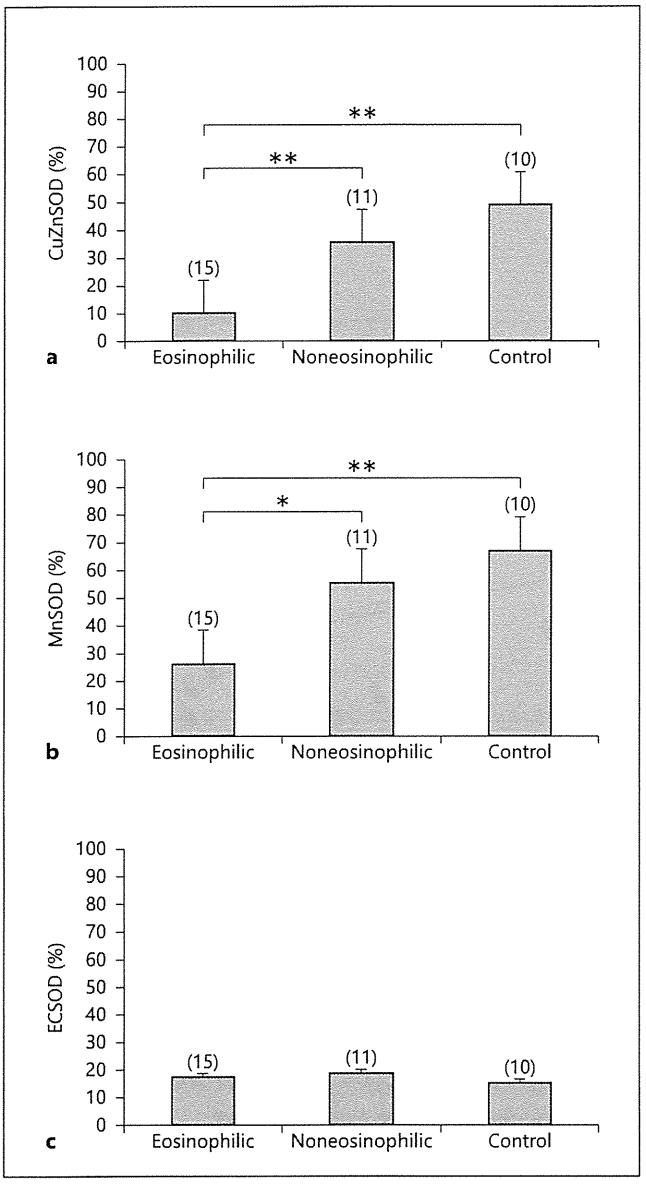

Fig. 3. The positive rate for SOD immunoreactivity in the epithelium. Numbers of samples are given in parentheses; * $p < 0.05$; ** $p < 0.01$.

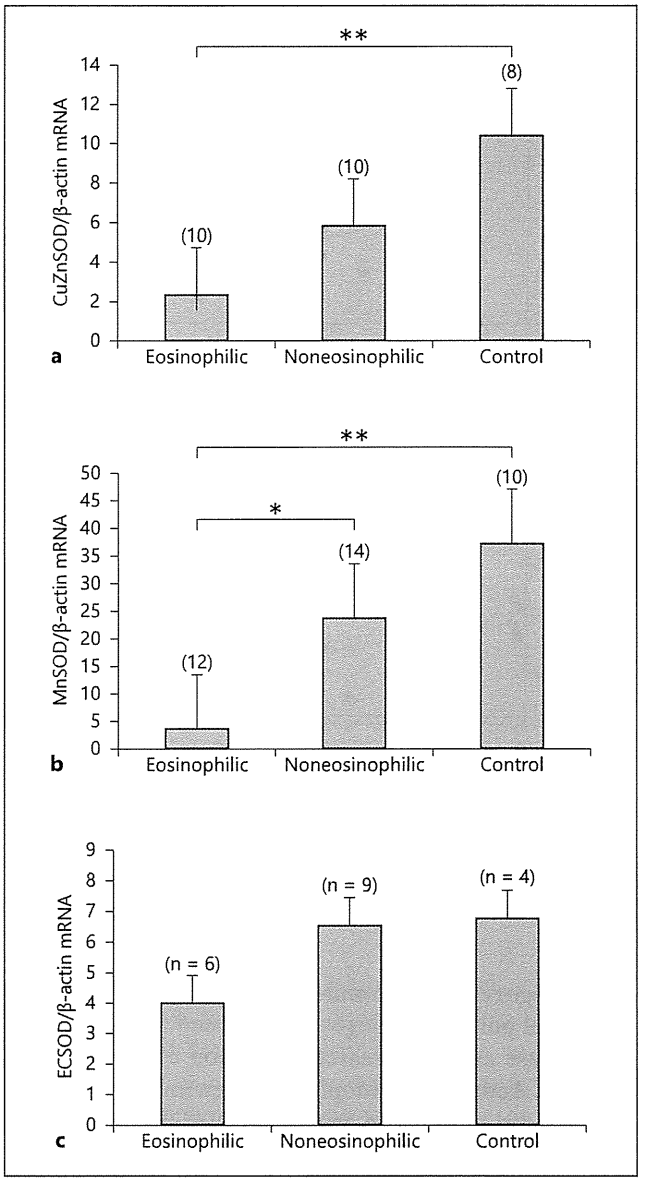

Fig. 4. Measurement of SOD mRNA by real-time RT-PCR. Numbers of samples are given in parentheses; * $p < 0.05$; ** $p < 0.01$.

of the SOD scavenger system for oxidative stress. The results can be summarized as follows: (i) SOD activity of the CRS groups was significantly decreased compared with that of the controls; (ii) immunostaining of both CuZnSOD and MnSOD of the eosinophilic group was significantly decreased compared with that of the noneosinophilic and control groups; (iii) CuZnSOD mRNA of the eosinophilic group was significantly decreased compared with that of the control group; (iv) MnSOD mRNA of the eosinophilic group was significantly decreased compared with those of the noneosinophilic and control groups; (v) neither immunoreactivity nor mRNA of ECSOD differed among the three groups, and (vi) the degree of epithelial damage and CT scores inversely correlated with CuZnSOD and MnSOD immunoreactivity.

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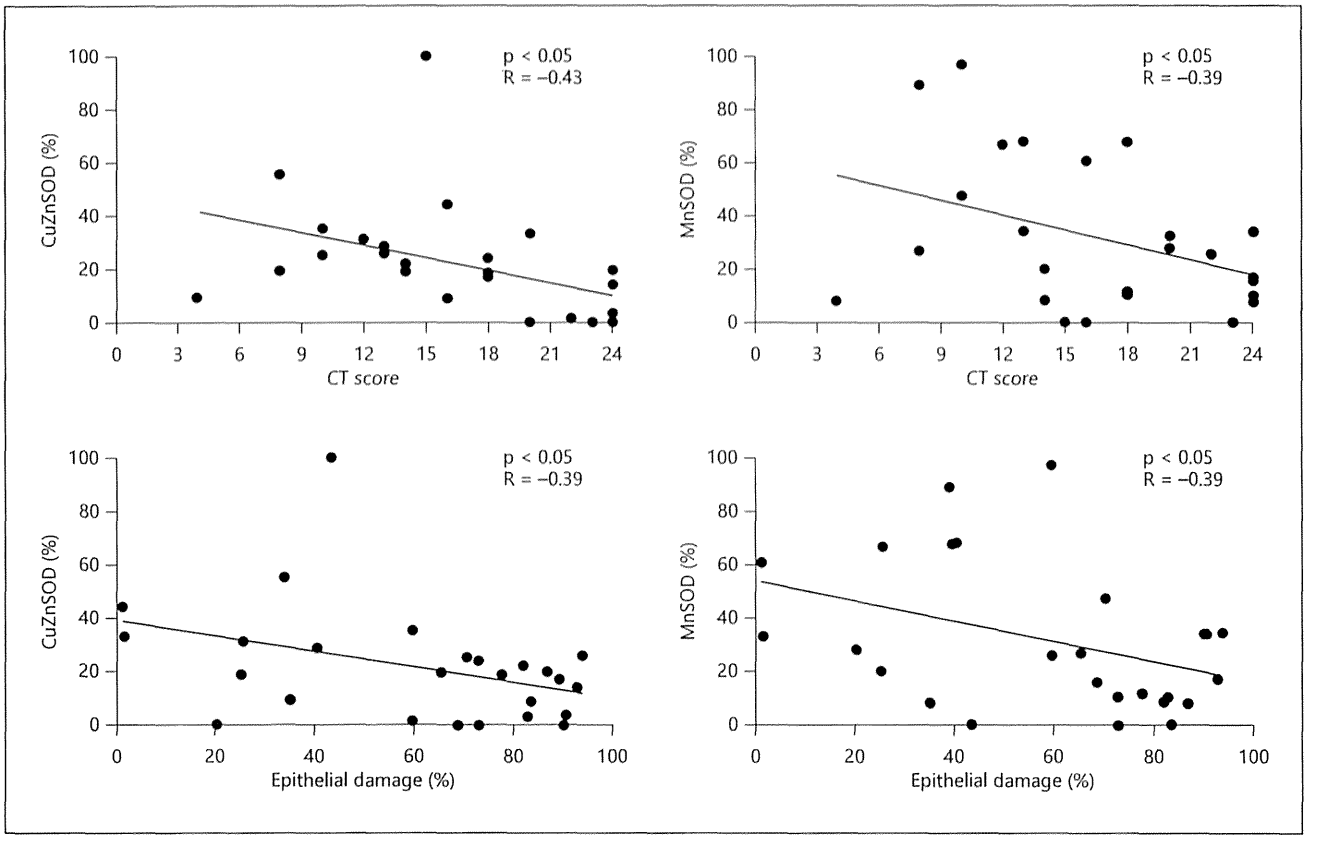

Fig. 5. Relevance of CT scores and epithelial damage to SOD activity.

A category of eosinophil-based inflammation in CRS with nasal polyps is thought to be related to more extensive disease and a decreased likelihood of surgical success [19]. Activated eosinophils can generate superoxides (O_2^-) via the membrane-associated NADPH-dependent complex. Subsequently, dismutation of O_2^- gives hydrogen peroxide (H_2O_2). O_2^- and H_2O_2 per se are moderate oxidants; however, both species are critical for the formation of potent cytotoxic radicals in biological systems that possess eosinophils. Eosinophil peroxidase catalyzes the oxidation of halides by H_2O_2 to form hypohalous acids. Hypohalous acid production is important in the host defense against infectious agents, which is one the exacerbating factors of eosinophilic CRS with nasal polyps [20]. However, during this reaction, the hydroxyl radical, which is a powerful and indiscriminate oxidant, is also produced. The oxidative injury caused by eosinophils can be substantial because the cells possess several times greater capacity to generate O_2^- and H_2O_2 than neutrophils [21], and the content of eosinophil peroxidase in eosinophils is 2–4 times higher than the amount of myeloperoxidase in neutrophils [22]. Thus, eosinophils infiltrating into the sinonasal mucosa are suggested to play a potential role in the pathogenesis of CRS with nasal polyps, particularly during exacerbations.

SOD is one of the essential first-line antioxidant enzymes, since catalytically converted O_2^- produced by eosinophils forms H_2O_2. The present finding that CuZnSOD expression in the nasal polyps characterized by a large amount of infiltrating eosinophils was reduced is well consistent with what is found in asthmatic patients [23]. However, the expressed forms of MnSOD are different between the upper and lower respiratory airways. The former show decreased expression of MnSOD in eosinophilic CRS compared with controls, whereas in the latter no difference was detected between asthmatic and control subjects [23].

The homeostasis of cellular functions during oxidative stress depends on the rapid induction of protective antioxidant enzymes. Naturally occurring antioxidants exist

to protect cells and tissue from the continuous production of reactive oxygen species. However, high levels of reactive species overwhelm the antioxidant defenses, resulting in loss of barrier function, increased permeability or destruction of epithelial cells [24]. In asthma, the lower SOD activity is a consequence of the increased oxidative stress, and thus is a sensitive marker of the airway redox status and asthma severity [25]. Similarly, the reduction in SOD activity in CRS with nasal polyps may influence the extent and severity of disease related to tissue eosinophilia.

The decreased SOD activity and protein may be brought about by (i) depletion of the enzyme due to consumption, (ii) inactivation of the enzyme, and (iii) effects of transcriptional regulation or gene mutation. It has been shown that oxidative modification/inactivation of MnSOD was present in asthmatic airway epithelial cells [26], indicating functional impairment in SOD activity due to oxidative processes. Although the promoter of CuZnSOD has been shown to be induced by oxidants and metal ions [27], most studies have shown that there was no upregulation of lung CuZnSOD induced by cytokines or oxidant stress in vivo or in vitro [28, 29]. In contrast, oxidants and cytokines generally cause MnSOD induction [30]. No experimental data have been reported in the upper airway, so far. We first demonstrated that mRNA of both CuZnSOD and MnSOD was downregulated in the eosinophilic CRS with nasal polyps. Our study suggests that there are differences in the expression patterns of SOD between the upper and lower airways. The impairment in oxidant-decomposing mechanisms in eosinophilic CRS with nasal polyps might involve genetic and environmental factors, which will require further research for clarification.

In conclusion, a disrupted balance between the oxidant generation mediated by recruited eosinophils and the oxidant defense system of SOD is suggested to play an important role in the formation and exacerbation of CRS with nasal polyps.

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JOHNS
特集●unified airwayからみた鼻副鼻腔病変●

気道疾患と鼻副鼻腔病変

鼻副鼻腔炎と気管支喘息

池田勝久*
Katsuhisa IKEDA

● Key Words ●好酸球性副鼻腔炎，気管支喘息，内視鏡下副鼻腔手術●

Ⅰ．下気道過敏性

喘息は鼻副鼻腔炎の共有疾患としてみなされており[1]，慢性鼻副鼻腔炎の約50％に喘息を合併していることが知られている[2]。近年の研究によると，上気道病変が下気道の症状や機能低下の原因因子や増悪因子となることが示唆されている[3]。上気道病変の適切な治療が上気道症状のみならず，下気道病変の改善につながることも徐々に証明されてきた。喘息を有しない慢性鼻副鼻腔炎患者でも効率にメサコリンによる下気道過敏性の亢進が認められている。

気道過敏性とは，気道が非特異的刺激に敏感なことを意味し，臨床的にはわずかな濃度のアセチルコリンやヒスタミンにより狭窄を生じる性質と定義され，喘息発作や重症度，慢性閉塞性肺疾患の肺機能に相関することが知られている。また無症候性の気道過敏性陽性例は高率に喘息へ移行し，下気道病変発症の予後因子となる。

明らかな下気道やアレルギー性鼻炎の症状を示さず，通常の呼吸機能検査正常の慢性鼻副鼻腔炎患者31名を対象に下気道過敏性検査を施行した。アストグラフ法によるメサコリンの連続吸入中の呼吸抵抗を測定し，呼吸抵抗の上昇し始めるメサコリンの閾値の累積をDminと定義して，過敏性の指標とした。慢性鼻副鼻腔炎患者は71％と高率に下気道過敏性を認めた。また下気道過敏性陽性の7症例において，内視鏡的副鼻腔手術により副鼻腔病変を治癒せしめるとDminは有意に改善しており，5症例ではほぼ正常範囲の気道過敏性となっていた（図1）[4]。

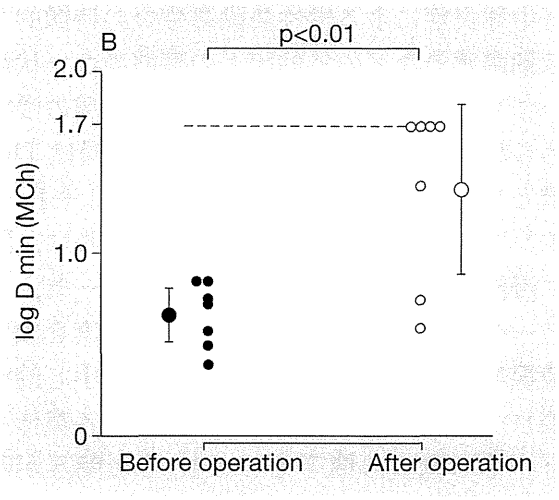

図1　慢性鼻副鼻腔炎症例における副鼻腔手術前後の下気道過敏性の変化

これらの所見から，上気道の炎症病変自体が下気道過敏性に直接影響を引き起こす因子であることを示唆している。Ponikauらは慢性鼻副鼻腔炎患者91％にメサコリンによる下気道過敏性を認めたと報告している[5]。慢性鼻副鼻腔炎患者の60％が病歴，呼吸機能検査，ヒスタミン誘発検査によって下気道病変を有していることも報告されている[1]。

Ⅱ．慢性鼻副鼻腔炎におけるTh1・Th2反応

慢性鼻副鼻腔炎は解剖学や局所免疫の異常などの宿主側に要因，微生物や環境因子との関連などの多因子による病態が複雑に関与している。慢性鼻副鼻腔炎は喘息と共通した上皮剥離，基底膜肥厚の病理像を呈している[1]，慢性鼻副鼻腔炎の粘膜のサイトカイン分布Th1優位とTh2優位の2グ

* 順天堂大学大学院医学研究科医学専攻課程耳鼻咽喉科学講座〔〒113-8421 東京都文京区本郷2-1-1〕

ループに分けて考えることができる。欧米における慢性鼻副鼻腔炎では鼻ポリープを伴わない場合に副鼻腔の自然口の閉塞による副鼻腔からの粘液の排泄障害を起こす病態で，慢性の細菌感染やTh1反応が関係する。鼻ポリープを伴う場合はTh2反応による好酸球炎主体の病態が考えられてきたが，嚢胞性線維症やアジア人では好中球性炎症の関与も判明してきている[6]。また鼻茸には好酸球や好中球に乏しく，少量の形質細胞やリンパ球の浸潤のみを示す症例も存在する。

好中球炎症による慢性鼻副鼻腔炎の特徴的な病態は細菌感染などの外的因子の曝露のない状態においても，副鼻腔洞内に持続的な好中球の浸出が認められることである。この好中球動員はTh1を基盤とした免疫反応によって，ICAM-1やE-セレクチンなどの接着因子を活性化し，さらに，副鼻腔に浸出した活性化好中球はエラスターゼ，プロテアーゼなどの蛋白分解酵素や活性酸素を放出し粘液線毛機能を低下させ，病態形成の中心的役割を演じている。慢性鼻副鼻腔炎における鼻汁中への好中球の動員の機序としてIL-8の関与が明らかになった[7]。

マクロライドの半量長期療法の作用機序の1つがIL-8分泌の抑制効果である，その結果，慢性鼻副鼻腔炎の遷延化の機序であるIL-8による好中球の動員の悪循環が打ち切れるのである[7]。

副鼻腔粘膜の病理組織学所見から，好酸球型と非好酸球型に区別することで慢性鼻副鼻腔炎の亜分類を試みたことから，好酸球性鼻副鼻腔炎の概念が生まれた[8]。1994年にNewman[9]は末梢血の好酸球の増多と高度な副鼻腔病変が関連なることを最初に報告した。その後，多くの研究で血中の好酸球数や副鼻腔の病的粘膜の好酸球浸潤の増加が病変の重症度や術後の予後不良に相関することが明らかになった[8]。

好酸球性鼻副鼻腔炎の病態はまだ十分に解明されていない。しかしながら，喘息を合併する場合が多く，また喘息の発症前の症例も多く含んでいるため，喘息との共通した病態が示唆されている。臨床的な特徴を**表1**にまとめた。好酸球性鼻副鼻腔炎の病因としては，

1）黄色ブドウ球菌などの内毒素由来のスーパー抗原

2）真菌のⅠ型アレルギー

3）真菌の非IgE依存性のアレルギー反応

4）アスピリン不応性

が提唱されている[8]。鼻ポリープ組織の黄色ブドウ球菌の内毒素が多く寄生しており，内毒素の特異的なIgE抗体の産生も認められている[10]。さらに，動物実験で黄色ブドウ球菌の内毒素の鼻腔内投与によって気管支と全身のTh2サイトカインの増加を伴ったアレルギー性鼻炎と喘息が悪化したことが報告され，細菌の内毒素が上下気道の共通した病因として提唱されている[11]。また真菌，特にアルテルナリアによって好酸球の脱顆粒が生じ，慢性鼻副鼻腔炎の病因に真菌の関与も提唱されている。

表1　好酸球性副鼻腔炎の診断基準（試案）

1. 多発性鼻ポリープ：特に嗅裂，中鼻道の病変
2. 喘息合併：肺機能，下気導過敏性検査，NO測定の併用
3. 著明な好酸球浸潤を伴う鼻ポリープ
4. 好酸球に富む粘稠な鼻漏，時にニカワ状（好酸球性ムチン）
5. 早期の嗅覚障害
6. ステロイド薬の全身投与による鼻ポリープの消退
7. 特徴的な画像診断：特にMRI

（日鼻誌46：37-38，2007より改変）

Ⅲ．内視鏡下副鼻腔手術による喘息への影響

内視鏡下副鼻腔手術（endoscopic sinus surgery：ESS）は慢性鼻副鼻腔炎の標準的な外科的治療であるが，合併する喘息の症状や薬物投与量の軽減をもたらす[2]。喘息を伴う慢性鼻副鼻腔炎の手術を21名の患者に行い，鼻汁，後鼻漏，鼻閉の鼻副鼻腔症状をスコア化すると，術後3カ月で71％，6カ月で86％の有意な改善を認めた。術前後6カ月間のピークフロー値を比較すると，術後で40～190 *l*/minの改善を認め，平均98 *l*/minと有意な値を示した（**図2**）。

このように喘息を合併している慢性鼻副鼻腔炎患者においては上気道の病変を手術的に治癒させ，鼻副鼻腔症状を改善させることによって，下気道の病態が改善するのである[16]。最近の前向き

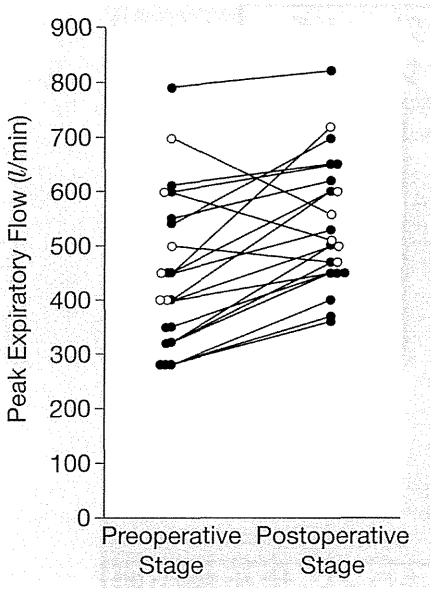

図 2　喘息合併の慢性鼻副鼻腔炎症例の副鼻腔手術前後（●）のピークフローの変化（○：対照）

の無作為試験によって慢性鼻副鼻腔炎の ESS 治療または薬物治療が喘息に奏功することが実証された[13]。以上より，合併する慢性鼻副鼻腔炎への積極的な治療は喘息の治療としても有用であると位置付けることができる。

ESS による慢性鼻副鼻腔炎の奏功率は喘息合併の有無で異なることが知られている。喘息合併症例では ESS の成功率が低く，術後の再発率が高くなる[2,14]。またアスピリン不耐性は ESS の予後不良因子である[15]。

Ⅳ．慢性鼻副鼻腔炎と喘息の連関の機序

上気道疾患が下気道病変を誘発させる下行説の機序を Irvin[16] らは C5a des arg で感作し，下気道過敏性の亢進を合併した鼻副鼻腔炎のウサギモデルを用いて，種々の観点から検討している。関節腔への感作では下気道過敏性は変化せず，血行性経路による下気道への炎症起炎物質の到達説は否定的である。動物を懸垂位にしたり気管挿管したりして，鼻汁の下気道への流入を防ぐと過敏性は生じないことより，鼻副鼻腔の受容体刺激→求心性線維→迷走神経刺激（遠心性線維）による気管支の収縮という経路の反射は否定された。この過敏性亢進動物の気道分泌液や組織像においては炎症所見が乏しいことから後鼻漏の下気道への直接

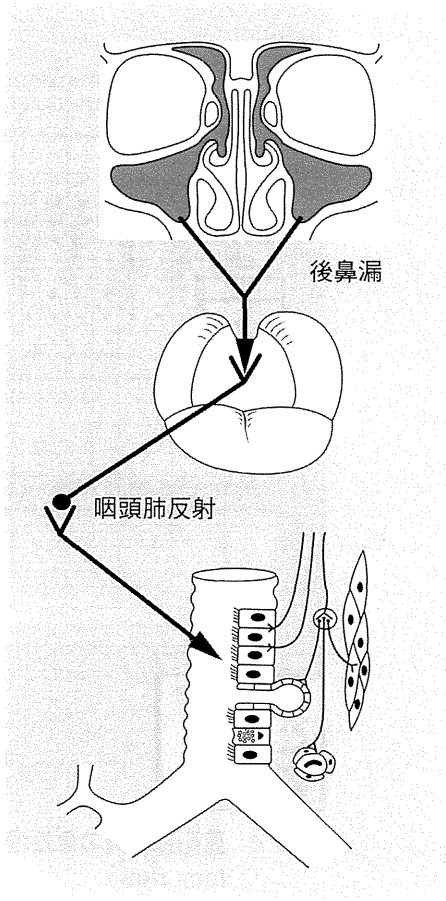

図 3　下行説の機序

流入は否定的である。これらの所見から，鼻副鼻腔から漏出した化学伝達物質が咽頭粘膜に到達して，咽頭肺反射を活性化し，下気道の収縮を引き起こすことが推定されている（**図 3**）。

臨床的には慢性鼻副鼻腔炎患者の副鼻腔に注入した放射性物質は後鼻漏となって下気道に流入することはないと証明されている[17]。さらに，前述した咽頭反射の亢進を示唆する咽頭粘膜の病理組織所見や上気道の過敏性に伴う後鼻漏が咽喉頭の受容体の亢進に関連する証拠が報告されている[18]。また Togias は局所で発生したアレルギー炎症の結果，接着因子の発現増強，循環する白血球の活性化，骨髄での白血球の前駆体の活性化などが生じ，離れた部位の炎症に発展することを提唱している[19]。Steinke は同様な考え方を好酸球性鼻副鼻腔炎と喘息における上下気道の現場でのアレルギー炎症の相互的な増強のメカニズムの説明に当てはめている[20]。

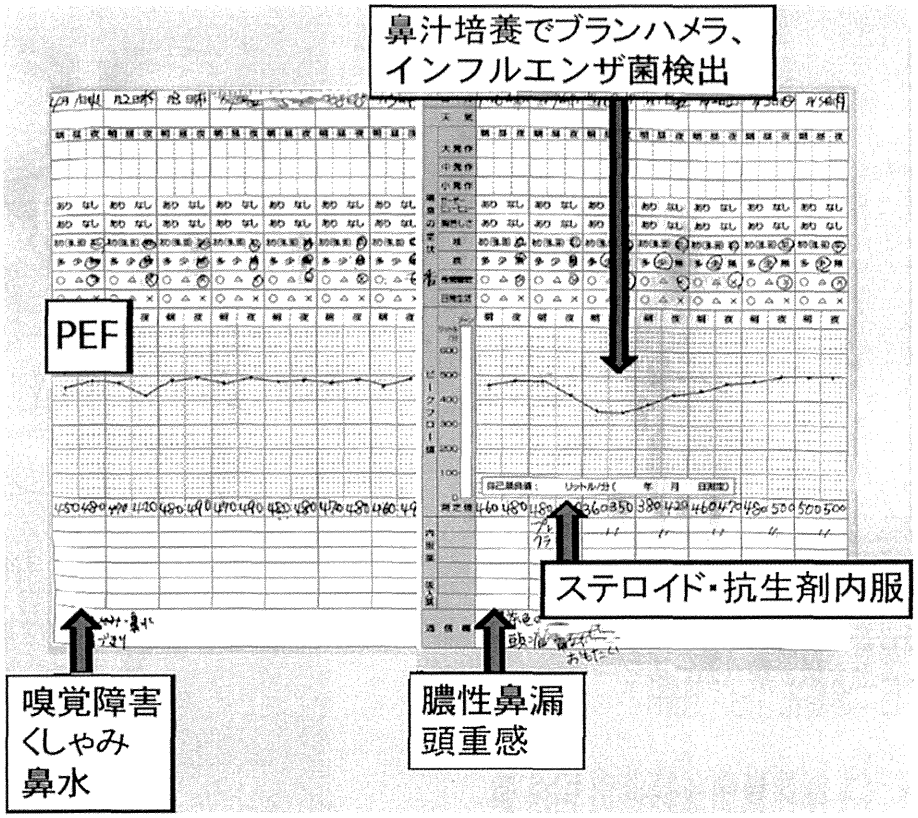

図 4　鼻副鼻腔炎の急性増悪による喘息の悪化の経過表（PEF：peak expiratory flow）

V．当科における喘息を合併した慢性鼻副鼻腔炎の治療戦略

一般に保存的治療法が第一選択であり，保存的治療に抵抗を示す重症例が手術適応となる。当科での好酸球性鼻副鼻腔炎の治療指針は，血中好酸球の増加，鼻汁スメアの好酸球の存在，篩骨洞・嗅裂部を中心とした高度粘膜病変，早期からの嗅覚障害などの臨床ならびに検査所見を呈する場合や鼻ポリープの生検で好酸球の集簇を認めた場合は好酸球性鼻副鼻腔炎と診断する。軽・中等症は抗ロイコトルエン拮抗薬，経口または局所ステロイド薬を行い，治療困難または再発を繰り返す症例ならびに重症例に対して，ESS を選択する。可能であれば術前後にステロイド薬を内服投与する。ESS はマイクロデブリッターを用いてポリープを除去し，上鼻甲介の下半分を切除し，嗅覚路を確保し，すべての副鼻腔を可及的に大きく開洞する。術後は抗アレルギー薬と鼻内吸入ステロイド薬の長期投与と鼻洗浄で管理する。

術後の管理にはニオイスティック（香水）を用いて嗅力の有無を自己判定・評価する。再発の徴候の判断は，嗅覚の低下と感染である。SST で嗅力の消退やニカワ様の好酸球性ムチンを示唆する鼻漏の出現時ではプレドニゾロン（0.5 mg/kg）を，膿性～膿粘性鼻漏が持続する場合では抗生剤（主にレスピラトリーキノロン）を頓服させる。また喘息合併症例や下気道の症状の合併症例ではピークフローメーターによる下気道の管理も指導している。上・下気道の所見を可能な限り日記として記載してもらい，指導管理の資料としている[21]。

40 症例で術後のフォローアップの経過中にポリープの再発を認めた 25 症例にステロイドの頓服を行った。その内，17 症例（80％）ではステロイドの感受性が悪かった。一方，喘息のステロイド感受性は応答が 19 例，不応答が 4 例であった。不応答症例の半数がアスピリン不耐性であった。今後の課題はステロイド不応性への対策であろう。

VI．副鼻腔手術の再発因子と喘息の増悪

再発の重要な要因として細菌感染がある。その

理由として，

1）副鼻腔の細菌叢が喘息の感染増悪の温床なること

2）急性鼻副鼻腔炎の3大起因菌（肺炎球菌，インフルエンザ菌，モラクセラ・カタラーリス）が喘息増悪の要因となること

3）喘息の70～75％で上気道にモラクセラ・カタラーリスを検出すること

である。

42症例の術後で膿性鼻漏などの再発徴候を示した37症例から83株の細菌が検出された。正常細菌または菌株なしは5症例であった。急性増悪の起因菌をして肺炎球菌，インフルエンザ菌，カタラリス菌は23株認めた。上気道炎を契機として，鼻副鼻腔炎の急性増悪を生じ，ピークフローメーター値（PEF）の低下を示した（図4）。ピークフローメーター計測した24症例中6症例に低下を認めた。全例で急性鼻副鼻腔炎の3大起因菌または緑膿菌を検出した[22]。以上より，鼻副鼻腔の細菌感染は副鼻腔病のみならず，喘息の再燃に関与することが判明し，喘息合併症例では鼻副鼻腔炎の再燃の防止は喘息の良好な経過にも貢献する。

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The Laryngoscope

Fungal Extracts Detected in Eosinophilic Chronic Rhinosinusitis Induced Cytokines From the Nasal Polyp Cells

Mikio Hirotsu, MD; Akihito Shiozawa, MD; Noritsugu Ono, MD; Masato Miwa, MD; Ken Kikuchi, MD; Katsuhisa Ikeda, MD

Objectives/Hypothesis: The role of fungi in chronic rhinosinusitis (CRS) is still controversial. The present study was conducted to detect and identify fungal species from the nasal polyp tissues of eosinophilic and noneosinophilic CRS, and to determine the role of fungal antigens in cytokine production.

Study Design: Prospective study.

Methods: Thirty-five specimens of nasal polyps were collected from patients with CRS and examined for fungus using culture, histology, and polymerase chain reaction analysis. The secretion of 14 cytokines stimulated by fungal extracts using dispersed nasal polyp cells (DNPCs) was determined by multiplex immunoassay.

Results: There was no microbiological growth (including fungus) in the cultures of homogenized nasal polyps. Furthermore, Grocott methanamine silver staining for all nasal polyps showed no fungal bodies. Sixteen of 35 samples of the nasal polyps showed amplification of fungal DNA. In none of the mucosa of the sphenoid sinus was fungal DNA detected. The number of eosinophils in the nasal polyps in which fungal DNA was detected was significantly higher than in the nasal polyps in which fungal DNA was not detected ($P < .01$). The extract of fungus enhanced the secretion of eosinophil-associated cytokines such as interleukine (IL)-5, IL-13, IL-17A, and RANTES (regulated on activation normal T-cell expressed and secreted), and proinflammatory cytokines such as IL-6, IL-8, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor from DNPCs.

Conclusions: The present study offers direct evidence supporting that fungal elements modify the inflammatory response in the nasal polyps of eosinophilic CRS.

Key Words: Fungus, nasal polyp, polymerase chain reaction, cytokine, eosinophil.

Level of Evidence: NA

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INTRODUCTION

Chronic rhinosinusitis (CRS) is considered to be a multifactorial disease within a heterogeneous group of diseases, with different underlying etiologies and pathophysiologies. CRS has been classified broadly as CRS with nasal polyps (CRSwNP), CRS without nasal polyps, and allergic fungal rhinosinusitis (AFRS). In particular, CRSwNP may have a complex pathogenesis and is thought to arise from multiple factors including allergy. Microorganisms have always been popularly suspected in the pathology of CRSwNP. There is recent evidence suggesting that 1) *Staphylococcus aureus* enterotoxins, 2) type I hypersensitivity to fungus, and 3) non-immunoglobulin E (IgE)-mediated hypersensitivity to fungus may play a role in the pathogenesis of eosinophilic inflammation.[1] However, the role of the microorganisms, particularly fungal pathogens, in the etiology of CRS remains largely unknown.

The role of fungi in CRS is still controversial. Conflicting with the prevailing belief that fungi were responsible for CRS in a selected group of patients with distinct pathophysiology, Ponikau et al.[2] and Braun et al.[3] observed that fungus is a ubiquitous intranasal presence, identified in close to 100% of both CRS patients and controls. The former group also detected fungi along with eosinophil and eosinophil-degraded products with mucus. Shin et al.[4] exposed peripheral blood mononuclear cells to fungal antigens in vitro and reported increased interleukin (IL)−5 and IL-13 production in 89% of CRS patients but not in controls. These observations formed the basis of the "fungal hypothesis of CRS." As further evidence, nasal mucus or tissue from CRS patients triggered eosinophil migration,[5] and *Alternaria* fungus in particular can directly induce eosinophil degranulation mediated by protease-activated receptor (PAR) activation.[6]

However, other investigators reported the absence of a universal hyper-responsiveness to fungal antigens in CRS patients.[7,8] Furthermore, a multicenter, randomized clinical trial of topical antifungal agents for CRS eventually failed to show any evidence of efficacy,[9] and a meta-analysis did not support the routine use of topical

From the Department of Otorhinolaryngology (M.H., A.S., N.O., M.M., K.I.), and Department of Infection Control Science (K.K.), Department of Bacteriology (K.K.), and Department of General Medicine (K.K.), Juntendo University Faculty of Medicine, Tokyo, Japan.

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Send correspondence to Katsuhisa Ikeda, MD, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail: ike@juntendo.ac.jp

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TABLE I.
Primers Used in the Study.

Primer	5′ → 3′
ITS-IF	GTC GTA ACA AGG TTA ACC TGC GG
ITS-4R	TCC TCC GCT TAT TGA TAT GC
NL-1	GCA TAT CAA TAA GCG GAG GAA AAG
NL-4	GGT CCG TGT TTC AAG ACG G
FF-2	GGT TCT ATT TTG TTG GTT TCT A
FR-1	CTC TCA ATC TGT CAA TCC TTA TT

antifungals for CRS.[10] Thus, the precise roles of fungi in the etiopathology of CRS remain unknown.

The present study was conducted to detect and identify fungal species from the nasal polyp tissues of eosinophilic and noneosinophilic CRS using Grocott methanamine silver staining and polymerase chain reaction (PCR) methods. Moreover, the effects of fungal extracts identified in the nasal polyps were examined by the ex vivo cellular responses of dispersed nasal polyp cells (DNPCs).

MATERIALS AND METHODS

Patients

Thirty-five patients with CRS with nasal polyps (21 males and 14 females, ranging in age from 23–77 years, mean age of 49 years) were consecutively recruited from the Department of Otorhinolaryngology of Juntendo University Hospital from April 2011 to March 2012. CRS with nasal polyps was diagnosed based on the criteria of the European position paper.[11] None of the patients was treated with antibiotics, systemic or topical corticosteroids, or other immune-modulating drugs for at least 1 month before the surgery. Subjects with AFRS were excluded from the present study. The criteria of AFRS of two positive findings, 1) specific IgE antibodies against fungi, and 2) the presence of fungi in the sinus effusion using Grocott methanamine cytological silver staining or microbiological examination. Serum fungus-specific IgE concentrations against *Alternaria, Aspergillus, Candida, Penicillium, Mucor, Cladosporium,* and *Pityrosporium* were measured. Patients with CRSwNP associated with current signs of purulent nasal discharge, chronic obstructive pulmonary disease, diffuse panbronchiolitis, fungal sinus disease, congenital mucociliary disease, or cystic fibrosis were excluded from this study. The control group consisted of 15 patients with pituitary tumor surgery (four males and 11 females, age range from 36 to 73 years, mean age of 55 years). The study was approved by the ethics committee of the Juntendo University Faculty of Medicine.

Sampling of Tissue and Pretreatment

Surgically removed human nasal polyps located in the middle meatus were obtained from the patients with CRSwNP, and the mucosa of the sphenoid sinus as a control were procured from patients with pituitary tumor. They were treated with 70% ethanol and physiologic saline to eliminate microorganisms outside of the nasal polyps. The samples were placed immediately in a 50% glycerol, then transferred to a −80°C freezer for storage until DNA extraction could be performed. At the same time, some of these samples were fixed in 10% formalin, embedded in paraffin wax, processed routinely, and stained with hematoxylin-eosin. According to our previous studies,[12,13] the eosinophilic and noneosinophilic groups were defined as eosinophil counts of the nasal polyps pf more and less 100/microacopic field (magnification ×400) using three fields, respectively. Eosinophils were quantified in the foci of the densest cellular infiltrate. The total number of eosinophils present with a 10 × 10-mm reticulate present in the eyepiece was determined as the count per high-power field. The samples for the DNPCs were treated within 30 minutes postoperatively.

Culture and Histology of Fungi

After the nasal polyps were disrupted with glass beads and 100 μL of Tris-EDTA (10 mM Tris-Cl, 1 mM ethylenediaminetetraacetic acid pH 8.0) using a homogenizer (BioMasher) (Takara Bio Inc., Otsu, Japan), and 1 μL of homogenized polyp samples were spread onto 5% sheep blood agar, chocolate agar, Columbia anaerobic blood agar, Drigalski agar, and Sabouraud agar, and incubated for 2 weeks at 35°C. All specimens were stained with Grocott methanamine silver staining for confirmation of the existence of fungal organisms.

PCR of Fungi in the Nasal Polyps

DNA was extracted from the tissue of homogenized nasal polyps. PCR amplification of fungal specific ribosomal DNA was performed using ITS-1F and ITS-4R primer, the PCR product covering the end of the 18SrDNA gene to the start of 26SrDNA gene and NL-1 and NL-4 primer, the PCR product covering D1/D2 26SrDNA (Table I). A universal fungal primer set designed by the National Institute for Occupational Safety and Health, FF2 and FR1 primers specific for the amplification of 18S rDNA, was also used. Amplification was performed in a TaKaRa PCR Thermal Cycler Dice Gradient (Takara Bio Inc., Otsu, Japan) according to the manufacturer's specifications.

Species Identification

PCR products were purified by the High Pure PCR Product Purification Kit (Roche, Indianapolis, IN). These amplifications were sequenced using ITS1F, ITS4R, NL1, NL4, FF2, and FR1 primers by a Big Dye Terminator V3.1 Cycle sequencing kit and ABI sequence analyzer 3730×I (Applied Biosystems Inc., Carlsbad, CA). Species identification was determined by a BLAST search(DNA Data Bank of Japan, Mishima, Japan).

Cell Cultures and Multiplex Immunoassay

DNPCs were prepared from nasal polyps of eosinophilic CRSwNPs in five randomly selected patients by enzymatic digestion, as described by Okano et al.[14] In flat-bottomed 24-well culture plates (Nunc, Roskiide, Denmark), 500 μL of 1×10^6/mL DNPCs were stimulated with serial concentrations (2 and 200 μg/mL) of *Candida parapsilosis, Rhodotorula mucilaginosa.* The crude extracts of *C parapsilosis* and *R mucilaginosa* were provided by Teikyo University Japanese Society for Medical Mycology Research Center. After the incubation, freeze-dried fungi were dissolved and sonicated in phosphate-buffered saline (PBS) with 0.2% NaN_3. Afterward, the extract was subjected to sterilizing filtration and lyophilization. The antigen extracts of fungus were dissolved with PBS (Sigma-Aldrich Inc., St. Louis, MO). As a control, DNPCs were cultured without antigen stimulation.

TABLE II.
Clinical Characteristics of Patients and Identified Fungal Species.

Patient	Sex/Age, yr	Blood Eosinophils	Blood IgE	RAST Fungus	Asthma	Eosinophilic Mucin	Tissue Eosinophils	Identified Fungal Species
1	M/47	420	38	−	+	+	58	*Candida parapsilosis*
2	M/36	508	332	+	+	+	148	*Candida parapsilosis*
3	F/74	301	65	−	+	−	111	*Rhodotorula mucilaginosa*
4	F/35	644	77	−	+	−	410	*Rhodotorula mucilaginosa*
5	M/45	328	162	−	+	−	184	*Rhodotorula mucilaginosa*
6	M/67	809	301	−	+	−	275	*Malassezzia restricta*
7	M/39	462	118	−	+	−	164	*Aspergillus* s/o
8	F/46	381	1,990	−	+	−	194	*Candida parapsilosis*
9	M/42	418	86	−	+	−	291	*Aspergillus gracillus*
10	M/44	675	51	−	+	−	650	*Candida parapsilosis*
11	M/49	428	517	−	−	−	198	*Rhodotorula mucilaginosa*
12	F/49	731	430	+	−	−	189	*Candida parapsilosis*
13	M/39	412	46	−	−	−	341	*Candida parapsilosis*
14	F/51	353	89	−	−	−	184	*Candida glabrata*
15	F/62	192	38	−	−	−	176	*Candida tropicalis*
16	F/55	280	1,882	+	−	−	266	*Candida parapsilosis*
17	M/49	420	104	−	+	+	232	None
18	M/45	328	162	−	+	−	124	None
19	F/60	468	191	−	+	−	158	None
20	M/32	538	25	−	+	−	175	None
21	M/66	271	83	−	−	−	18	None
22	F/34	795	5	−	−	−	91	None
23	F/56	99	24	−	−	−	14	None
24	M/25	152	118	−	−	−	12	None
25	F/60	1,372	191	−	−	+	158	None
26	F/58	413	65	−	−	+	186	None
27	M/63	567	515	+	−	+	58	None
28	F/57	413	104	−	−	−	153	None
29	M/42	246	250	−	−	−	111	None
30	M/44	292	91	−	−	−	128	None
31	F/52	270	287	−	−	−	36	None
32	M/76	7	11	−	−	−	27	None
33	F/63	384	528	+	−	−	146	None
34	F/34	30	1,409	+	−	−	148	None
35	M/43	150	500	+	−	−	66	None

F = female; IgE = immunoglobulin E; M = male; RAST = radioallergosorbent test.

The culture supernatant was collected after 72 hours and stored at −80°C, after which the levels of IL-4, IL-5, IL-6, IL-8, IL-13, IL-17A, IL-23, IL-25, IL-33, eotaxin, RANTES (regulated on activation normal T-cell expressed and secreted), tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured by Bio-Plex Suspension Array System (Bio-Rad Laboratories, Inc., Hercules, CA). Data were expressed as the fold change relative to that without stimulus of five cell cultures. Viability was assessed by the exclusion of trypan blue stain.

Statistical Analyses

Values are given as means ± standard errors for the multiplex immunoassay. Differences between the values were determined using the Student *t* test. Tukey's hinge was used in the comparison of eosinophils within nasal polyps. *P* values <.05 were considered significant. All analyses were conducted using Statmate IV for windows (ATMS Co., Ltd., Tokyo, Japan).

RESULTS

Culture and Histology of Fungi

There was no microbiological growth (including fungus) in the culture media of the homogenized nasal polyps. Furthermore, Grocott methanamine silver staining for the all nasal polyps showed no fungal bodies.

Detection and Identification of Fungal DNA

Sixteen of 35 samples of the nasal polyps showed amplification of fungal DNA. Using the D1/D2 domain of

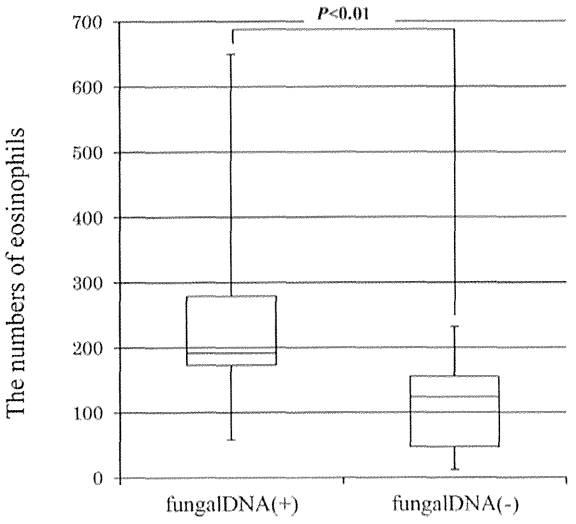

Fig. 1. Comparison of the numbers of tissue eosinophils with nasal polyps between positive samples and those negative for fungal DNA. The number of eosinophils in three fields with cell clusters were counted using light microscopy (×400 magnification). The box and whisker plots show the median and the interquartile range of the number. The whiskers extend to the maximum and the minimum data points.

the large subunit (26S) ribosomal DNA primer, NL1 / NL4, we found that 16 cellular tissues of the homogenized nasal polyps (100%) were positive for fungal DNA, whereas using the 18S rDNA primer, ITS1F/ITS4R amplified sequences four of the 16 (25%) were positive. On the other hand, using the universal fungal primer set, FF2 and FR1, no fungal DNA was detected. Incidentally, in none of the mucosa of the sphenoid sinus was fungal DNA detected. The amplification products obtained from 16 patients were sequenced to identify the detected species in the tissue of the homogenized nasal polyps, such as *C parapsilosis, R mucilaginosa,* and *Aspergillus* sp. Table II summarizes the data for all patients. The average number of eosinophils within the nasal polyps in which fungal DNA was detected was significantly higher than that in the absence of fungal DNA ($P < .01$, Fig. 1).

Cytokine Secretion Stimulated by Fungal Extracts

To determine the biological and immunological roles of the identified fungi in CRSwNP in the present study, five samples of DNPCs were stimulated by extracts of *C parapsilosis* and *R mucilaginosa*. The expression levels of each cytokine and chemokine are summarized and compared with those of the unstimulated group in Figure 2). The secretion levels of the cytokines were set to the maximal level of each standard curve because the levels were above the maximal levels of detection.

C parapsilosis stimulation of DNPCs significantly induced the secretion of IL-5, IL-6, IL-8, IL-13, RANTES, TNF-α, and GM-CSF. The most remarkable upregulation was observed in IL-6 (approximately 100-fold) and IL-8 (approximately 60-fold), followed by IL-5, TNF-α, RANTES, IL-13, and GM-CSF. IL-4, IL-17A, IL-23, and eotaxin showed no significant increase from stimulation by *C parapsilosis* extracts. The expression of IL-25, IL-33 and IFN-γ showed a decreasing trend. No significant differences were seen in the responses between 2 and 200 μg/mL of any cytokines.

The stimulation of DNPCs by *R mucilaginosa* resulted in a significant induction of IL-5, IL-8, IL-13, IL-17A, RANTES, TNF-α, and GM-CSF. Similar to *C parapsilosis*, *R mucilaginosa* most strongly upregulated the production of both IL-6 and IL-8, which was followed by IL-5, TNF-α, RANTES, IL-13, IL-17A, and GM-CSF. IL-4, IL-23, IL-25, IFN-γ, and eotaxin showed no significant production with stimulation of *R mucilaginosa* extract. On the other hand, *R mucilaginosa* inhibited IL-33 production. There was no significant difference in the cytokine releases induced by 2 and 200 μg/mL.

DISCUSSION

In the present study, we found fungal DNA in the nasal polyps in spite of the lack of detection of fungus using histology and culture. A variety of studies agree that PCR is superior to both culture and Grocott methanamine silver staining for detecting fungal elements.[15] Because the PCR studies showed the ubiquitous presence of fungi such as *Aspergillus, Penicillium, Cladosporium, Candida, Aureobasidium,* and *Alternaria* in the nose and paranasal sinuses of both CRS patients and healthy controls,[16–18] it is unlikely that fungal species and fungal load play a role in disease development.[19] Gosepath et al.[20] reported that fungal DNA was detected in all 27 CRS specimens with universal PCR primers. The differences in the detection rates of fungal DNA between Gospath et al.'s report and our study may be due to the sampling method, surgical specimens, or environmental factors. On the other hand, Rao et al.[21] reported that the proper design of PCR primers for fungi and the meticulous harvesting of nasal mucosa resulted in a low but significant incidence of fungi in CRS. The primer pairs NL1/NL4, which encode the D1/D2 domain of large subunit (26S) ribosomal DNA, have been widely used for the detection of fungal DNA and the identification of fungal species, as compared with two other primers, ITS1F/ITS4R and FF2/FR1.[22,23] No PCR products could be obtained from normal mucosa as negative controls. Positive and negative controls were correctly amplified in every experiment. Therefore, the three PCR amplified systems used here were appropriately operated. The exact reason why the detection rates of three PCR systems showed different values is not known. The large amount of human DNA extracted from the nasal polyps might have inhibited several fungus-specific PCR products, because fungi share the same class of ribosomal RNA with humans. We detected PCR products derived from four species of fungi including *Candida, Rhodotorula, Malasezzia,* and *Aspergillus* in the nasal polyps, which were never detected in the normal sinus mucosa. The *Rhodotorula* species are ubiquitous saprophytic yeast that can be recovered from many environmental sources. Previously reported as nonpathogenic, *Rhodotorula* species have emerged as opportunistic

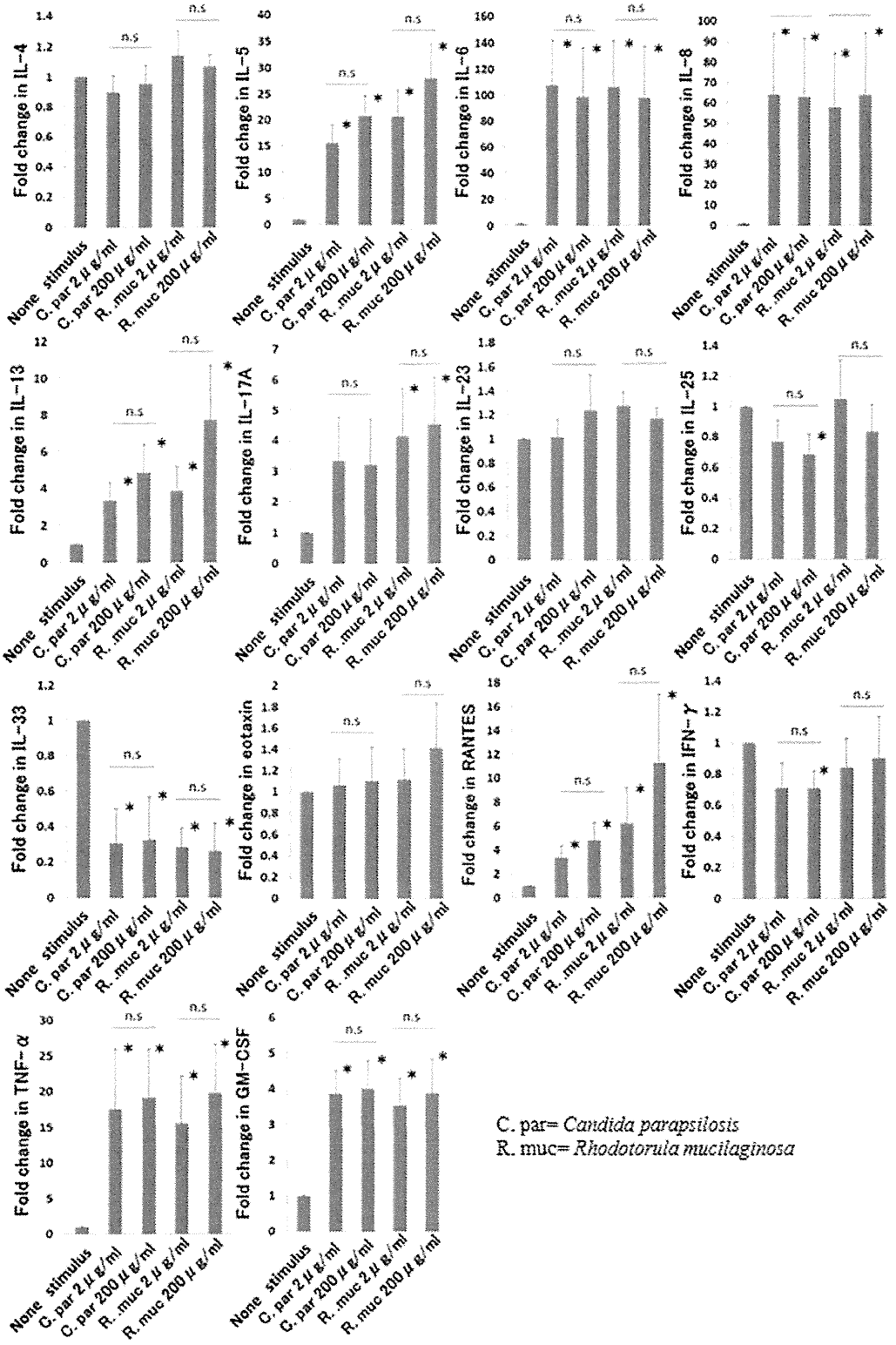

Fig. 2. Effect of extracts of *Candida parapsilosis* and *Rhodotorula mucilaginosa* on interleukin (IL)−4, IL-5, IL-6, IL-8, IL-13, IL-17A, IL-23, IL-25, IL-33, eotaxin, RANTES, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) production by dispersed nasal polyp cells. Five hundred microliters of 1×10^6/mL dispersed nasal polyp cells were collected after 72 hours, and the levels of the cytokines were determined by multiplex immunoassay. Data were expressed as the fold change relative to that without stimulus of five cell cultures (the means ± standard errors). Significant differences (*) were considered if *P* values were <.05. RANTES = regulated on activation normal T-cell expressed and secreted.

pathogens with the ability to colonize and infect susceptible patients.[24] *Malasezzia* is a monophyletic genus of fungi detected as a ubiquitous component of the human skin microbiome and is associated with a myriad of skin problems.[25] Although we attempted to observe the localization of the DNA of *Candida* and *Rhodotorula* using in situ hybridization in the nasal polyps, the experimental results failed to detect the fungal element, presumably due to the use of inappropriate probes. The fungal DNA would be identified within bacterial biofilms[26] and/or phagocytic vesicles of antigen-presenting cells such as macrophages and neutrophils in the nasal polyps.[27]

The detection rate of fugal DNA was significantly elevated in the eosinophilic CRS as compared with non-eosinophilic CRS, suggesting that the presence of fungi is closely related with eosinophil accumulation. Eosinophils are known to be prominent in the reaction to parasitic infections. A concentration-dependent increase in eosinophil migration toward both CRS nasal mucin and CRS nasal tissue extract was augmented as compared with the mucin of healthy controls.[5] Exposure of peripheral blood mononuclear cells to fungal antigen in vitro increased IL-5 and IL-13 production, whereas cells from normal controls did not respond.[4] A component of *Alternaria* was shown to degranulate eosinophils from CRS patients by acting on PARs. Moreover, activation of nasal epithelial cells with fungi resulted in the upregulation of PAR2 and PAR3 mRNA.[28] These findings may lead to a hypothesis that fungi on the sinus mucosal surface induce the production of cytokines, which promote eosinophil migration through the epithelial cells and other constitutive cells of the nasal polyps. Although we did not employ quantitative assays of PCR products, it was considered that the patients with more fungal DNA would have significantly higher eosinophil counts.

The final series of experiments aimed to evaluate immunological aspects of the detected fungi in the induction of eosinophilic inflammation. The fungal extracts derived from *Candida* and *Rhodotorula*, which were detected from the nasal polyps, apparently and remarkably upregulated a Th1/Th2 and Th1Th2/Th17 cytokine profile, respectively, in the ex vivo models of the nasal polyp in the present study. It would be desirable to have stimulated the cytokine-producing cells from healthy sphenoid control tissue. However, it is well known that healthy tissue does not contain eosinophils and very minimal amounts of lymphocytes if any. Thus, no quantitative comparison would have been useful. Protease activity contained in fungi can activate epithelial cells via their PARs. Kaufman et al.[29] showed that the interaction of protease present in fungal extracts from the inferior nasal conchae of nonatopic subjects led to morphologic change, cell desquamation, and the induction of proinflammatory Th1 cytokines. In addition, PAR2 stimulation in CRS patients did not induce the release of eosinophil attracting cytokines like eotaxins or RANTES.[30] Recent investigations are focusing on the contribution of Th17 cells to the pathology of and resistance to fungi. Zelante et al.[31] found that by affecting fungal clearance and by promoting chronic inflammation and tissue damage, Th17 responses at the mucosal surface had a detrimental effect on the course of fungal infections. Our previous study revealed that the infiltration of cells positive for both CD4 and IL-17A (Th17 cells) showed a significant correlation with the numbers of eosinophils and mucosal remodeling in Japanese CRSwNP.[12]

Another underlying mechanism that might explain the fungus-mediated secretion of cytokines and chemokines, such as IL-6 and IL-8, from DNPCs may involve toll-like receptors (TLRs) expressed on the sinonasal mucosa. The recognition of various pathogen-molecular patterns by the TLRs, including mannan, Candida and CpG DNA induces a cascade of downstream signaling that provokes an inflammatory cytokine profile.[32] A quantitative increase in TLR2 mRNA was seen in cystic fibrosis polyps as well as in CRS,[33,34] whereas in some studies[35,36] a decrease in mucosal TLR2 and TLR9 mRNA in samples from CRSwNP occurred. Despite inconsistencies in previous data, TLR signaling appears to play an important role in mediating host inflammation, with potential derangements contributing to the development of CRS.[37] Moreover, nucleotide-binding oligomerization domain-like receptors (NLRs) are newly discovered cytosolic receptors belonging to the pattern-recognition receptor family. NLR mRNA was found to be higher in nasal polyps than in normal nasal mucosa.[38] Further studies are required to elucidate the role of innate immunity in fungus-related CRS.

CONCLUSION

The present study offers direct evidence to support the notion that fungal elements modify the inflammatory responses in the nasal polyps of eosinophilic CRS, though the underlying mechanisms responsible for the eosinophil-based inflammation require further examination.

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Combinations of two odorants of smell identification test for screening of olfactory impairment

Hideaki Shiga [a,*], Junpei Yamamoto [a], Miwa Kitamura [a], Hideaki Nakagawa [b,c], Tomoko Matsubasa [d], Atsuko Seo [d], Takaki Miwa [a]

[a] Department of Otorhinolaryngology, Kanazawa Medical University, Ishikawa 920-0293, Japan
[b] Department of Epidemiology and Public Health, Kanazawa Medical University, Ishikawa 920-0293, Japan
[c] Health Control Center, Kanazawa Medical University Hospital, Ishikawa 920-0293, Japan
[d] Tokyo Gas Corporation Ltd., Tokyo 105-8527, Japan

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ABSTRACT

Objective: To determine whether combinations of two odorants of the Open Essence smell identification test can be used to screen for olfactory impairment in Japanese people.
Methods: A total of 243 Japanese subjects (142 males, 101 females; mean age, 37.5 years; age range, 20–62 years) were enrolled in the study. The main outcome measures were the results of olfactory testing by using the full 12 odorants (condensed milk, cooking gas, curry, cypress wood (Japanese cypress, *hinoki*), India ink, Japanese orange (*mikan*), menthol, perfume, roasted garlic, rose, sweaty-smelling clothes, and wood) of the Open Essence test as well as combinations of two odorants of the Open Essence test, and the results of self-reported questionnaires addressing awareness of a smell disorder, history of sinunasal disease, self-reported nasal obstruction, and history of smoking.
Results: In screening with combinations of two odorants, the highest positive likelihood ratio (19.1) was obtained with the cypress wood and India ink odorants. All subjects correctly identified the curry odorant. Combinations of other odorants also had high positive likelihood ratios (India ink and sweaty-smelling clothes, 17.6; perfume and sweaty-smelling clothes, 14.7; cypress wood and roasted garlic, 14.1; cypress wood and rose, 13.2; cypress wood and perfume, 11.0; cypress wood and wood, 10.7).
Conclusion: The combination of cypress wood and India ink odorants may be useful for detecting individuals with olfactory impairment among subjects who can correctly identify the curry odorant.

1. Introduction

Impaired olfaction significantly increases the risk of serious accident or disease [1]. Simple odor presentation devices have been developed for the screening of olfactory impairment in Japan. The Odor Stick Identification Test for the Japanese (OSIT-J; Daiichi Yakuhin Sangyo, Tokyo, Japan) [2] has proven useful for identifying patients with olfactory impairment in otorhinolaryngological clinics [3,4]. The OSIT-J consists of 12 odorants that are familiar for Japanese people [2]. Miwa et al. [5,6] have shown that correct identification of the rose, curry, or sweaty-smelling clothes odorants in the OSIT-J has a statistically significant relationship with assessments made using the T&T olfactometer threshold test, which is the standard olfactory function test used in Japan. Furthermore, the curry odorant in the OSIT-J is more effective than the rose or sweaty-smelling clothes odorants, or a combination of these two odorants, for the detection of olfactory impairment in Japanese people [7]. However, screening for olfactory impairment is not widely conducted in Japan because the OSIT-J could hardly be applied for the self-reported test.

The Open Essence smell identification test (Wako, Japan) was developed to address the deficiencies of the OSIT-J [8,9]. Both the Open Essence test and the OSIT-J use the same 12 odorants and a four-plus alternative forced-choice paradigm; however, the Open Essence test is suitable for self-reporting because the odorants are contained within simple, sealed test cards rather than in sticks (Fig. 1). Subjects and experimenters reported the Open Essence test to be easier, shorter, more interesting, and more convenient to conduct than the OSIT-J [9]. In Japanese subjects, the scores from

* Corresponding author at: Department of Otorhinolaryngology, Kanazawa Medical University, 1-1 Daigaku, Uchinadamachi, Kahokugun, Ishikawa 920-0293, Japan. Tel.: +81 76 218 8147; fax: +81 76 286 5566.
E-mail address: shigah@kanazawa-med.ac.jp (H. Shiga).

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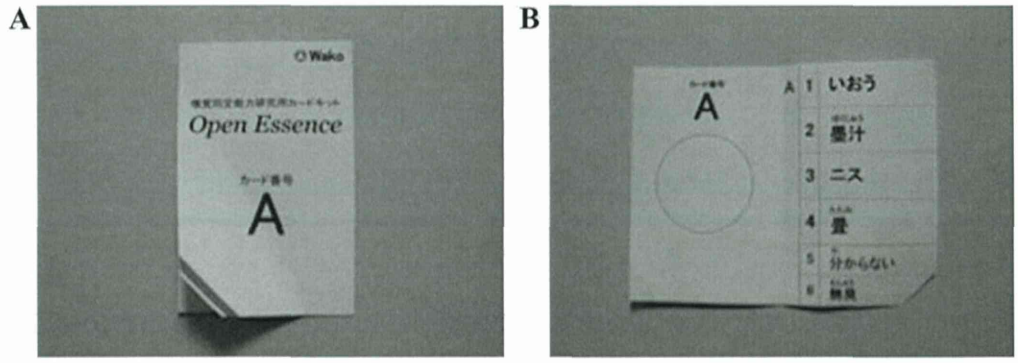

Fig. 1. Photographs of an Open Essence smell identification test card. (A) Front and (B) inside: left, the area within the printed circle is impregnated with the odorant; right, four odor name choices as well as "do not know" and "no smell detected" are listed in Japanese.

the Open Essence test are significantly correlated with the odor recognition threshold obtained by using the T&T olfactometer threshold test [8,9].

Here, we assess the odorants used in the Open Essence test for their usefulness in screening for olfactory impairment in Japanese subjects. We show that screening with the combination of cypress wood and India ink odorants of the Open Essence test is the most effective combination for identifying olfactory impairment in Japanese subjects who can correctly identify the curry odorant.

2. Subjects and methods

2.1. Subjects

A total of 243 Japanese subjects (142 males, 101 females; 74 recipients of an executive check-up at Kanazawa Medical University Hospital, 110 medical workers employed at Kanazawa Medical University Hospital, and 59 medical students enrolled at Kanazawa Medical University; mean age, 37.5 years; age range, 20–62 years) participated in this study. The protocol for this study was reviewed and approved by the clinical research ethics committee of Kanazawa Medical University Hospital. Informed consent was obtained from all subjects prior to the study.

2.2. Open Essence

The Open Essence test (Fig. 1) uses 12 odorants that are familiar to Japanese people [8,9]. These odorants are described as condensed milk, cooking gas, curry, cypress wood (Japanese cypress, *hinoki*), India ink, Japanese orange (*mikan*), menthol, perfume, roasted garlic, rose, sweaty-smelling clothes, and wood. Subjects received the odor cards from the experimenter, opened them, and then sniffed and identified the odorant in a four-plus alternative forced-choice paradigm. The order in which the odorants were presented was randomized.

2.3. Self-reported questionnaires

The subjects were asked to complete questionnaires addressing awareness of a smell disorder, history of sinunasal disease, self-reported nasal obstruction, and history of smoking.

2.4. Statistical analysis

Fisher's exact test (two-tailed) (Prism 4; GraphPad, San Diego, CA, USA) was used to determine the differences between scores for each odorant, screening with combinations of two odorants, and scores for the full 12 odorants. Fisher's exact test (two-tailed) was also used to compare the results of the identification of cypress wood and India ink odorants with the results of the self-reported questionnaires. A P value less than 0.05 was considered statistically significant.

Positive likelihood ratios (Prism 4, GraphPad) were calculated to determine how many times more likely subjects who could not correctly identify one odorant or a combination of two odorants of the Open Essence test are to have scores of 7 correct answers or less for the full 12 odors of the Open Essence test compared with subjects who could correctly identify the odorant or the combination of two odorants.

3. Results

3.1. Distribution of the total number of correct answers in the full Open Essence screening

The distribution of the total number of correct answers in the full Open Essence screening of 243 subjects is shown in Fig. 2 (mean ± SD, 8.97 ± 1.63). It was previously reported that the mean total score for the full 12 odors of the Open Essence test in Japanese subjects with normal olfactory function was greater than 7 correct answers, whereas that in Japanese subjects with slight, moderate, or severe hyposmia, or anosmia was 7 correct answers or less than 7 correct answers [8]. Furthermore, Fujio et al. [10] have suggested that scores of 8 or higher on the Open Essence test should be judged as normal. Therefore, subjects with 7 correct answers or less were classified as having olfactory impairment and subjects with 8 correct answers or more were classified as having normal olfaction.

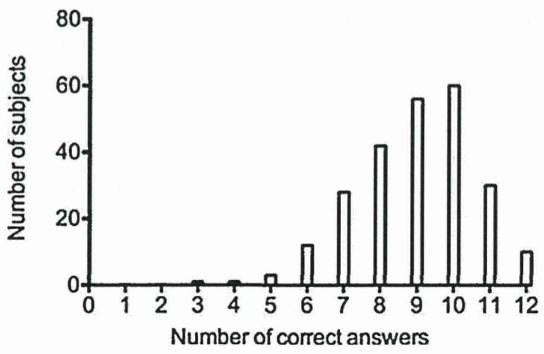

Fig. 2. Distribution of correct answers in the Open Essence screening. Subjects with 7 of 12 correct answers or less were classified as having olfactory impairment. Patients with 8 of 12 correct answers or more were classified as having normal olfaction.

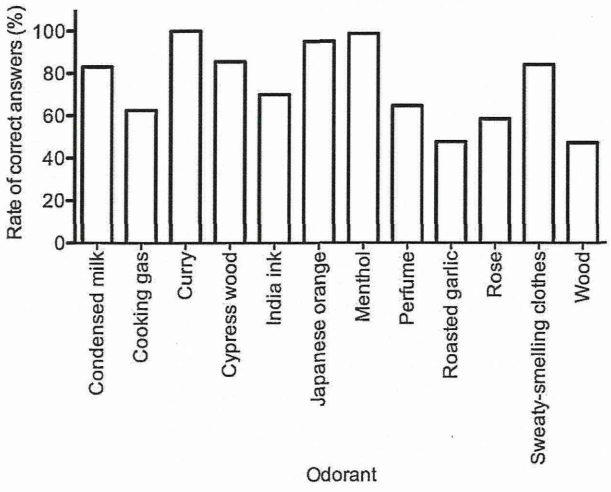

Fig. 3. Rate of correct answers for each odorant of the Open Essence test. All 243 subjects assessed in the present study correctly identified the curry odorant.

3.2. Rate of correct answers for each odorant of the Open Essence test

The rate of correct answers for each odorant of the Open Essence test is shown in Fig. 3. All 243 subjects assessed in the present study gave the correct answer when tested with the curry odorant. Three of the 243 subjects did not correctly identify the menthol odor, and these three subjects all scored 7 correct answers or less when screened with the full 12 odors.

3.3. Correlation between the scores for each odorant and the scores for the full 12 odorants of the Open Essence test

The identification of each odorant of the Open Essence test was correlated with the scores for the full 12 odors (Table 1). The correlation between the identification of the curry or menthol odorants and the full 12 odorants was not assessed because all 243 subjects gave correct answers when tested with the curry odorant and most of them gave correct answers when tested with the menthol odorant. Screening with the cypress wood odorant had the highest positive likelihood ratio (5.2; sensitivity, 42.2%; specificity, 91.9%).

3.4. Screening with combinations of two odorants of the Open Essence test

The positive likelihood ratios obtained by screening with combinations of two odorants from 10 odorants (excluding curry and menthol) of the Open Essence test are listed in Table 2. Screening with the cypress wood and India ink odorants had the highest positive likelihood ratio (19.1; sensitivity, 28.9%; specificity, 98.5%). Combinations of other odorants also had high positive likelihood ratios (India ink and sweaty-smelling clothes, 17.6; perfume and sweaty-smelling clothes, 14.7; cypress wood and roasted garlic, 14.1; cypress wood and rose, 13.2; cypress wood and perfume, 11.0; cypress wood and wood, 10.7). In the 240 participants who could correctly identify the menthol odorant, a high positive likelihood ratio was obtained for the screening with the cypress wood and India ink odorants (20.4; sensitivity, 31.0%; specificity, 98.5%).

Table 1
Screening with single odorants of the Open Essence test in 243 subjects.

Odorant	Positive likelihood ratio	Sensitivity (%)	Specificity (%)
Cypress wood	5.2	42.2	91.9
Japanese orange	3.1	11.1	96.5
India ink	2.7	62.2	77.3
Cooking gas	2.5	73.3	70.7
Sweaty-smelling clothes	2.5	31.1	87.4
Condensed milk	2.3	31.1	86.4
Rose	2.2	75.6	66.2
Perfume	2.1	62.2	70.7
Wood	1.8	82.2	54.0
Roasted garlic	1.7	77.8	53.5

The positive likelihood ratios obtained by screening with combinations of cypress wood, India ink and one odorant from eight odorants (excluding curry and menthol) of the Open Essence test were also assessed. Combinations of three odorants including cypress wood and India ink did not have high positive likelihood ratios (cypress wood, India ink and sweaty-smelling clothes, 5.5; cypress wood, India ink and roasted garlic, 6.0; cypress wood, India ink and rose, 6.2; cypress wood, India ink and perfume, 6.1; cypress wood, India ink and wood, 6.2; cypress wood, India ink and Japanese orange, 5.6; cypress wood, India ink and cooking gas, 6.0; cypress wood, India ink and condensed milk, 5.8).

Our results suggest that amongst subjects who could identify the curry odorant, screening with a combination of cypress wood and India ink odorants is the most reliable method for the detection of those subjects who might have low scores when screened with the full 12 odorants of the Open Essence test.

3.5. Correlation between the identification of cypress wood or India ink odorants with the results of the self-reported questionnaire

The correlations between the identification of cypress wood or India ink odorants with the results of the self-reported questionnaire addressing awareness of a smell disorder, history of sinunasal disease, self-reported nasal obstruction, and history of smoking are shown in Table 3. Correct identification of the combination of the

Table 2
Screening with combinations of two odorants in 243 subjects (positive likelihood ratio).

	India ink	Wood	Perfume	Japanese orange	Cooking gas	Rose	Cypress wood	Sweaty-smelling clothes	Condensed milk	Roasted garlic
India ink		4.6	4.1	2.2	5.7	6.6	**19.1**	17.6	8.8	5.1
Wood			4.6	7.3	5.0	3.8	**10.7**	4.8	3.1	2.8
Perfume				8.8	6.1	4.4	**11.0**	**14.7**	5.1	6.0
Japanese orange					Low	8.8	Low	Low	Low	4.4
Cooking gas						8.4	9.7	3.7	8.8	3.7
Rose							**13.2**	4.0	3.5	3.6
Cypress wood								Low	4.4	**14.1**
Sweaty-smelling clothes									4.4	4.0
Condensed milk										4.4

Low: <1.0.

Table 3
Comparison of the identification of cypress wood or India ink odorants with the results of the self-reported questionnaire in 243 subjects.

Self-reported question	Cypress wood or India ink	Cypress wood and India ink	*P* value
	No. correct	No. incorrect	
Awareness of a smell disorder			
(+)	29	5	0.06
(−)	198	11	
History of sinunasal disease			
(+)	64	3	0.57
(−)	157	13	
Self-reported nasal obstruction			
(+)	16	1	>0.99
(−)	208	15	
Smoking history			
(+)	40	5	0.19
(−)	186	11	

(+), yes; (−), no.

cypress wood and India ink odorants was not significantly correlated with awareness of a smell disorder ($P = 0.06$), history of sinunasal disease ($P = 0.57$), self-reported nasal obstruction ($P > 0.99$), or history of smoking ($P = 0.19$).

4. Discussion

The main result of the present study is that screening with the combination of the cypress wood and India ink odorants of the Open Essence test is effective for the detection of subjects who might have low scores (7 correct answers or less than 7 correct answers) when screened with the test's full 12 odorants.

Nishida et al. [9] reported that the full Open Essence test (12 odorants) takes about 5 min to conduct both in patients with olfactory impairment and in healthy volunteers. Therefore, to reduce the total screening time, instead of using the full 12 cards of the Open Essence test package, examiners could instead use combinations of two odorants of the OSIT-J.

Furthermore, a test that uses a smaller number of the individually sealed Open Essence test cards would not only be quick to conduct but would be suitable for self-reporting. Since the prevalence of olfactory impairment is yet to be studied in the Japanese population, a large-scale population-based study of olfactory impairment could be conducted by mailing Open Essence test cards to participants. Therefore, we propose that individual Open Essence test cards be made available in the future.

In the present study, we did not assess subjects with a T&T olfactometer after the Open Essence test screening. Previous reports suggest that scores of 8 or higher on the Open Essence test should be judged as normal [8,10]. Further investigation with a T&T olfactometer is warranted in the participants who did not identify the cypress wood and India ink odorants to determine whether of not they had hyposmia or anosmia.

Previously, it was shown that the curry odorant of the OSIT-J is the most effective of the 12 OSIT-J odorants for detecting individuals with olfactory impairment in a screening of 83 Japanese participants at an executive check-up [7]. However, all 243 subjects assessed in the present study correctly identified the curry odorant. The mean age (37.5 years) of the 243 subjects assessed in the present study was younger than the mean age (50.0 years) of the 83 Japanese participants at the check-up previously assessed [7]. Okutani et al. [11] have shown that all participants recognized the curry odorant of the Open Essence test in a cohort of young, healthy volunteers. Curry odor may be familiar for most Japanese people because curry is a very popular food in Japan, and it is possible that the curry odorant contains odors that intensely stimulate olfactory neurons. Further investigation is warranted to test whether the curry odorant of the Open Essence test is effective for the detection of older individuals with olfactory impairment and whether it can be used as first-line screening to detect severe hyposmia or anosmia in Japanese people.

Screening with the cypress wood odorant had the highest positive likelihood ratio in the screening with a single odorant of the Open Essence test. The rate of correct identification of the cypress wood odorant was 85.6% in the 243 subjects assessed. Previously, it was shown that there was no significant difference between US and Japanese subjects' identification rates for the cypress wood odorant of the OSIT-J, even though only 40% of the US subjects selected the correct answer as "wood" [12]. Cypress wood odor may be familiar for most Japanese people because cypress wood is a very popular building material in Japan. A simple odor identification test that includes a cypress wood odorant warrants further investigation with subjects from outside of Eastern Asia.

Previously, it was shown that there was a significant difference between US and Japanese subjects' identification rates for the India ink odorant of the OSIT-J [12]. Therefore, the India ink odorant of the Open Essence test should be replaced with another odorant for subjects from outside of Eastern Asia.

In conclusion, screening with a combination of two odorants of the Open Essence test, namely the cypress wood and India ink odorants, is useful for the detection of Japanese individuals with olfactory impairment among subjects who can correctly identify the curry odorant.

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Conflict of interest

The authors declare that they have no competing financial interests.

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