Yamazaki H, Yamamoto R, Moroto S, Yamazaki T, Keizo Fujiwara K, Nakai M, Ito J, Naito Y	Cochlear implantation in children with congenital cytomegalovirus infection accompanied by psycho-neurological disorders	Acta Otolaryngol (accepted for publication)			2012
Wakabayashi K, Fujioka M, Kanzaki S, Okano HJ, Shibata S, Yamashita D, Masuda M, Mihara M, Ohsugi Y, Ogawa K, Okano H.	Blockade of interleukin-6 signaling suppressed cochlear inflammatory response and improved hearing impairment in noise-damaged mice cochlea.	Neuroscience Res.	66(4)	345-352	2010
Cui Y, Sun G, Yamashita D, Kanzaki S, Matsunaga T, Fujii M, Kaga K, Ogawa K.	Noise-induced apoptosis in fibrocytes of the cochlear spiral limbus of mice.	European Arch. Oto.	268 (7)	973-978	2011
Shibata T, Yamashita D, Hasegawa S, Saito M, Otsuki N, Hashikawa K, Tahara S, Nibu K.	Oral candidiasis mimicking tongue cancer.	Auris Nasus Larynx	38(3)	418-420	2011
豊田英樹, 下郡博明, 菅原一真, 吉田周平, 山下裕司	AMPAによる末梢前庭障害に対するFGLM+SSSRの効果	頭頸部自律神経	25	20-21	2011
吉田周平, 菅原一真, 豊田英樹, 金川英寿, 下郡博明, 山下裕司	SSSR、FGLM-NH2による有毛細胞保護効果について	頭頸部自律神経	25	22-23	2011
Nakamoto T, Mikuriya T, Sugahara K, Hirose Y, Hashimoto T, Shimogori H, Takii R, Nakai A, Yamashita H.	Geranylgeranylacetone suppresses noise-induced expression of proinplammatory cytokinesin the cochlea.	Auris Nasus Larynx		in press	2011
Toyota H, Shimogori H, Sugahara K, Yamashita H.	Topical application of substasnce P facilitates vestibular functional recovery induced by AMPA in the guinea pig.	The Bulletin of Yamaguchi Medical School		in press	2011

Ⅳ. 研究成果の刊行物・別刷

Original Paper

Audiology & Neurotology

Audiol Neurotol 2010;15:168–174
DOI: 10.1159/000241097

Received: April 14, 2009
Accepted after revision: July 15, 2009
Published online: September 24, 2009

The Performance of Cochlin-Tomoprotein Detection Test in the Diagnosis of Perilymphatic Fistula

Tetsuo Ikezono[a] Susumu Shindo[a] Satomi Sekiguchi[b] Toshio Morizane[c]
Ruby Pawankar[a] Atsushi Watanabe[d] Masakazu Miura[e] Toshiaki Yagi[a]

[a]Department of Otorhinolaryngology, Nippon Medical School, [b]R&D and Business Development Segment, Mitsubishi Chemical Medience Corporation, Tokyo, [c]Department of Medicine, Kanagawa Dental College, Kanagawa, [d]Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, and [e]Department of Clinical Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa, Japan

Key Words
Diagnostic accuracy · Perilymphatic fistula · Hearing loss · Vertigo · Perilymph · *COCH* gene · Cochlin isoform · Cochlin tomoprotein · Human · Specificity · Sensitivity

Abstract
Background: Perilymphatic fistula (PLF), defined as an abnormal communication between the inner and middle ear, presents with a symptomatology of hearing loss and vestibular disorder that is indistinguishable from a number of other inner ear diseases. Methods of diagnosis remain controversial. We have previously shown that Cochlin-tomoprotein (CTP) is selectively detected in the perilymph. To establish a definite diagnostic test for PLF using CTP as a biochemical marker, we examined the diagnostic performance of the CTP detection test. ***Methods:*** CTP detection test was performed by Western blot using recombinant human CTP (rhCTP) as a spiked standard. We evaluated the specificity of the CTP detection test by testing non-PLF cases. To describe the limitations of the test, we tested samples from patients with middle ear infection. We also studied the stability of CTP protein by storing the samples at room temperature (25°C) or 4°C for 55 days. The effects of repeated freezing and thawing were also evaluated. Serially diluted perilymph was tested to find out the detection limit of CTP. ***Findings:*** We have established a standardized CTP detection test using high (0.27 ng) and low (0.13 ng) spiked standards of rhCTP in Western blotting. Middle ear lavages (MEL) from 54 of 55 non-PLF cases were negative in the CTP detection test, i.e. the specificity of the test is 98.2%. MEL from 43 out of 46 cases with chronic suppurative otitis media or middle ear cholesteatoma were negative for CTP. CTP is a stable protein and detection was not affected by the storage, or freezing and thawing. The detection limit of perilymph was 0.161 μl/lane in an average of 5 samples. ***Interpretation:*** CTP is a stable perilymph-specific protein, and this CTP detection could be the first clinically established diagnostic tool to detect PLF with a high specificity. PLF is surgically correctable by sealing the fistula. Appropriate recognition and treatment of PLF can improve hearing and balance in afflicted patients.

Introduction

Perilymphatic fistula (PLF) is defined as an abnormal communication between perilymph in the labyrinth and the middle ear. Representative symptoms of PLF are

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2009 S. Karger AG, Basel
1420–3030/10/0153–0168$26.00/0

Accessible online at:
www.karger.com/aud

Tetsuo Ikezono, MD, PhD
Department of Otorhinolaryngology, Nippon Medical School
1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603 (Japan)
Tel. +81 3 3822 2131, ext. 6746, Fax +81 3 5685 0830
E-Mail ctp16kdaplf@nms.ac.jp

sudden onset and/or progressive hearing loss with episodic attacks of vertigo; however, reports in the literature have suggested PLF to be putatively involved in a broad spectrum of hearing loss symptoms and balance disorders. PLF can be congenital or acquired, and in the latter it is associated with a traumatic or barotraumatic event resulting in labyrinthine fracture, iatrogenic artifacts (ear surgery), or a disruption of the membranes of the round and/or oval window(s) [Goodhill, 1971; House et al., 1991; Fitzgerald, 2001; Minor, 2003; Weber et al., 2003].

Unlike other causes of sensorineural hearing loss, PLF is surgically correctable by sealing the fistula. Appropriate recognition and treatment of PLF can improve hearing and balance, and hence the quality of life of the afflicted patients. However, despite extensive efforts to establish definitive methods for PLF detection, such as audiometry, electrocochleogram, electronystagmogram and radiological examination, there is as yet no widely accepted specific test for diagnosing PLF [Podoshin et al., 1994; Wall and Rauch, 1995; Nomura, 1994; Black et al., 1992]. The conventional definitive diagnosis of PLF depends on the direct visualization of the perilymphatic leak and fistula, but this is both difficult and highly subjective. The difficulty of making a definitive diagnosis of PLF has caused a long-standing debate regarding its prevalence, natural history, management, and even its very existence [Hughes et al., 1990; Schuknecht, 1992; Friedland and Wackym, 1999].

Previously, by proteomic analysis of inner ear proteins, we found very unique properties of cochlin (encoded by the *COCH* gene and mutated in DFNA9 – a hereditary form of hearing loss), which is expressed abundantly in the inner ear [Robertson et al., 1998; Ikezono et al., 2005; Robertson et al., 2006; Shindo et al., 2008]. We detected 3 cochlin isoforms, p63s, p44s and p40s, in the inner ear tissue and a short 16-kDa isoform named Cochlin-tomoprotein (CTP) in the perilymph [Ikezono et al., 2001, 2004]. Since cochlin was found to be highly specific to the inner ear [Robertson et al., 1994; Abe et al., 2003; Li et al., 2005], we tested the expression specificity of CTP in perilymph; CTP was indeed selectively expressed only in the perilymph, and not in CSF, saliva or serum [Ikezono et al., 2009]. In addition, we reported the molecular mechanisms that regulate the perilymph-specific expression of CTP [Sekine et al., 2009].

In order to establish CTP as a diagnostic marker of PLF, we standardized the CTP detection test using spiked standards of recombinant human CTP (rhCTP) in Western blotting. We evaluated the specificity of the CTP detection test by testing samples from non-PLF cases. To describe the limitations of the test, we evaluated the influence of middle ear infection on the test results. We also studied the stability of CTP protein when samples were stored at room temperature (25°C) or 4°C for as long as 55 days. The effects of repeated freezing and thawing were also evaluated. Serially diluted perilymph was tested to find out the detection limit of CTP. The present study showed that CTP could be the first clinically established biochemical marker to allow a definitive diagnosis of PLF-related hearing loss.

Methods

Standardization of the CTP Detection Test by Western Blot

For Western blot analysis, the rabbit polyclonal anti-CTP antibody (formerly anti-LCCL-C Ab) was prepared as previously described [Ikezono et al., 2004]. In brief, a 14-mer peptide (LSRWSASFTVTKGK) corresponding to residues 114–127 in the LCCL domain was used to generate the antibody. Rabbits were immunized by repeated subcutaneous injections of the KLH-coupled peptides. The serum was purified by a protein A column, followed by peptide-affinity chromatography. The specificity of the antibodies for the corresponding antigenic peptides was confirmed by dot blot analysis and a peptide absorption test (data not shown). The rhCTP was used as a spiked standard in the Western blot. The exact N- and C-terminal sequence of CTP is not yet known. However, a putative CTP sequence predicted from our previous study [Ikezono et al., 2004], located at positions 101–403 of the cDNA and corresponding to amino acid residues 32–132, was amplified by PCR from a human-expressed sequence tag clone, Image ID 27789 (Kurabo); rhCTP was produced using pCR/T7/TOPO/TA expression kits (Invitrogen).

Samples were loaded onto 15% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Membranes were blocked overnight at 4°C in 5% skimmed milk and 0.2% polyoxyethylene sorbitan (Tween-20) dissolved in PBS (pH 7.5). Membranes were then incubated in PBS containing 1% skimmed milk and 0.1% Tween-20 for 2 h at room temperature with the primary antibody (anti-CTP antibody) diluted at 1:1000. After washing with 0.1% Tween-20 in PBS, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Dako) diluted at 1:1000 in the same buffer used for the primary antibody reaction. They were washed again, and the reaction was developed with a chemiluminescence reaction kit (ECL advance, Amersham) and then analyzed by an image analyzer LAS-3000 (Fuji Film). Tests were performed and analyzed by well-trained personnel who did not have any information on the clinical background of the patients, to avoid any biased judgments. Test results were expressed as positive or negative by the presence or absence of the anti-CTP antibody reacting protein with the molecular weight that exactly matched the molecular weight of native CTP (16 kDa) on Western blotting.

Method of Sampling

In our previous study, we showed that CTP is selectively expressed in the perilymph, and not in samples of the body fluids, serum, CSF or saliva. The ultimate purpose of this test is to be able to detect the presence of leaked PLF in the middle ear cavity preoperatively in the outpatient clinic. We aimed at establishing an easy-to-perform sampling method. Samples were collected by lavaging the middle ear cavity 3–4 times with the same bolus of 0.3 ml saline and recovering the fluid, and these were defined as middle ear lavage (MEL). MEL was collected from non-PLF cases and those with suppurative otitis media or middle ear cholesteatoma. Samples were centrifuged at 1250 *g* for 1 min, and the supernatants were frozen and stored at –80°C until use; 16 μl MEL was mixed with 8 μl of 3 times concentrated sample buffer (0.188 M Tris buffer, 2.39 mM SDS, 30% glycerol, and 15% of 2-mercaptoethanol) for Western blot analysis.

To test the stability and detection limit of CTP, perilymph was collected from 5 cases of cochleostomy for cochlear implant surgery. We collected the leakage from the cochleostomy using a 27-gauge (0.22 mm internal diameter) blunt-end fine needle. All patients gave their full informed consent, and the study was approved by the Ethics Committee of Nippon Medical School.

Non-PLF Cases

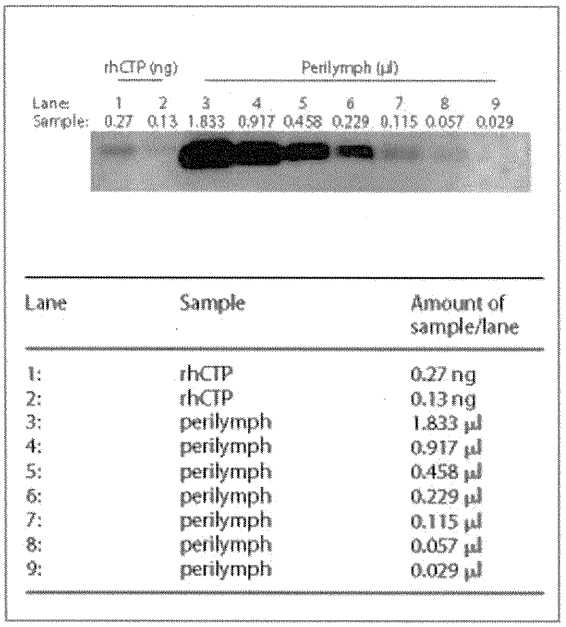
In order to evaluate the specificity of the CTP detection test, we examined MEL from non-PLF cases. In this study, we defined 'non-PLF' as those cases with otosclerosis (which had undergone stapedectomy), profound deafness (cochlear implant surgery) or conductive hearing loss (exploratory tympanotomy). We took MEL prior to the stapedectomy or cochleostomy, or prior to surgical treatment for conductive hearing loss. These cases did not have any symptoms or test results suggestive of PLF (including high-resolution temporal bone target CT scans and intraoperative findings, such as microscopic visualization of perilymph leakage and/or fistula). Patients who had revision stapedectomy, revision cochlear implantation, ossified cochlea or infection of the middle ear were excluded.

Effect of Middle Ear Infection on CTP Detection Test

It is well known that protein-rich samples, such as pus, can cause nonspecific signals on a Western blot. Therefore, we further clarified the influence of the infection in the middle ear on the test results. The MEL from surgically treated chronic suppurative otitis media cases (n = 12) and middle ear cholesteatoma cases (n = 34) were evaluated. None of these cases had any symptoms or test results suggestive of PLF.

Testing the Stability of CTP

In everyday clinical settings, collected samples may not be frozen immediately. We therefore evaluated if the results of the CTP detection test were affected by storage conditions that could lead to protein degradation. We tested diluted perilymph (1:20 with saline) kept at room temperature (25°C) or in a refrigerator at 4°C for 1, 2, 6, 8, 9, 12, 13, 15, 16, 19, 20, 23, 27, 34, 41, 48 or 55 days; 4 μl diluted saline was mixed with sample buffer (24 μl total volume) and 22 μl sample, i.e. 0.18 μl of perilymph/lane, was loaded on to the gel. In addition, MEL could be tested multiple times by Western blotting or by an alternative method to confirm the test results. We performed the CTP detection test of diluted perilymph after repeatedly freezing (–70°C) and thawing (25°C) for 10 times.



Lane	Sample	Amount of sample/lane
1:	rhCTP	0.27 ng
2:	rhCTP	0.13 ng
3:	perilymph	1.833 μl
4:	perilymph	0.917 μl
5:	perilymph	0.458 μl
6:	perilymph	0.229 μl
7:	perilymph	0.115 μl
8:	perilymph	0.057 μl
9:	perilymph	0.029 μl

Fig. 1. The detection limit of serially diluted perilymph samples using a standardized CTP detection test to define spiked standards. We loaded rhCTP as high and low spiked standard (lanes 1, 2) and serially diluted perilymph samples (lanes 3–9). When the intensity of the band in samples tested was below the high standard signal, the result was considered to be negative. The intensity of the band in lane 8 is below the high spiked standard (lane 1); thus, lane 8 was considered to be negative. The detection limit of CTP in the diluted perilymph (0.115 μl/lane; lane 7) is shown.

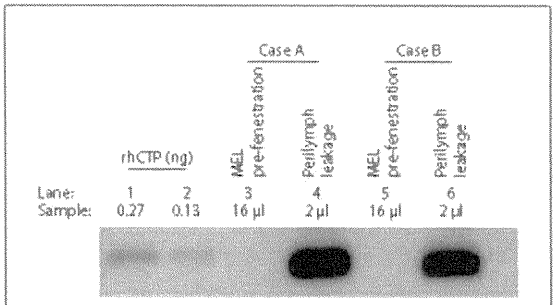
Detection Limit

Five serially diluted perilymph samples were tested independently to establish the detection limit of CTP. We mixed 4 μl perilymph with 28 μl saline and 16 μl of 3 times concentrated sample buffer. This mixture was serially diluted with sample buffer. Diluted samples were heated to 100°C for 10 min. Then 22 μl of these samples were loaded onto the gel and the volume of loaded perilymph samples was calculated as follows: 1.833, 0.917, 0.458, 0.229, 0.115, 0.057, 0.029 (μl/lane).

Results

Standardized CTP Detection System

As previously reported, the detection limit of the serially diluted rhCTP was between 0.27 and 0.13 ng/well. These 2 amounts of rhCTP were set as the high and low spiked standards, respectively, and were the amounts electrophoresed each time when we tested the samples



Lane	Sample	Amount of sample/lane
1:	rhCTP	0.27 ng
2:	rhCTP	0.13 ng
3:	Case A: MEL pre-fenestration	16 µl of MEL
4:	Case A: Perilymph leakage	2 µl of perilymph
5:	Case B: MEL pre-fenestration	16 µl of MEL
6:	Case B: Perilymph leakage	2 µl of perilymph

Fig. 2. The result of CTP detection from non-PLF cases and the perilymph (samples from 2 cochlear implant surgery cases). MEL taken prior to the fenestration and the perilymph leakage from the cochleostomy were subjected to the CTP detection test. MEL taken before fenestration did not have any signal, whereas CTP was detected at 16 kDa in perilymph samples.

Table 1. CTP detection in non-PLF samples

	Total	CTP positive	CTP negative
Prior to stapedectomy	35	1	34
Prior to cochleostomy	12	0	12
Exploratory tympanotomy	8	0	8
Total	55	1	54

Table 2. Effect of middle ear infection on CTP detection test

	Total	CTP positive	CTP negative
Chronic suppurative otitis media	12	1	11
Middle ear cholesteatoma	34	2	32
Total	46	3	43

(fig. 1). When a high standard was detected, we accepted the result; otherwise, samples were re-evaluated. When the intensity of the band in samples tested was below the high-standard signal, the result was considered to be negative. Low spiked standard was used to estimate of the protein transfer efficiency. The molecular weight of rhCTP exactly matched that of native CTP (16 kDa) on Western blot. Inter-assay and intra-assay reproducibility was tested and confirmed (data not shown).

CTP Detection from non-PLF Cases

MEL from 34 of 35 cases prior to stapedectomy, 12 of 12 cases prior to cochleostomy, and 8 of 8 cases during exploratory tympanotomy were negative for CTP. In total, 54 MEL from 55 non-PLF cases were negative for CTP (table 1); therefore, the specificity of the CTP detection test for the diagnosis of PLF is 98.2%.

Figure 2 shows the results of CTP detection from non-PLF cases and the perilymph. Samples of MEL taken prior to fenestration and the perilymph leakage from the cochleostomy of 2 cochlear implant surgery cases were subjected to the CTP detection test. MEL taken before fenestration did not have any signal, whereas CTP was detected at 16 kDa in perilymph samples.

Effect of Middle Ear Infection on the CTP Detection Test

MEL from 11 out of 12 cases with chronic suppurative otitis media and 32 of 34 cases of middle ear cholesteatoma were negative for CTP (table 2). Thus, the specificity of the CTP detection test is 93.5%.

Stability Test of CTP

We tested samples stored at 25°C or 4°C for 1, 2, 6, 8, 9, 12, 13, 15, 16, 19, 20, 23, 27, 34, 41, 48, 55 days. In the Western blot, CTP was detected in all 34 samples tested. The intensity of CTP signals did not change remarkably. After repeated freezing and thawing (10 times), the intensity of CTP signals did not change (data not shown). These results suggest that CTP is a stable protein, and the results of CTP detection test by Western blotting would not be altered by storage conditions within this rage.

Detection Limit of CTP

Five serially diluted perilymph samples were tested to show the detection limit. Detection limits were 0.229 µl/lane (2 samples) and 0.115 µl/lane (3 samples), which gives an average of 0.161 µl/lane (fig. 1). This detection limit could be useful in the clinical application of CTP as a diagnostic marker of PLF.

Discussion

We previously analyzed the expression of CTP in various human bodily fluids, including the serum, CSF, saliva and perilymph [Ikezono et al., 2009]. All bodily fluid samples, except the perilymph, were negative for CTP. These results strongly suggest that CTP is expressed specifically and exclusively in the perilymph, from amongst these 4 kinds of bodily fluids that may be present in a healthy or diseased middle ear, and that CTP can be considered to be a specific biochemical marker for PLF. Recently, we reported the molecular mechanisms that regulate the perilymph-specific expression of CTP [Sekine et al., 2009]. We performed RNA ligation-mediated amplification of cDNA ends (RLM-RACE) using RNA isolated from the inner ear and spleen of rats, which are known to express abundant cochlin mRNA. We detected a novel short mRNA (a spliced variant), which includes the LCCL domain. This short mRNA was detected in the inner ear, and not in spleen.

The conventional gold standard of PLF detection is the intraoperative microscopic visualization of perilymph leakage and fistula, which ostensibly confirms the existence of PLF. If the patient does not have PLF, leakage will not be detected. However, since the surgical procedure itself can induce seepage that accumulates in the concave-shaped round and oval window niches, this could be misinterpreted as perilymph leakage [Nomura, 1994; Friedland and Wackym, 1999]. The difficulty of making a definitive diagnosis of PLF has caused a long-standing debate regarding PLF [Hughes et al., 1990; Schuknecht, 1992; Friedland and Wackym, 1999].

The appropriate recognition and treatment of PLF can improve hearing and balance in the afflicted patients. Our ultimate goal has been to establish a clinical test to allow a definitive diagnosis of PLF using CTP as a biochemical marker. It should be a clinically useful and specific test for the 'preoperative' diagnosis of PLF, in order to avoid unnecessary exploratory surgery. At the same time, this method has to be applied to a variety of clinical scenarios in PLF, wherein the leakage could take place in the oval or round window, fractured bony labyrinth, or minor fissures [Kohut et al., 1986]. Moreover, the leakage could be intermittent, ongoing or could have ceased with the leaked perilymph pooled in the middle ear. Therefore, we used MEL for collecting the samples from the middle ear in which the sampling was easily performed in an outpatient setting, only by the conventional method of myringotomy under local anesthesia. Saline lavage should include all the perilymph from wherever the perilymph leaked out or became pooled.

Detection of the target protein in a Western blot is affected by the efficiency of protein transfer. Transfer efficiency depends on factors such as the composition of the gel, complete contact of the gel with the membrane, the position of the electrodes, the transfer time, size and composition of proteins, field strength and the presence of detergents. In the present study, we have standardized the CTP detection test through defining high and low spiked standards as 0.27 and 0.13 ng rhCTP, respectively. When a high standard was detected, we accepted the result; otherwise, samples were re-evaluated. When the intensity of the band in samples tested was below the high standard signal, the result was considered to be negative. The average detection limit of CTP in 5 serially diluted perilymph samples was 0.161 μl/lane. This means that the test can detect CTP if there is 3.3 μl perilymph in 0.3 ml MEL (amount of perilymph contained in the diluted sample of the detection limit: $0.161 \times 24/22 = 0.176$ μl; perilymph in the total MEL: $0.176 \times 300/16 = 3.3$ μl). This detection limit could be used in the clinical application of CTP as a diagnostic marker of PLF.

MEL should contain middle ear mucosal secretion and other substances normally expressed in the middle ear cavity. Since these substances may cause false-positive reactions to the antibody, we tested MEL from non-PLF cases. In this study, we defined 'non-PLF' as those cases with otosclerosis (who had undergone stapedectomy), profound deafness (cochlear implant surgery), or conductive hearing loss (exploratory tympanotomy). We took MEL prior to the stapedectomy or cochleostomy, or prior to surgical treatment of conductive hearing loss. None of these cases had any symptoms or test results suggestive of PLF (including high-resolution temporal bone target CT scans and intraoperative findings). We detected anti-CTP antibody reacting protein at 16 kDa in 1 otosclerosis case. The diagnostic performance of CTP detection test for the diagnosis of PLF was found to have a specificity of 98.2%. We are now trying to evaluate the sensitivity of the test by performing the CTP detection test in 'definite PLF cases', such as traumatic stapes injury.

There are limitations to this test. Analysis of MEL collected from patients with middle ear infections can give a false-positive result (as in this study), where the high protein concentration of the thick pus was the most likely cause. Specificity of CTP detection test decreases to 93.5% when testing infected ears. We have reported that CTP was not detectable in 28 serum samples [Ikezono et

al., 2009], and was not detected in multiple hemolyzed samples (data not shown). However, to ensure the accuracy of the test, MEL samples should ideally be kept frozen after removing the cells or tissue debris by the centrifuge to provide the minimum protein concentration.

Protein markers such as CTP may become degraded through the process of storage prior to the detection test or during the handling of the samples. The result of the test may vary if the marker is easily degradable protein. We have tested the stability of CTP by storing the diluted sample (1:20 with saline) at room temperature or at 4°C for 17 time points maximum of 55 days. CTP was detected in all 34 samples tested, without remarkable changes in the intensity of CTP signals. In addition, CTP was stable after repeated freezing (–70°C) and thawing (25°C) for 10 repetitions. CTP has enough stability in the various storage conditions in hospitals, and it is responsive to multiple measurements after thawing.

Conclusion

CTP is a stable perilymph-specific protein, for which we have established a standardized CTP detection test. This is the first clinically established diagnostic tool for the detection of PLF with a high specificity. In PLF, inner ear damage is affected by the speed, duration of the perilymph leakage, the site of the leakage and other biological factors. Hence, these patients' symptoms, physiological test results and outcomes of treatment are widely variable. Using this CTP detection test, a definitive diagnosis of PLF can be made and appropriate therapeutic options for this surgically correctable disease taken into consideration. Further studies will be needed to provide insight into the etiology, pathomechanisms, prevalence and natural history of PLF, and these may lead to the development of therapeutic and preventative strategies for acute, late-onset and debilitating neuro-otological problems.

Acknowledgments

We thank Dr. James Shelhamer, NIH, CCMD for a critical review of the manuscript. We thank Dr. Shin-Ichi Haginomori, Department of Otorhinolaryngology Osaka Medical College and Dr. Hideki Matsuda, Department of Otorhinolaryngology, Yokohama City University School of Medicine, for their generous cooperation. None of the persons acknowledged received any financial compensation for their work.

This study was supported by Health and Labor Sciences Research Grants in Japan (Research on Measures for Intractable Diseases, Researches on Sensory and Communicative Disorders) and a grant from the Ministry of Education, Culture, Sports, Science and Technology.

References

Abe S, Katagiri T, Saito-Hisaminato A, Usami S, Inoue Y, Tsunoda T, Nakamura Y: Identification of CRYM as a candidate responsible for nonsyndromic deafness, through cDNA microarray analysis of human cochlear and vestibular tissue. Am J Hum Genet 2003;72:73–82.

Black FO, Pesznecker S, Norton T, Fowler L, Lilly DJ, Shupert C, Hemenway WG, Peterka RJ, Jacobson ES: Surgical management of perilymphatic fistulas: a Portland experience. Am J Otol 1992;13:254–262.

Fitzgerald DC: Perilymphatic fistula and Meniere's disease: clinical series and literature review. Ann Otol Rhinol Laryngol 2001;110:430–436.

Friedland DR, Wackym PA: A critical appraisal of spontaneous perilymphatic fistulas of the inner ear. Am J Otol 1999;20:261–276.

Goodhill V: Sudden deafness and round window rupture. Laryngoscope 1971;81:1462–1474.

House JW, Morris MS, Kramer SJ, Shasky GL, Coggan BB, Putter JS: Perilymphatic fistula: surgical experience in the United States. Otolaryngol Head Neck Surg 1991;105:51–61.

Hughes GB, Sismanis A, House JW: Is there consensus in perilymph fistula management? Otolaryngol Head Neck Surg 1990;102:111–117.

Ikezono T, Omori A, Ichinose S, Pawankar R, Watanabe A, Yagi T: Identification of the protein product of the Coch gene – hereditary deafness gene – as the major component of bovine inner ear protein. Biochim Biophys Acta 2001;1:258–265.

Ikezono T, Shindo S, Ishizaki M, Li L, Tomiyama S, Takumida M, Pawankar R, Watanabe A, Saito A, Yagi T: Expression of cochlin in the vestibular organ of rats. ORL J Otorhinolaryngol Relat Spec 2005;67:252–258.

Ikezono T, Shindo S, Li L, Omori A, Ichinose S, Watanabe A, Kobayashi T, Pawankar R, Yagi T: Identification of a novel Cochlin isoform in the perilymph: insights to Cochlin function and the pathogenesis of DFNA9. Biochem Biophys Res Commun 2004;314:440–446.

Ikezono T, Shindo S, Sekiguchi S, Charuk Hanprasertpong, Li L, Pawankar R, Morizane T, Baba S, Koizumi Y, Sekine K, Watanabe A, Komatsuzaki A, Murakami S, Kobayashi T, Miura M, Yagi T: Cochlin-tomoprotein (CTP), a novel perilymph-specific protein and a potential marker for the diagnosis of perilymphatic fistula. Audiol Neurootol 2009;14:338–344.

Kohut RI, Hinojosa R, Budetti JA: Perilymphatic fistula: a histopathologic study. Ann Otol Rhinol Laryngol 1986;95:466–471.

Li L, Ikezono T, Watanabe A, Shindo S, Pawankar R, Yagi T: Expression of full-length Cochlin p63s is inner ear specific. Auris Nasus Larynx 2005;32:219–223.

Minor LB: Labyrinthine fistulae: pathobiology and management. Curr Opin Otolaryngol Head Neck Surg 2003;11:340–346.

Nomura Y: Perilymph fistula: concept, diagnosis and management. Acta Otolaryngol Suppl 1994;514:52–54.

Podoshin L, Fradis M, Ben-David J, Berger SI, Feiglin H: Perilymphatic fistula: the value of diagnostic tests. J Laryngol Otol 1994;108:560–563.

Robertson NG, Khetarpal U, Gutierrez-Espeleta GA, Bieber FR, Morton CC: Isolation of novel and known genes from a human fetal cochlear cDNA library using subtractive hybridization and differential screening. Genomics 1994;23:42–50.

Robertson NG, Lu L, Heller S, Merchant SN, Eavey RD, McKenna M, Nadol JB, Miyamoto RT, Linthicum FH, Lubianca Neto JP, Hudspeth AJ, Seidman CE, Morton CC, Seidman JG: Mutations in a novel cochlear gene cause DFNA9, a human nonsyndromic deafness with vestibular dysfunction. Nat Genet 1998; 20:299–303.

Robertson NG, Cremers CW, Huygen PL, Ikezono T, Krastins B, Kremer H, Kuo SF, Liberman MC, Merchant SN, Miller CE, Nadol JB, Sarracino DA, Verhagen WI, Morton CC: Cochlin immunostaining of inner ear pathologic deposits and proteomic analysis in DFNA9 deafness and vestibular dysfunction. Hum Mol Genet 2006; 15:1071–1085.

Schuknecht HF: Myths in neurotology. Am J Otol 1992;13:124–126.

Sekine K, Ikezono T, Matsumura T, Shindo S, Watanabe A, Li L, Pawankar R, Nishino T, Yagi T: Expression of cochlin mRNA splice variants in the inner ear. Audiol Neurotol 2010;15:88–96.

Shindo S, Ikezono T, Ishizaki M, Sekiguchi S, Mizuta K, Li L, Takumida M, Pawankar R, Yagi T: Spatiotemporal expression of Cochlin in the inner ear of rats during postnatal development. Neurosci Lett 2008;444:148–152.

Wall C, Rauch SD: Perilymph fistula pathophysiology. Otolaryngol Head Neck Surg 1995; 112:145–153.

Weber PC, Bluestone CD, Perez B: Outcome of hearing and vertigo after surgery for congenital perilymphatic fistula in children. Am J Otolaryngol 2003;24:138–142.

Acta Oto-Laryngologica, 2011; 131: 937–944

informa
healthcare

ORIGINAL ARTICLE

Cochlin-tomoprotein (CTP) detection test identifies traumatic perilymphatic fistula due to penetrating middle ear injury

TETSUO IKEZONO[1], SUSUMU SHINDO[1], KUWON SEKINE[1], KYOKO SHIIBA[1], HAN MATSUDA[1], KAORU KUSAMA[1], YASUO KOIZUMI[1], KAZUKI SUGIZAKI[1], SATOMI SEKIGUCHI[2], RYOHEI KATAOKA[1], RUBY PAWANKAR[1], SHUNKICHI BABA[1], TOSHIAKI YAGI[3] & KIMIHIRO OKUBO[1]

[1]*Department of Otorhinolaryngology, Nippon Medical School, Tokyo,* [2]*R&D and Business Development Segment, Mitsubishi Chemical Medience Corporation, Tokyo and* [3]*Chiba Kashiwa Rehabilitation College, Chiba, Japan*

Abstract

Conclusions: The cochlin-tomoprotein (CTP) detection test can be used to make a definite, objective diagnosis of traumatic perilymphatic fistula (PLF), and therefore offers valuable information on patient selection for surgical treatment. *Objectives:* Penetrating middle ear injury can cause traumatic PLF, which is a surgically treatable otologic emergency. Recently, we have reported on CTP, a novel perilymph-specific protein. The purpose of this study was to determine if the CTP detection test is useful for the diagnosis of traumatic PLF. *Methods:* This was a prospective study of CTP detection in penetrating middle ear injury cases with tympanic membrane perforation and hearing loss. *Results:* A total of seven individuals were included in this study. CTP was detected in three of four cases with posterosuperior quadrant perforation of the tympanic membrane. In one of these three cases, even though the high resolution CT scan was not suggestive of PLF and the perilymph leakage could not be visualized intraoperatively, the CTP detection test was able to detect PLF. In two cases, the preoperative positive test results enabled us to make a diagnosis of PLF and a decision for surgical treatment. CTP was not detected in the cases with anterior or inferior tympanic membrane perforation.

Keywords: *Middle ear trauma, hearing loss, COCH gene, cochlin isoform*

Introduction

Penetrating trauma of the middle ear can occur as a result of the introduction of a variety of foreign bodies, for example, a knitting needle, hairpin, bullet, twig of a tree, ear pick, cotton-tipped applicators, stone, and iatrogenic damage [1–9]. Trauma associated with the ossicular chain is a frequent finding in these cases and carries a good prognosis. Inner ear damage, however, while less frequent, exposes the patient to permanent disabilities in hearing and vestibular function.

Etiologies of inner ear damage due to penetrating middle ear injury include labyrinthine concussion, acoustic trauma, and perilymphatic fistula (PLF) [10–12]. Among these conditions, PLF is an otologic emergency and is surgically correctable by sealing the fistula. Appropriate recognition and treatment of PLF can improve hearing and balance in the afflicted patients; otherwise, permanent deafness may result [2]. In the absence of high resolution CT scan (HRCT) findings suggestive of PLF, such as otic capsule fracture, stapes luxation or pneumolabyrinth, it is difficult to make a definite, objective diagnosis of PLF.

Recently, we reported our findings on cochlin-tomoprotein (CTP), a novel perilymph-specific protein. By proteomic analysis of inner ear proteins, certain unique properties of cochlin isoforms were discovered. We detected three cochlin isoforms, p63s, p44s, and p40s, in the inner ear tissue and a short

Correspondence: Tetsuo Ikezono MD PhD, Department of Otorhinolaryngology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. Tel: +81 3 3822 2131, Tel: ext. 6746. Fax: +81 3 5685 0830. E-mail: ctp16kdapt@nms.ac.jp

(Received 2 February 2011; accepted 20 March 2011)

ISSN 0001-6489 print/ISSN 1651-2251 online © 2011 Informa Healthcare
DOI: 10.3109/00016489.2011.575795

16 kDa isoform, named cochlin-tomoprotein (CTP), in the perilymph [13,14]. CTP was selectively expressed only in the perilymph, not in cerebrospinal fluid (CSF), saliva or serum on testing 65, 60, 29, and 28 samples, respectively [14 16].

We have reported a standardized CTP detection test for the diagnosis of PLF, using a spiked standard of recombinant human (rh)CTP on Western blot [17].

Using this diagnostic method, we detected CTP in three of seven cases of penetrating middle ear injury. To the best of our knowledge this is the first definite diagnosis of traumatic PLF using CTP as the diagnostic marker.

Material and methods

Study design and participants

The participants in this investigation were derived from the patients who had been consecutively referred to the first author's outpatient clinic. Enrolment took place from June 2003 to December 2007, and patients were included based on a history of insertion of an object into the middle ear, tympanic membrane perforation, and accompanying hearing loss with or without balance disorders. A total of seven patients were enrolled in this study. An ear pick caused injury while removing cerumen (a common practice in Japan) in cases 1 5 and 7. A screwdriver was accidentally inserted into the ear of case 6.

We performed physiological testing by pure tone audiometry and nystagmoscopy with infrared radiation. HRCT was performed in all cases. In this study, HRCT findings suggestive of PLF are defined as otic capsule fracture, pneumolabyrinth (vestibulum or cochlea), ossicular dislocation of the stapes or incudostapedial joint. Fistula test (applying positive and negative pressure to the middle ear) was not performed to avoid further damage of the inner ear.

Standardized CTP detection test by Western blot

Samples were tested by the standardized CTP detection test [17] with minor modifications. For Western blot analysis, the rabbit polyclonal anti-CTP antibody (formerly anti-LCCL-C Ab) was prepared as described previously. In brief, a 14-mer peptide (LSRWSASFTVTKGK) corresponding to residues 114 127 in the LCCL domain was used as an antigenic peptide to generate antibody. The rhCTP was used as a spiked standard on Western blot. A putative CTP sequence predicted from our previous study, the 101 to 403 positions of the cDNA corresponding to amino acid residues 32 132, was amplified by PCR from a human expressed sequence tag clone, IMAGE ID 27789 (Kurabo, Japan). rhCTP was produced using pCR/T7/TOPO/TA Expression Kits (Invitrogen).

Samples were loaded onto 15% polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked overnight at 4°C in 5% skim milk and 0.2% polyoxyethylenesorbitan (Tween-20) dissolved in phosphate-bufferd saline (PBS; pH 7.5). Membranes were then incubated in PBS containing 1% skim milk and 0.1% Tween-20 for 2 h at room temperature with the primary antibody (anti-CTP antibody) diluted at 1:2000. After washing with 0.05% Tween-20 in PBS, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Dako, Japan) diluted at 1:10 000 in the same buffer as used for the primary antibody reaction. They were washed again and the reaction was developed with a chemiluminescence reaction kit (ECL Advance, Amersham), and then analyzed by an image analyzer LAS-3000 (Fuji Film, Japan). Tests were performed and analyzed by well-trained personnel who did not have any information on the clinical background of the patients.

The detection limit of the serially diluted rhCTP was between 0.27 and 0.13 ng/well. These two amounts of rhCTP were set as the high and low spiked standards, respectively, and were the amounts electrophoresed each time when we tested the samples. When the intensity of the band in the samples tested was below the high standard signal, the result was considered to be negative. The low spiked standard was used to estimate the protein transfer efficiency.

Test results were expressed qualitatively (positive or negative) by the presence or absence of the anti-CTP antibody reacting protein with the molecular weight, which exactly matched the molecular weight of native CTP (16 kDa) on Western blot. The result of the CTP detection test was provided to the clinic within 4 days of sampling.

Performance of the CTP detection test

The performance of the CTP detection test was described previously [17]. In brief, the detection limit of perilymph was 0.161 µl per lane in an average of five samples. This means that the test can detect CTP if there is 3.3 µl of perilymph in 0.3 ml of middle ear lavage (MEL) (explained in detail by Ikezono et al. [17]). We also studied the stability of CTP protein when samples were stored at room temperature (25°C) or 4°C for as long as 55 days. The effect of repeated freezing and thawing was also evaluated. The

results showed that CTP is a stable protein and detection is not affected by the storage condition or repeated freezing and thawing [16].

We also reported the specificity of the CTP detection test by testing non-PLF cases [17]. We defined 'non-PLF' as those cases with otosclerosis (that had undergone stapedectomy), profound deafness (cochlear implant surgery), or conductive hearing loss (exploratory tympanotomy) without any sign of inflammation or infection. We took MEL before the stapedectomy or cochleostomy, or before surgical treatment of conductive hearing loss. The MEL in 54 of 55 non-PLF cases was negative with the CTP detection test, i.e. the specificity of the test was found to be 98.2%. To further elucidate the limitations to this test, we analyzed the MEL collected from patients with middle ear infections, which can give a false-positive result. The MEL in 43 of 46 cases with chronic suppurative otitis media or middle ear cholesteatoma was negative for CTP. The specificity of the CTP detection test decreases to 93.5% when applied to inflamed and infected ears. The high protein concentration of the thick pus present with infection was the most likely cause. In the present investigation inflamed ears were studied, so the specificity is thought to be due to the latter. These findings indicate that CTP can be considered a marker for the diagnosis of PLF.

Sampling method

We aimed to establish an easy-to-perform sampling method. Samples were collected by lavaging the middle ear cavity four times with the same bolus of 0.3 ml of saline and recovering the fluid. This was defined as middle ear lavage (MEL). In the outpatient clinic, MEL was collected through a perforated tympanic membrane (cases 3 7), or at myringotomy in case 2 whose traumatic tympanic membrane perforation had been closed during the 27 day period between the onset and the first visit to our clinic. MEL was also collected during surgery in cases 1 3. Hence, in cases 2 and 3, MEL was collected twice (before and during operation) and we could test whether the leakage continued during these two sampling time points. Samples were centrifuged at 1250 *g* for 1 min and the supernatants were frozen and stored at 80°C until use. Sixteen µl of MEL were mixed with 8 µl of three times concentrated sample buffer (150 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.3% bromophenol blue, 300 mM DTT) for Western blot analysis. All patients gave their full informed consent and the study was approved by the Ethics Committee of Nippon Medical School.

Therapeutic procedure

All cases except for cases 4 and 5 received tapering doses of oral prednisolone (60 30 mg) down to 5 mg in an 8 14 day period depending on the patient's general condition and any other systemic illnesses. Surgery (exploratory tympanotomy and PLF repair surgery) was performed under general anesthesia in cases 1 3, in order to inspect and seal the potential perilymph leak from the fistula and to reconstruct the ossicular chain (explained in detail in the Results section). Conservative treatment (medication and bedrest) was selected in cases 4 7 and tympanic membrane perforation was closed with patching.

Evaluation of the therapeutic outcome

Pure tone audiogram was obtained before and after treatment. The pure tone average (PTA) hearing thresholds of bone conduction and air conduction were determined by four frequencies (500, 1000, 2000, and 4000 Hz). The patients were followed up regularly and PTA was performed until complete recovery, or for more than 2 months (average 8 months). Hearing improvement after treatment was gauged by PTA of air conduction using the following criteria; CR, complete recovery (final audiogram ≤20 dB HL, or improvement to the same degree of hearing as the unaffected ear); RI, remarkable improvement (improvement >30 dB HL); I, improvement (improvement of 10 30 dB HL); NC, no change or deterioration (improvement <10 dB HL) (modified from Kanzaki et al. [18]). Successful hearing improvement was defined as complete recovery or remarkable improvement.

Results

The patient information and test results (age, sex, affected side, HRCT suggestive of PLF, CTP test results, onset to MEL sampling (days), site of perforation, pre- and post-treatment PTA, air bone gap, hearing improvement, nystagmus) are summarized in Table I. Representative Western blot analysis of CTP detection in MEL from cases 2, 3, and 7 are shown in Figures 1 and 2. None of these cases presented with infection in the middle ear. The contralateral ear exhibited normal hearing in all cases. Successful hearing improvement (complete recovery or remarkable improvement) was achieved in all cases except in case 7. Vertigo, as a sign of inner ear damage, was observed in cases 1 4, and disappeared in all cases during the follow-up period. In cases 5 7, perforation was not in the posterosuperior quadrant (PSQ) and CTP was negative (Figure 2), suggesting

Table I. Summary of patient information and test results.

Parameter	Case no.						
	1	2	3	4	5	6	7
Age	54	29	7	27	28	39	48
Sex	F	F	M	F	F	M	M
Affected side	L	L	R	L	R	L	L
HRCT suggestive of PLF	+	+					
CTP test results	+	+, +	+, +				
Onset to MEL sampling (days)	8	27, 35	2, 9	1	4	1	1
Site of perforation	PSQ	PSQ	PSQ	PSQ	Inferior	Anterior	Anterior
Pretreatment bone PTA	33	20	5	35	14	20	35
Pretreatment air PTA	65	70	20	56	40	58	56
Pretreatment ABG	32	50	15	21	26	38	21
Post-treatment Bone PTA	20	19	3	24	11	15	28
Post-treatment air PTA	23	33	10	36	15	19	36
Post-treatment ABG	3	14	8	13	4	4	9
Hearing improvement	RI	RI	CR	CR	CR	CR	I
Nystagmus	S	S, P	S	S, P, BPPV		P	S

Middle ear lavage (MEL) was collected only once except in cases 2 and 3, where MEL was taken twice preoperatively and during the operation. +, +, CTP detection test was performed twice and both results were positive. ABG, air bone gap; BPPV, benign paroxysmal positional vertigo; CR, complete recovery; CTP, cochlin-tomoprotein; HRCT, high resolution CT scan; I, improvement; MEL, middle ear lavage; NC, no change or deterioration; P, rotatory and horizontal, paretic; PLF, perilymphatic fistula; PSQ, posterosuperior quadrant; PTA, pure tone average; RI, remarkable improvement; S, rotatory and horizontal, stimulatory.

that the inner ear damage could be attributed to non-PLF causes. Therefore, in the following section we focus on discussing cases 1–4.

Case 1 had mixed hearing loss and developed severe rotatory vertigo 36 h after the traumatic event. HRCT exhibited pneumolabyrinth in the vestibulum and dislocation of the incudostapedial joint. The stapedial footplate seemed to be in an intact position. The intraoperative findings showed that the stapes annular ligament was lax with excess mobility of the stapes. The stapedial footplate was slightly elevated (probably an ear pick with a spoon-like tip lifted the stapes when the patient pulled it out after penetration, as shown by Kobayashi and Gyo [1]). Perilymph leakage was detected, the stapes was replaced after coverage of the oval window with fascia, and the ossicular chain was reconstructed. The MEL performed intraoperatively was positive for CTP. The PTA demonstrated remarkable hearing improvement after surgery (Figure 3a).

Case 2 was referred to our clinic because of sustained conductive hearing loss and vertigo for 1 month post injury. HRCT revealed stapes luxation and air in the vestibulum. MEL was taken on the 35th day and CTP was detected (Figure 1), suggesting that the perilymph had been continuously leaking out and/or had pooled in the middle ear cavity for 35 days without remarkable exacerbation of the sensorineural component of hearing loss. During surgery, perilymph leakage was detected and the stapes was smoothly taken out through the oval window. The ossicular chain was reconstructed with a Teflon piston and the oval window was covered with fascia to seal the perilymphatic leakage. MEL taken intraoperatively was positive for CTP. The PTA demonstrated remarkable hearing improvement post surgery (Figure 3b).

Case 3 had severe rotatory vertigo 10 h after the traumatic event and mild conductive hearing loss (Figure 3c). HRCT did not exhibit any findings suggestive of PLF. MEL taken on the second day after the event showed positive CTP expression (Figure 1). Due to the traumatic PLF and the possibility that the hearing might worsen, surgery was performed on the ninth day. Intraoperatively, the stapes annular ligament was lax with excess mobility of the stapes, and there was slight dislocation of the incudostapedial joint. Granulation was observed around the stapes without identifiable leakage of perilymph. MEL taken intraoperatively was positive for CTP. The oval window was sealed with fascia and fibrin glue and the incudostapedial joint was reconstructed. The audiometric findings were normal and there was no vertigo post surgery (Figure 3c). The

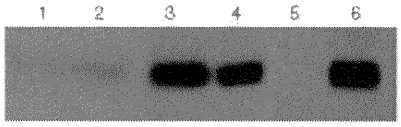


Figure 1. The middle ear lavage (MEL) samples collected preoperatively from cases 2 and 3 were tested by Western blot using anti-cochlin-tomoprotein (CTP) antibody to detect CTP. The MEL from both cases 2 and 3 shows a positive result. Lane 1, rhCTP low spiked standard (0.13 ng/well), CTP-negative; 2, rhCTP high spiked standard (0.27 ng/well), CTP-positive; 3, 16 µl of MEL from case 3, CTP-positive; 4, 2 µl of perilymph (positive control), CTP-positive; 5, blank, CTP-negative; 6, 16 µl of MEL from case 2, CTP-positive.

MEL samples taken preoperatively and at surgery were both positive for CTP, indicating that the perilymph was still leaking out and/or had pooled in the middle ear cavity for 8 days.

In case 4, perforation was in the PSQ, but the HRCT finding was not suggestive of PLF and the CTP was negative. The vertigo and hearing loss resolved with conservative treatment.

Discussion

Penetrating middle ear injuries are reportedly commonly caused by the insertion of various kinds of foreign objects. These foreign objects result in perforation of the tympanic membrane, ossicular dislocation or, most seriously, direct damage to the stapes. In Japan, removing the cerumen by an ear pick is a common practice, and there were 20 cases of penetrating middle ear injuries reported over a 10-year period [1]. The current report comprises the largest number of middle ear injury cases (seven cases) in a single study.

We would like to emphasize that even though it is not a common trauma, penetrating middle ear injury is a very important condition for understanding the pathomechanisms of traumatic inner ear injuries. An improved understanding will enable therapeutic and preventative strategies for acute, late onset, and debilitating neuro-otological problems of patients with similar pathomechanics, such as temporal bone fracture, pneumolabyrinth or iatrogenic injuries during otologic surgery.

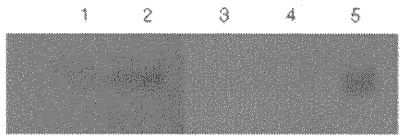


Figure 2. The middle ear lavage (MEL) sample from case 7 was tested by Western blot using anti-cochlin-tomoprotein (CTP) antibody to detect CTP, which shows a negative result. Lane 1, rhCTP low spiked standard (0.13 ng/well), CTP-negative; 2, rhCTP high spiked standard (0.27 ng/well), CTP-positive; 3, blank, CTP-negative; 4, 16 µl of MEL from case 7, penetrating middle ear injury, CTP-negative; 5, 0.25 µl of perilymph (positive control), CTP-positive.

Symptoms commonly associated with this type of injury include acute hearing loss, vertigo, tinnitus, and pressure sensation in the affected ear. The severity of the injury was first analyzed by the symptoms and test results such as HRCT findings, audiogram, and nystagmus. Inner ear damage can have various etiologies, such as labyrinthine concussion, acoustic trauma or PLF [10–12]. Conservative treatment is adequate for labyrinthine concussion and acoustic trauma, but traumatic PLF is an otologic emergency that may require surgical treatment. Appropriate recognition and treatment of PLF is especially important and it can improve hearing and balance, and hence the quality of life of the afflicted patients.

However, differential diagnosis among these various etiologies is not a simple task. This has led to a series of research efforts to identify an endogenous marker of perilymph [14]. Previously beta2-transferrin was thought to be a marker; however, a more recent study showed that, because of the relative amount of serum and perilymph in a mixed sample, electrophoretic separation of the transferrin variant might not be diagnostic [19]. As explained in the Methods section, we have shown that CTP was selectively detected in the perilymph and established a standardized CTP detection test for the diagnosis of PLF, using a spiked standard of rhCTP on Western blot [15–17]. We have reported the performance of the CTP detection test, including the specificity of the test and the stability of the CTP protein.

In the present study, we have shown the usefulness of the CTP detection test for the diagnosis of traumatic PLF in the clinical setting. In four middle ear trauma cases suspected to have stapes injury due to the symptoms and the site of perforation (i.e. the PSQ), three cases had a positive CTP detection test (cases 1–3). When the site of perforation was in the PSQ and HRCT detected pneumolabyrinth, a diagnosis of traumatic PLF due to the direct injury to the stapes was made, as in cases 1 and 2. Even HRCT was not clearly indicative of PLF, since it was the CTP detection test that enabled the detection of PLF in case 3. If the site of perforation and HRCT findings are not suggestive of PLF, a negative CTP result can thus help to exclude PLF, as in cases 5–7.

The conventional gold standard for PLF detection is the intraoperative microscopic visualization of perilymph leakage and fistula [17]. By this intraoperative microscopic visualization, a definitive diagnosis of

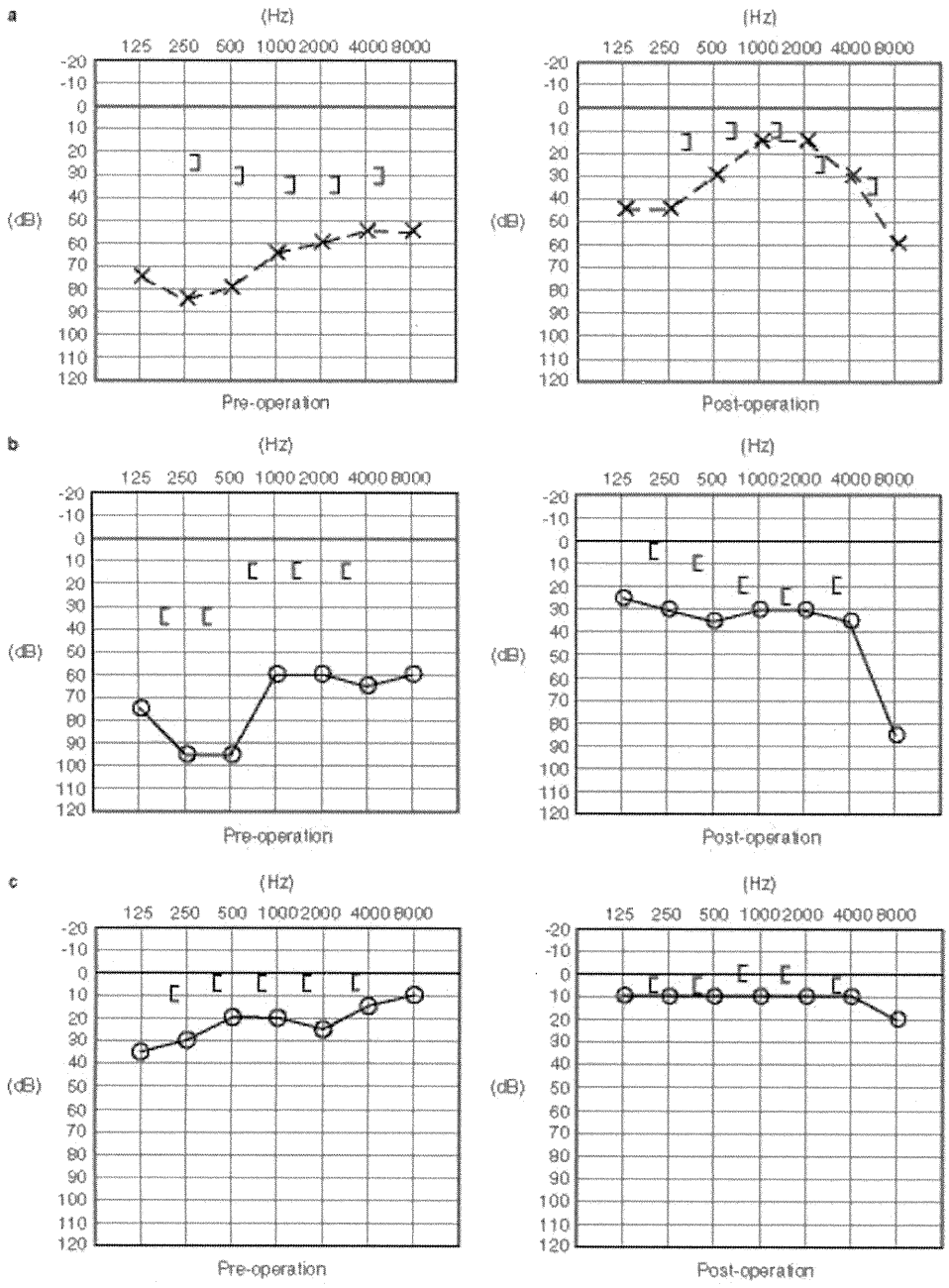


Figure 3. Pre- and post-treatment pure tone audiograms of case 1 (a), case 2 (b), and case 3 (c), with positive CTP detection test results.

traumatic PLF was made in both cases 1 and 2, and the CTP positive result confirmed this. In case 3, the oval window fistula and leakage were not apparent, even though the annular ligament was identified as lax with excess mobility. Even in this type of ambiguous condition, the CTP detection test was able to clearly identify PLF. In these cases with a positive CTP test result, severe rotatory vertigo was one of the main complaints. Even if the hearing loss was stable or had not deteriorated remarkably, the presence of

vestibular symptoms is important for an accurate diagnosis.

The preoperative CTP detection test was valuable for the decision as to the need for surgery in cases 2 and 3. In chronic cases such as case 2, the key question is whether the perilymph leakage persists or not due to spontaneous healing with granulation formation in the oval window. When the leakage ceases, conservative treatment is indicated. When the leakage persists, surgical treatment (sealing the fistula and reconstruction of ossicular chain) is needed to prevent inner ear infection, meningitis or further deterioration of the inner ear dysfunction. On the other hand, with mild traumatic PLF as in case 3, sealing the damaged annular ligament can be performed without serious complications.

We were able to achieve improvement in both hearing and vestibular symptoms in cases 1 3. However, surgical treatment is still a controversial issue in these types of stapes injury. Hearing and vestibular function vary after surgical treatment due to the initial degree of inner ear damage caused by the injury, and the additional effect caused by the surgical procedure itself, which may further damage the inner ear.

Conclusion

The CTP detection test enabled a definite diagnosis of traumatic PLF among penetrating middle ear injury cases. These PLF cases, which were definitely diagnosed with the positive CTP test results, revealed the patient symptoms and physiological test results to be widely variable. The inner ear damage may be dependent upon the rapidity of onset, duration of the perilymph leakage, the site of the leakage, and other biological factors. Using this CTP detection test, a definitive diagnosis of PLF can be made and appropriate therapeutic options for this surgically correctable disease can be taken into consideration. Further studies will be needed to provide insight into the etiology and pathomechanisms, and such insight may lead to the development of therapeutic and preventative strategies for acute, late onset, and debilitating neuro-otological problems.

Acknowledgments

We thank Dr Shin-Ichi Haginomori, Department of Otorhinolaryngology Osaka Medical College, Dr Hideki Matsuda, Department of Otorhinolaryngology, Yokohama City University School of Medicine, and Dr Hiroshi Ogawa, Department of Otorhinolaryngology, Fukushima Medical University, for their generous cooperation. This study was supported by Health and Labor Sciences Research Grants in Japan (Research on Measures for Intractable Diseases, Researches on Sensory and Communicative Disorders), a grant from the Ministry of Education, Culture, Sports, Science and Technology. Nippon Medical School and Mitsubishi Chemical Corporation have Japanese patent on this test. Nippon Medical School has Chinese, Australian, European, and US patent. None of the authors have received any financial compensation for their work.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Kobayashi T, Gyo K. Earpick injury of the stapes. Am J Otolaryngol 2000;21:340 3.
[2] Vanderstock L, Vermeersch H, De Vel E. Traumatic luxation of the stapes. J Laryngol Otol 1983;97:533 7.
[3] Herman P, Guichard JP, Van den Abbeele T, Tan CT, Bensimon JL, Marsinowski R, et al. Traumatic luxation of the stapes evidenced by high-resolution CT. AJNR Am J Neuroradiol 1996;17:1342 4.
[4] Yamasoba T, Amagai N, Karino S. Traumatic luxation of the stapes into the vestibule. Otolaryngol Head Neck Surg 2003; 129:287 90.
[5] Lao WW, Niparko JK. Assessment of changes in cochlear function with pneumolabyrinth after middle ear trauma. Otol Neurotol 2007;28:1013 17.
[6] Hatano A, Rikitake M, Komori M, Irie T, Moriyama H. Traumatic perilymphatic fistula with the luxation of the stapes into the vestibule. Auris Nasus Larynx 2009;36:474 8.
[7] Snelling JD, Bennett A, Wilson P, Wickstead M. Unusual middle-ear mischief: trans-tympanic trauma from a hair grip resulting in ossicular, facial nerve and oval window disruption. J Laryngol Otol 2006;120:793 5.
[8] Ederies A, Yuen HW, Chen JM, Aviv RI, Symons SP. Traumatic stapes fracture with rotation and subluxation into the vestibule and pneumolabyrinth. Laryngoscope 2009;119:1195 7.
[9] Meriot P, Veillon F, Garcia JF, Nonent M, Jezequel J, Bourjat P, et al. CT appearances of ossicular injuries. Radiographics 1997;17:1445 54.
[10] Schuknecht HF. Mechanism of inner ear injury from blows to the head. Ann Otol Rhinol Laryngol 1969;78: 253 62.
[11] Fitzgerald DC. Head trauma: hearing loss and dizziness. J Trauma 1996;40:488 96.
[12] Ohlemiller KK. Recent findings and emerging questions in cochlear noise injury. Hear Res 2008;245:5 17.
[13] Ikezono T, Omori A, Ichinose S, Pawankar R, Watanabe A, Yagi T. Identification of the protein product of the Coch gene hereditary deafness gene as the major component of bovine inner ear protein. Biochim Biophys Acta 2001;3:258 65.
[14] Ikezono T, Shindo S, Li L, Omori A, Ichinose S, Watanabe A, et al. Identification of a novel Cochlin isoform in the perilymph: insights to Cochlin function and the pathogenesis of DFNA9. Biochem Biophys Res Commun 2004; 6:440 6.

[15] Ikezono T, Shindo S, Sekiguchi S, Hamprasertpong C, Li L, Pawankar R, et al. Cochlin-tomoprotein (CTP), a novel perilymph-specific protein and a potential marker for the diagnosis of perilymphatic fistula. Audiol Neurotol 2009; 14:338–44.

[16] Ikezono T, Sugizaki K, Shindo S, Sekiguchi S, Pawankar R, Baba S, et al. Temporal analysis of profuse fluid leakage (gusher) from cochleostomy. Acta Otolaryngol 2010;130: 881–7.

[17] Ikezono T, Shindo S, Sekiguchi S, Morizane T, Pawankar R, Watanabe A, et al. The performance of CTP detection test for the diagnosis of perilymphatic fistula. Audiol Neurootol 2009;15:168–74.

[18] Kanzaki J, Inoue Y, Ogawa K, Fukuda S, Fukushima K, Gyo K, et al. Effect of single-drug treatment on idiopathic sudden sensorineural hearing loss. Auris Nasus Larynx 2003;30:123–7.

[19] Rauch SD. Transferrin microheterogeneity in human perilymph. Laryngoscope 2000;110:545–52.

Acta Oto-Laryngologica, 2010; Early Online, 1–7

informa
healthcare

ORIGINAL ARTICLE

CTP (Cochlin-tomoprotein) detection in the profuse fluid leakage (gusher) from cochleostomy

TETSUO IKEZONO[1], KAZUKI SUGIZAKI[1], SUSUMU SHINDO[1], SATOMI SEKIGUCHI[2], RUBY PAWANKAR[1], SHUNKICHI BABA[1] & TOSHIAKI YAGI[1]

[1]*Department of Otorhinolaryngology, Nippon Medical School and* [2]*R&D and Business Development Segment, Mitsubishi Chemical Medience Corporation, Tokyo, Japan*

Abstract

Conclusions: By testing 125 samples, we confirmed that Cochlin-tomoprotein (CTP) is present in the perilymph, not in cerebrospinal fluid (CSF). Perilymph and CSF exist in two distinct compartments, even in the case of a malformed inner ear with a bony defect in the lamina cribrosa, as described here. Cochleostomy might have suddenly decreased the perilymph pressure, allowing the influx of CSF into the inner ear resulting in profuse fluid leakage, first perilymph then CSF. *Objectives:* The first purpose of this study was to further confirm the specificity of the perilymph-specific protein CTP that we reported recently. Secondly, we assessed the nature of the fluid leakage from the cochleostomy using the CTP detection test. *Methods:* A standardized CTP detection test was performed on 65 perilymph and 60 CSF samples. Samples of profuse fluid leakage collected from cochleostomy during cochlear implantation surgery of one patient with branchio-oto-renal (BOR) syndrome were also tested by the CTP detection test. *Results:* CTP was detected in 60 of 65 perilymph samples but not in any of the CSF samples. The leaked fluid was shown to contain CTP, i.e. perilymph, at the outset, and then the CTP detection signals gradually disappeared as time elapsed.

Keywords: *Perilymph, CSF, cochlear implant, COCH gene, Cochlin isoform, branchio-oto-renal syndrome, sensorineural hearing loss*

Introduction

Normally the cerebrospinal fluid (CSF) in the subarachnoid space extends laterally into the internal auditory canal (IAC) as far as the lateral fundus, where it is separated from the perilymph by the bony plate of the lamina cribrosa, the nerves that pass through the lamina cribrosa and the spiral ganglion. Profuse leakage of perilymph and CSF is the result of an abnormal bony defect in the lamina cribrosa, rather than enlargement of the cochlear aqueduct. In some congenitally dysplastic ears there is a deficiency in this barrier, allowing direct confluence between the CSF and perilymph [1,2].

In a large series of cochlear implants, the incidence of this profuse leakage of perilymph and CSF, i.e. a 'gusher', was approximately 1% of all cases. The term gusher is widely used in the literature to describe egress of clear fluid from the cochleostomy site. However, the term is variably applied and bears no explicit relation to the amount of fluid leaking, time elapsed until the flow ceases, pressure of the fluid column or origin of the fluid. The fluid may be CSF, perilymph, or a mixture of the two [3]. Even though the current level of diagnostic imaging technology is considerably advanced, at present it is unclear whether the perilymph and CSF spaces are separate and function as two distinct compartments or not. The fragile membranous tissue at the fundus of the IAC can be perforated after the fenestration of the cochlea due to a pressure decrease in the perilymphatic space resulting from the cochleostomy.

Correspondence: Tetsuo Ikezono MD PhD, Department of Otorhinolaryngology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. E-mail: ctp16ikdsph@nms.ac.jp

(Received 28 September 2009; accepted 19 November 2009)

ISSN 0001-6489 print/ISSN 1651-2251 online
DOI: 10.3109/00016480903508910

Alternatively, these liquid compartments may be mixed preoperatively because of a defect in the lamina cribrosa. The confluence of these two fluids could be one of the causes of the deterioration of hearing [2].

Recently, we reported the features of Cochlin-tomoprotein (CTP), a novel perilymph-specific protein. By proteomic analysis of inner ear proteins, we found certain unique properties of the cochlin isoforms. We detected three cochlin isoforms, p63s, p44s and p40s, in the inner ear tissue, and a short 16 kDa isoform, named Cochlin-tomoprotein (CTP), in the perilymph [4,5]. Since COCH gene/cochlin is known to be highly specific to the inner ear, we examined CTP expression in body fluid. CTP was selectively expressed in the perilymph of all 20 perilymph samples tested, but not in 77 samples of the body fluids, i.e. serum (28 samples), CSF (20 samples) or saliva (29 samples) [6]. Using CTP as a biomarker, we have established a standardized CTP detection test for the diagnosis of perilymphatic fistula (PLF), using a spiked standard of recombinant human (rh)CTP in Western blotting [7].

In the present study, we further analysed the specificity of CTP expression in body fluids, testing 65 perilymph and 60 CSF samples. The results confirm that CTP is present in the perilymph, but not in the CSF. Perilymph and CSF are distinguished by the presence or absence of a particular protein, CTP. Then we tested the samples of the profuse fluid leakage collected temporally from one case of cochleostomy during cochlear implantation surgery. The leaked fluid at the beginning of the cochleostomy contained CTP, i.e. perilymph, and the CTP signal gradually disappeared as time elapsed. These results indicate that even in the malformed inner ear of the case presented here, with a defect in the bony barrier at the fundus, the perilymph and CSF fluid spaces function as two separate compartments. Cochleostomy might have suddenly decreased the perilymph pressure and allowed CSF influx into the inner ear, resulting in profuse fluid leakage (perilymph then CSF) from the cochleostomy site. This is the first report of a temporal analysis of the CTP protein in gusher fluid.

Material and methods

Analysis of CTP expression in CSF and perilymph: collection and processing of body fluid samples

For the assessment of the specificity of CTP expression in body fluids, we collected perilymph during stapedectomy for otosclerosis or during cochleostomy for cochlear implant surgery. CSF was purchased from Biotech (Valley Center, CA, USA). The CSF was collected from consenting donors at an FDA-licensed and registered facility. No adverse events were observed during sample collection. The samples were centrifuged at 1250 *g* for 1 min and the supernatants were frozen and stored at −80°C until use. All patients gave their full informed consent and the study was approved by the ethics committee of Nippon Medical School.

Samples of perilymph or CSF (4 μg) were mixed with 5 μl of sample buffer (150 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.3% bromophenol blue, 300 mM DTT) after normalization per average protein concentration (perilymph 200 mg/dl, CSF 40 mg/dl) [8], then analysed by Western blot.

Analysis of CTP expression in the profuse fluid leakage from cochleostomy

We performed cochlear implantation in a 50-year-old male patient who was diagnosed as having branchio-oto-renal (BOR) syndrome (without renal manifestation) based upon the diagnostic criteria [9]. The patient started to use a hearing aid at the age of 8 years, and his hearing had progressively deteriorated. At the time of his first visit to our office, he had profound bilateral sensorineural hearing loss, right preauricular sinus and a slightly malformed left auricle. The tympanic membranes showed no anatomical abnormalities. High resolution computed tomography (HRCT) of the temporal bones (Fig. 1) presented findings typically seen in BOR cases; hypoplastic apical turn of the cochlea, a funnel-shaped IAC. The fundus of the IAC was wide, the lamina cribrosa was hypoplastic and no bony structure was seen [10]. Echogram and HRCT revealed no renal malformation. An 18-year-old daughter of the patient had bilateral profound sensorineural hearing loss and a history of surgical removal of bilateral cervical fistulas. She had similar HRCT findings. The patient's father also had bilateral progressive hearing loss.

During cochlear implantation in this case, profuse fluid leakage from the cochleostomy (gusher) was observed. We collected the leakage samples in a temporal manner at the following time points: 0 (immediately after fenestration), 0.5, 2, 3, 6, 15, 25, 35 and 40 min. It is the policy of this department to wait until the flow of the leakage weakens and then seal the cochleostomy with fascia and fibrin glue to avoid lumbar drainage. As a control, middle ear lavage (MEL), i.e. lavaging the middle ear cavity three to four times with the same bolus of 0.3 ml of saline and recovering the fluid, was

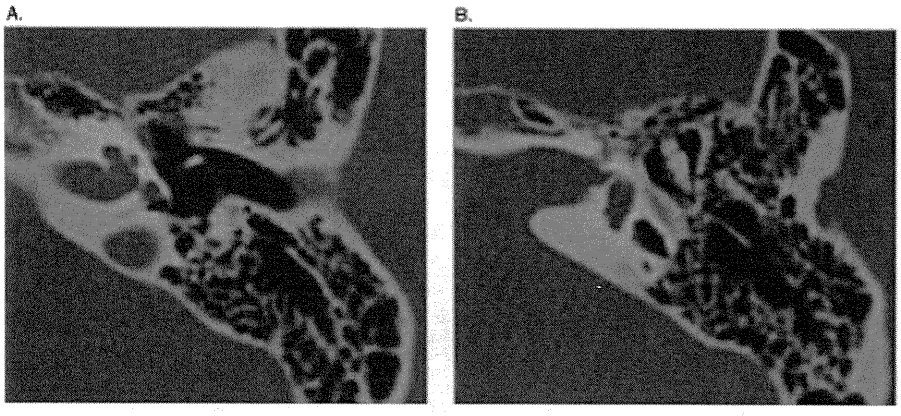


Figure 1. HRCT of the right ear in the axial plane. Both ears had findings typically seen in BOR cases: (a) the hypoplastic apical turn of the cochlea, the fundus of the internal auditory canal was wide, the lamina cribrosa was hypoplastic and no bony structure was seen; (b) the funnel-shaped internal auditory canal (IAC).

taken from the middle ear before cochleostomy. MEL (16 µl) was mixed with 8 µl of 3× concentrated sample buffer (150 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.3% bromophenol blue, 300 mM DTT) for Western blot analysis.

Standardized CTP detection test by Western blot

Samples were tested by the standardized CTP detection test [6,7], with minor modifications. For Western blot analysis, the rabbit polyclonal anti-CTP antibody (formerly anti-LCCL-C Ab) was prepared as described previously. In brief, a 14-mer peptide (LSRWSASFTVTKGK) corresponding to residues 114–127 in the LCCL domain was used as an antigenic peptide to generate antibodies.

Samples were loaded onto 15% polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked overnight at 4°C in 5% skim milk and 0.2% polyoxyethylenesorbitan (Tween-20) dissolved in PBS (pH 7.5). Membranes were then incubated in PBS containing 1% skim milk and 0.1% Tween-20 for 2 h at room temperature with the primary antibody (anti-CTP antibody) diluted to 1:2000. After washing with 0.05% Tween-20 in PBS, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labelled goat anti-rabbit IgG antibody (Dako, Tokyo, Japan) diluted to 1:10 000 in the same buffer as used for the primary antibody reaction. They were washed again and the reaction was developed with a chemiluminescence reaction kit (ECL Advance, Amersham) and then analysed with an image analyser LAS-3000 (Fuji Film, Tokyo, Japan). Tests were performed and analysed by well-trained personnel who did not have any information on the clinical background of the patients, to avoid any biased judgments. Test results were expressed qualitatively (positive or negative) by the presence or absence of the anti-CTP antibody reactive protein, with a molecular weight that exactly matched the molecular weight of native CTP (16 kDa) on Western blot.

rhCTP was used as a spiked standard on the Western blot. A putative CTP sequence predicted from our previous study, the 101–403 positions of the cDNA corresponding to amino acid residues 32–132, was amplified by PCR from a human expressed sequence tag clone, IMAGE ID 27789 (Kurabo, Japan). rhCTP was produced using pCR/T7/TOPO/TA expression kits (Invitrogen). To establish a clinical test for the diagnosis of perilymph leakage and avoid test variability, we standardized the CTP detection test using high (0.27 ng) and low (0.13 ng) spiked standard levels of rhCTP on Western blot. When the intensity of the band in the samples tested was below the high-level standard signal, the result was considered to be negative. The low spiked standard was used to estimate the protein transfer efficiency.

Diagnostic performance of the CTP detection test

To evaluate the specificity of the CTP detection test in the clinical setting, we previously reported the test results of samples from non-PLF cases. In that study, we defined non-PLF as those cases with otosclerosis (that had undergone stapedectomy), profound deafness (cochlear implant surgery), or conductive hearing loss (exploratory tympanotomy), without