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G．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

H．健康危険情報について
なし

新須磨病院における
外リンパ瘻疑い例と確実例の経験

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はじめに

外リンパ瘻は稀な疾患と考えられてきたが、池園の CTP の発見により外リンパ瘻が生化学的にも証明され、報告頻度は増加している。我々はこれまでの外リンパ瘻の経験から、外リンパ瘻はよくある疾患であると認識し、さらにその中には潜在化している外リンパ瘻が多いのではないかと考えている。そこで今回は新須磨病院における外リンパ瘻の疑い例と確実例を集計した。併せて外リンパ瘻確実例のうち、フラツキが主訴で聴力の左右差がない症例で、再手術時の検体から CTP 陽性となった症例を報告し、潜在化している外リンパ瘻の可能性について述べる。

対象と方法

当科での外リンパ瘻は 2004 年から 2011 年の 8 年間で、外リンパ瘻の診断基準平成 2 年度（案）に基づく、当初の外リンパ瘻疑い例は 314 例であった。一方、外リンパ瘻確実例は全体で 259 例で、そのうち外リンパ瘻疑い例からの移行症例は 42 例であった。

外リンパ瘻確実例のうち、フラツキが主訴で聴力の左右差がない症例で、他院では前庭神経炎と診断されていた症例で、当科で手術を施行し外リンパ漏出を確認して内耳窓閉鎖によりフラツキが消失したが、症状再発し再手術の際の検体から CTP 陽性と認められた症例の経過を併せて報告する。

結果

当科での外リンパ瘻は 2004 年から 2011 年の 8 年間に診断基準に基づく外リンパ瘻疑い例は 314 例で、そのうち手術を施行して外リンパ漏出を確認し、外リンパ瘻確実例となった症例は 42 例（13.4%）であった。

一方、手術で外リンパ漏出を確認した外リンパ瘻確実例は 259 例であった。そのうち外リンパ瘻疑い例からの移行症例は 42 例でその比率は外リンパ瘻確実例全体の 16.2%であった。

症例提示

CTP 陽性の外リンパ瘻確実例を提示する。症例は 67 歳女性で、主訴はフラツキと気分不良である。

家族歴：娘（35 歳）が低音障害の左感音性難聴を繰り返していた。最初は左突発難聴と左耳鳴（ザワザワ）で A 耳鼻咽喉科に受診した。そこで左突発性難聴と診断され、点滴によるステロイドパルス療法を受け、回復した。9 ヶ月後に左難聴、左耳鳴（ザワザワ）が再発し、B 耳鼻咽喉科に受診してステロイド内服処方を受け、聴力は再度

回復した。さらに 3 ヵ月後に左難聴と左耳鳴（ザワザワ）が再発し、B 耳鼻咽喉科でステロイド内服処方されるも改善せず、さらにメニエール病の可能性も考慮されイソソルビド内服を処方されたが聴力改善は認められなかった。精査加療目的で当科に紹介受診となった。当科でも入院の上、点滴によるステロイドパルス療法を行ったが、左難聴と左耳鳴（ザワザワ）は改善せず左内耳窓閉鎖術を施行した。その後、左難聴と左耳鳴（ザワザワ）は消失している。

病歴と症状経過：当科受診の 7 ヶ月前に C 病院整形外科で足関節の手術を受け、入院中に強い回転性眩暈が出現持続し、C 病院耳鼻咽喉科で前庭神経炎と診断された。その後も仰臥位で左耳を下にした時に眩暈が出現するため、左側臥位では眠れなくなっていた。また朝起きるときは気分不良が生じるようになった。歩行時も左側にふらついて寄って行き、自転車も乗れなくなっていた。投薬を受けるも症状改善せず、娘の勧めで当科に受診した。

純音聴力検査では聴力の左右差を認めず、DPOAE でも DP レベルに左右差を認めなかった。仰臥位の純音聴力検査を施行すると、低音域を中心として左耳の聴力改善と右耳聴力低下があり左右差が出現した。坐位臥位聴力検査を別の日に 2 回行ない、同様の結果を得た。眼振は日によって変化し、仰臥位左耳下頭位で強くなる傾向が認められた。以上より左外リンパ瘻を疑い手術を施行したところ、左卵円窓と左正円窓の両窓より外リンパ漏出を確認し側頭筋膜にて充填閉鎖した。手術後、フラツキや眩暈、気分不良は消失し、自転車にも乗れるようになっていた。

初回手術の 8 ヵ月後に夜行バスで旅行した後から、左側臥位になると眩暈が出現するようになった。その後も症状改善せず、初回手術の 1 年 2 ヵ月後に再手術を施行した。その再手術の際に得られた検体で CTP 検査をお願いしたところ、CTP 陽性との結果が判明した。手術後めまいは改善し現在に至っている。

考察

外リンパ瘻はこれまで稀な疾患と捉えられがちであったが、池園の CTP の発見により外リンパ瘻が生化学的にも証明され、その報告頻度は増加している。

当科での外リンパ瘻を、外リンパ瘻の診断基準平成 2 年度（案）に基づいて診断したところ、2004 年から 2011 年の 8 年間で外リンパ瘻の疑い例と確実例の合計は 531 例となり、年間平均は約 66 例である。この年間平均約 66 例という数値は、外リンパ瘻が決して少ないものではないことを反映しているといえる。また当初の外リンパ瘻疑い例は 314 例で、そのうち手術を施行して外リンパ瘻確実例となった症例は 42 例（13.4%）で、外リンパ瘻全体からみると手術に至る症例は少ないのではないかと考えられる。

一方、外リンパ瘻確実例は全体で 259 例あり、そのうち外リンパ瘻疑い例からの移行症例は 42 例で、外リンパ瘻確実例全体の

16.2%を占めるのみであった。これは外リンパ瘻全体を考えると、外リンパ瘻の診断基準の疑い例に該当しない症例が多数存在することを示唆している。

当科での CTP 陽性症例でも外リンパ瘻の診断基準の疑い例に該当していなかった。その娘の外リンパ瘻確実例も繰り返す低音障害の感音性難聴を呈していたが、やはり診断基準の疑い例には該当していなかった。さらにその母親の CTP 陽性症例では、フラツキや気分不良が主症状であり、通常の純音聴力検査では左右差が認められないため、他の耳鼻咽喉科では前庭神経炎と診断されてしまっていた。以上の結果より、診断基準から外れて潜在化している外リンパ瘻が相当に存在しているのではないかと考える。今後、CTP 検査などを含めて、潜在化している外リンパ瘻の存在を明らかにしてゆく必要がある。

結論

新須磨病院での外リンパ瘻は、2004 年から 2011 年の 8 年間に診断基準に基づく外リンパ瘻疑い例は 314 例で、そのうち手術を施行して外リンパ漏出を確認し、外リンパ瘻確実例となった症例は 42 例（13.4%）であった。一方、手術で外リンパ漏出を確認した外リンパ瘻確実例は 259 例であった。そのうち外リンパ瘻疑い例からの移行症例は 42 例でその比率は外リンパ瘻確実例全体の 16.2%であった。

CTP 陽性症例やその娘の外リンパ瘻確実例を併せて考慮すると、潜在化している外リンパ瘻が多数存在している可能性が示唆された。

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Ⅲ.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

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

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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

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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

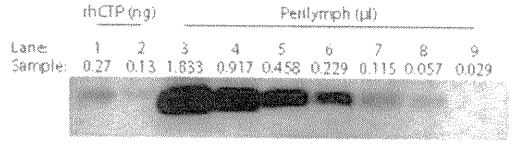
In order to evaluate the specificity of the CTP detection test, we examined MEL from non-PLF cases. In this study, we defined 'non-PLF' as those cases with otosclerosis (which had undergone stapedectomy), profound deafness (cochlear implant surgery) or conductive hearing loss (exploratory tympanotomy). 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.

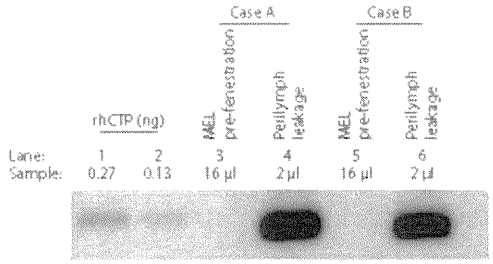
Detection Limit

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.

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