregating together with the disease in the family. First, a family with many affected family members is collected. To investigate linkage for autosomal dominant inherited diseases such as otosclerosis, as a rule of thumb, more than 10 affected family members and unaffected sibs are required to reach statistical significant linkage. In the case of otosclerosis, researchers usually first investigate whether the family is linked to any of the known loci. Today, 7 loci have already been localized (*OTSC1–5*, *7*, and *8*) (109–115). One locus name, *OTSC6*, has been reserved by the Human Genome

TABLE 1. *Monogenic otosclerosis loci*

Locus	Region	Family	Maximum LOD score	Publications
OTSC1	15q25–26/14.5 cM	South Indian	3.4	113,116–118
OTSC2	7q34–36/16 cM	Belgian (3 families)	3.54	115–120
OTSC3	6p21.3–22.3/17.4 cM	Cypriot	3.83	111,118
	—	Tunisian	2.04	—
OTSC4	16q21–23.2/10.1 Mb	Israeli	3.97	110,118
OTSC5	3q22–24/15.5 Mb	Dutch	3.46	114,118,121
OTSC6	—	—	—	Not reported
OTSC7	6q13–16.1/13.5 cM	Greek	7.5	112,122,123
		Dutch	1.96	
OTSC8	9p13.1–q21.11/34.2 Mb	Tunisian	4.13	109,118

LOD indicates logarithm of the odds.

Organisation Nomenclature Committee but has not been published yet (Table 1). When linkage to the known regions is excluded, a whole genome linkage scan can be performed. Approximately 500 microsatellite markers or at least 2,000 single nucleotide polymorphisms (SNPs) can be used as genetic markers. Single nucleotide polymorphisms are small variations in the DNA sequence in which 1 nucleotide (A, T, G, or C) is substituted for another. Single nucleotide polymorphisms are very frequent across the genome, and on average, 1 SNP occurs every 1,000 base pairs. For most SNPs, the nucleotide at that position can be 1 of 2 nucleotides. These 2 possible nucleotides are called alleles. This means that an individual who has 2 copies of each autosomal chromosome can have 2 alleles for each SNP. The combination of these 2 alleles is called a genotype.

When linkage is found in a certain chromosomal region, this locus can be refined with additional markers. Subsequently, genetic databases are checked to see which genes reside in the linked region. Good candidate genes in this linked interval are subjected to mutation analysis to identify the causal mutation. Good candidate genes for otosclerosis can be genes that are directly or indirectly involved in bone metabolism. However, to date, none of the otosclerosis-causing genes have been identified despite the known chromosomal localization of 7 of them.

Complex Forms of Otosclerosis

Many otosclerosis patients do not have a familial history of otosclerosis or do not show a clear Mendelian segregation of the disease. These patients represent the complex form of otosclerosis caused by environmental and genetic factors.

Human Leukocyte Antigen

As for many diseases, associations with the human leukocyte antigen (HLA) system have been performed for otosclerosis. The HLA system represents the major histocompatibility complex in humans and is encoded by a complex DNA segment on chromosome 6. This group of genes encodes cell-surface antigen-presenting proteins. Human leukocyte antigen is an important factor associated with many diseases, especially diseases with an immunologic component. Analysis of the HLA antigenic determinants showed association in some studies (124–127), whereas in other studies, no association could be detected (128–131). Identification of the disease-causing gene of the *OTSC3* locus might help to settle this issue because the linkage interval covers the HLA region (111). However, mutation analysis is extremely difficult because of the genetic complexity of this region.

Association Studies

The strategies and methods to investigate complex genetic diseases have evolved enormously during the past 10 years. A genetic case-control association study is currently the most popular form to investigate these diseases. The genetic markers that are most commonly used in these association studies are SNPs.

The HAPMAP project has provided an inventory of most SNPs in the human genome (132,133). Currently, HAPMAP databases contain approximately 1 SNP per 2,000 base pairs. Additional information can be found such as allele frequencies in different populations and linkage disequilibrium (LD) patterns. Linkage disequilibrium is a term that explains why SNPs that are lying close to each other are often not independent, and some combinations of alleles are more frequent in the population than would be expected. Because of LD, not all SNPs in the human genome need to be analyzed in genetic studies. A certain subset of SNPs (called tagSNPs) can be selected, ensuring that most of the variation in the human genome is covered.

Genetic association studies are performed to determine whether a genetic variant (here an SNP) is associated with a disease or trait. When association is present, it means that a certain allele, genotype, or haplotype (combination of alleles of different neighboring SNPs) is seen more often in patients than could be expected by chance. Although there are some family-based designs for association studies, case-control designs are the easiest and most popular choice. In these case-control studies, the allele, genotype, or haplotype frequencies of the tagSNPs are compared between large groups of cases and controls, and a statistically significant difference in frequency between the 2 groups indicates that the associated variant may increase or decrease the risk of developing the disease.

In most cases, the associated SNP is not the causal one, but is in LD, with the true causal variant somewhere in that region.

An important issue in case-control association studies is that the controls must be matched with the cases, especially for ethnicity. This matching avoids false-positive associations due to population stratification because SNPs often have different allele frequencies in different populations.

There are 2 strategies in case-control genetic association studies: the candidate gene–based association study and the genome-wide association (GWA) study. In a candidate gene–based association study, a gene is selected on the basis of a hypothesis regarding a possible involvement in the disease pathology. When you have a good candidate gene, tagSNPs and functional SNPs (SNPs with a predicted biologic function) are selected to cover the gene completely, and these SNPs are analyzed in all cases and controls. However, for this type of study, a priori knowledge or assumption of the disease-causing gene is necessary, and this is not always easy. Recently, new technologies have made it possible and affordable to perform association studies on a genome-wide level such as linkage studies. A set of 500,000 to 1 million SNPs spread over the entire genome are put on a single SNParray, and these SNParrays are genotyped in all cases and controls. To conclude whether a SNP is statistically significant associated with a disease, you have to take into account that many SNPs are tested, and that you have to correct for multiple testing. Multiple testing correction is an important but controversial issue in genetic studies, and the critical *p* value for genome-wide significance is still a matter of debate.

When association is found, either via a candidate gene–based or via a GWA study, it is important to replicate the results in an independent population to minimize false-positive results. Although it is generally accepted today that a correction for multiple testing has to be taken into account, replication of association in independent populations is generally seen as more important than a very low *p* value (134). Nonreplication, however, can have many causes and does not necessarily rule out a true association. Small samples sizes reduce the power of the study to pick up real signals and may lead to nonreplication. Another reason for nonreplication may be that different disease-causing alleles predominate in different populations (135).

When an association is proven by solid replication, the disease-causing variants need to be identified. Because of LD, the associated SNPs are, in most cases, not the causal variant. In this case, DNA resequencing of the associated region is the way to find the true causal variant. Functional analyses will eventually have to be performed to define the role of these variants in the disease pathology. However, this type of studies may be very complex and time-consuming. For otosclerosis, a few candidate gene–based association studies have already been performed to date, and they will be discussed in the succeeding sentences.

Collagens

The first genetic case-control association study for otosclerosis investigated the association with collagen Types I and II genes (81). The *COL2A1* gene was first analyzed because of its abundance within the globuli interossei and the hypothesis that an autoimmune reaction to Type II collagen could be involved in otosclerosis (136–138). No association has been detected to date (81,139). *COL1A1* and *COL1A2* associate in a 2:1 ratio to form a collagen Type I triple helix. *COL1A1* was also a good candidate gene because of its role in 2 other diseases where conductive hearing loss is a part of the phenotype: OI Type 1 and osteoporosis (described in Supplementary Data 1). In 1998, McKenna et al. (81) reported the first association between *COL1A1* and otosclerosis in a small American population of European descent living in Massachusetts. The association was reproduced in 2007 by Chen et al. (140) in the same population from Massachusetts and in an additional small German population. The latter study identified association of specific haplotypes of *COL1A1* with otosclerosis. In vitro analysis of the haplotype with a higher frequency in patients in an osteoblast cell line showed an increased promoter-reporter activity by affecting the binding of certain transcription factors (leading to an increased production of collagen α1 (I) homotrimers). This higher COL1A1 expression may be causally related to the development of otosclerosis. However, there is still some controversy regarding the *COL1A1* association because another study could not confirm this result. A study by Rodriguez et al. (139) investigated only 2 SNPs in *COL1A1* in a Spanish population but could not replicate the result.

Transforming Growth Factor–β1

Recently, *TGFB1* was found to be associated in 2 large independent populations (141). Transforming growth factor–β1 is the prototype of the TGF-β superfamily and plays a major role in the development and maintenance of both cartilage and bone (142). It also plays an important role in the embryonic development of the otic capsule (29). In human otosclerotic bone cell cultures, it can modify the phenotypic expression of GAG, fibronectin, and collagen of the extracellular matrix (57). A case-control association study was preformed using tagSNPs and amino acid–changing SNPs covering the entire gene in a large Belgian-Dutch (632 cases and 632 matched controls) and French (457 cases and 497 controls) population. Analysis of the data revealed that the amino acid–changing SNP p.Thr263Ile (c.788C<T) was associated in both populations, and that the T allele coding for Ile was more frequent in controls (7%) compared with cases (2.6%), which points to a protective role of I263 (combined p value = 9.2×10^{-6}). Functional analysis showed that the protective variant p.Ile263 of TGF-β1 was more active than the wild type (21.2%). It is hypothesized that p.Ile263 decreases susceptibility to otosclerosis by inhibiting osteoclast differentiation and activation in the first otospongiotic phase of otosclerosis (141). Recent studies

have shown that not only common but also rare variants can be involved in complex diseases. Therefore, DNA sequencing of the coding part of *TGFB1* was conducted in 755 otosclerosis patients and 877 control samples from Belgian, Dutch, and French origin. The study revealed 3 new different amino acid–changing variants (p.Glu29, p.Ala29, and p.Ile241) in 4 otosclerosis patients (143). In silico analysis showed that these variations could have an influence on TGF-β1 function and activity, suggesting that multiple rare amino acid–changing variants (p.Glu29, p.Ala29, and p.Ile241) and more common variants (p.Thr263Ile) in TGF-β1 may contribute to the susceptibility of otosclerosis (143).

BMP2 and BMP4

To look for other susceptibility genes, a larger study was set up by Schrauwen et al. (144) using the same study populations as the *TGFB1* study. Thirteen new candidate genes were selected based on their association with the TGF-β1 interacting network, function in the metabolism or chondrogenesis of the otic capsule, involvement in syndromic or nonsyndromic forms of stapes fixation, and other hypotheses regarding the cause of otosclerosis. A total of 92 tagSNPs and functional SNPs were genotyped in the Belgian-Dutch population. Associated SNPs were analyzed in the French population to confirm the positive result. In both populations, *BMP2* and *BMP4* were the only genes with a significant association showing the same effect. Individuals that possess the T allele of a SNP located in the 3′ untranslated region of *BMP2* (g.6700201T<C) have a higher risk of developing otosclerosis. The 3′ untranslated region of a gene can be involved in the regulation of gene expression by controlling nuclear export, polyadenylation status, subcellular targeting, rates of translation, and degradation of mRNA (145). The other associated SNP was an amino acid–changing SNP (p.Ala152Val) lying in exon 4 of *BMP4*. Although LD of these SNPs with yet unidentified causative variations elsewhere cannot be excluded, it is tempting to speculate that these SNPs could have a direct effect on protein expression or function (144).

Renin-Angiotensin-Aldosterone System

The most recently published association for otosclerosis is with the renin-angiotensin-aldosterone (RAA) system (146). This hypothesis was seen in relation to the female predominance in otosclerosis and pregnancy as a risk factor and was based on the fact that during pregnancy, the RAA system is stimulated. In a French population, Imauchi et al. (146) investigated 3 functional polymorphisms in 3 different genes in this system: the p.Met235Thr polymorphism in the angiotensinogen, an insertion/deletion polymorphism in the angiotensin converting enzyme, and the p.Ala1166Cys variant in the angiotensin II receptor Type I gene. A significant association was found for the first 2 polymorphisms. However, these results could not be replicated in a larger study with higher statistical power (147). Nevertheless, association of the RAA system with otosclerosis cannot be ruled out completely because in both studies, not all variation in these genes was captured and analyzed (135).

Gene Expression Analysis

An alternative for genetic association studies to identify molecular contributors to a disease is to perform a microarray analysis of gene expression in diseased tissue. This was recently performed by Ealy et al. (148) for otosclerosis. In this study, they used RNA from 9 stapes footplates of otosclerosis patients and compared the gene expression to 7 control stapes footplates derived from patients undergoing labyrinthine surgery for vestibular schwannomas. The analysis showed that 110 genes were differentially expressed, of which 92 were up-regulated and 18 were down-regulated. The genes with the largest difference in expression were related to TGF-β signaling. The gene with the highest up-regulation in otosclerosis samples was *PF4* (platelet factor 4). *PF4* inhibits the binding of TGF-β1 to Type I TGF-β1 receptor (149), and its increased expression in otosclerosis samples may result in a stimulation of bone resorption by inhibiting the TGF-β1 signaling. *IBSP*, the gene encoding for the bone sialoprotein, was the most down-regulated gene. Increased TGF-β1 signaling increases the expression of *IBSP* (150). Therefore, a down-regulation of this gene in otosclerosis patients could be due to an overall decreased TGF-β1 signaling. Gene ontology analysis of the differentially expressed genes suggested a number of new pathways that could be involved in otosclerosis, including interleukin signaling and inflammation. Other identified genes were receptors, transcription factors and signaling molecules, and genes involved in biologic processes such as signal transduction. Genes that were previously described to be differentially expressed were not confirmed in the study of Ealy et al.: *BMP2*, *BMP4*, and *BMP7* (151); *TNF-α* (152); *OPG* (38); and parathyroid hormone–parathyroid hormone–related peptide receptor (153). Failure to replicate these study results could be explained by the limited number of samples used, the relatively large variance of the microarray results, or the complex nature of the disease (148).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Thanks to a good understanding of the bone metabolism of the otic capsule and recent developments in the methods to study complex diseases, the past year has seen a remarkable progress in the identification of genes involved in otosclerosis. From a genetic viewpoint, otosclerosis can be seen as a complex disease where both genetic and environmental factors confer disease susceptibility, with rare autosomal dominant forms in which 1 gene causes otosclerosis. However, autosomal dominant otosclerosis families large enough to perform a genetic linkage study are rare, and often, factors such as reduced penetrance complicate the analysis. To date, for the monogenic form, 7 autosomal dominant loci (*OTSC1–5*, *7*, and *8*)

have already been published, but none of the disease-causing genes have been identified.

For the complex form of otosclerosis, different genes (*TGFB1*, *BMP2*, *BMP4*, *COL1A1*, *AGT*, *ACE*) and the HLA system were reported to be associated. For otosclerosis, only the associations with *TGFB1*, *BMP2*, and *BMP4* replicated convincingly, whereas for the other studies, results have been contradictory. Most importantly, this review demonstrates that both genetic association studies and gene expression analysis for otosclerosis point to the same direction, namely, the TGF-β1 pathway. The association study shows that an increased TGF-β1 activity protects against otosclerosis, whereas the gene expression study showed evidence for decreased TGF-β1 signaling in otosclerotic bone. How all the different pathways of TGF-β1 really interact to produce otosclerosis is difficult to understand at this moment because the TGF-β1 signaling is very complex. What we can conclude is that TGF-β1 and genes involved in the TGF-β1 signaling are important in the pathogenesis of otosclerosis, and further research in this area will be of major importance.

What could be the mechanism behind the development of otosclerosis? The bone turnover is highly suppressed in normal otic capsules, probably due to a local inner ear mechanism for which OPG seems to be an important factor. It is most likely that otosclerosis develops due to a relief of this specific inhibition of bone remodeling, rather than a more general activation of bone turnover, because the increased bone turnover, seen in otosclerosis, only influences the otic capsule and is not found elsewhere in the human skeleton.

Thanks to the availability of new information from the Human Genome Project, high-density SNP maps such as the HAPMAP and new genotyping platforms, GWA studies have become possible. Genome-wide association studies are a powerful strategy to identify new genes in a hypothesis-free manner. Disease pathways that are currently not implicated in the disease pathophysiology can be identified. Although GWA studies are still very expensive, they are clearly the way to go for otosclerosis in the future.

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