分子病理学教員　指導に当たって

指導教員：北澤荘平、北澤理子、原口竜摩

北澤荘平（教授）

愛大に 2010 年 6 月に赴任して来たときには、医科学研究（基礎配属実習）の学生は誰もいない状態でした。初年度の病理学総論・各論講義実習を行った学年が、次の年からの医科学研究受入の第一号となりました。幸い病理には希望者が多く、精一杯頑張って 9 名の学生を受け入れ、スタートすることとなりました。指導に当たる教員は、当初私と、助教であった原口先生だけでしたが、皆やる気のある優秀な学生が集まってくれ、なんとかスタート出来ました。学生研究員制度が始まる前でありましたが、スタート当初より全員が学会発表、論文発表を最終目的として、各自が独立した個別のテーマで研究・症例解析に取り組むこととなりました。私は、そもそも研究者としての才能やセンスは、持って生まれたものが大きく、教育が関与できることは限られていると思っております。個人の「独創性」を他者が「教育」によって指導すると言うことは矛盾したことではないでしょうか。指導者として出来ること、大切なことは、研究方法や研究室の正しいルールと作法をきちんと伝えること、そして、論文を書くときには、雑誌の投稿規定、即ち相手のルールに従って、基本的な構文で定型的な英作文することを伝えることであると思っております。私は、『詩人が詩人であるためには、「俺の心の中には詩があふれている」ではだめで、詩集を出してこそ、初めて詩人として認識されるのですよ』と学生に言っています。多くの医学生が、やがて臨床医としての道を進むものと思います。山中伸弥先生の例を見ても、早くから研究に親しむことだけが、研究者としての才能・独創性を伸ばすことにはならないとは思いますが、学生には、医学研究者あるいは医学研究を行う期間をもつという人生の選択肢を持ち続けてもらいたいと願っております。9 名の学生は、これまでに 8 名が学生研究員になり、既に 6 名の論文が英文誌に掲載されています。このような体験を通じて学生の選択肢が広がることを願っております。最後に、このようなシステムをサポートしていただきました田中潤也先生をはじめとする諸先生方に心より感謝申し上げます。

http://www.m.ehime-u.ac.jp/school/pathology1/index.html

北澤理子（特任教授）

私の初夢

当時の 3 年生 9 名が分子病理学分野で活動を始めた頃、私はまだ、愛媛と神戸を往復して暮らしていた。教授が着任して 2 年目、「君たちが主力になってほしい」と研究室の命運を託された学生達は、ほどなく、免疫染色を習得し、病理組織標本からの PCR クローニング･DNA シークエンスを行うようになった。その前年度は、改修工事の間に神戸の研究室で原口先生達と技術研修を行い、機材を愛媛大学に移送した。研究拠点の移動に

は労力を要したので、愛媛大学で若い世代が襷を繋いでくれることは、本当に有り難い。また病理学会などの学生発表にも同行し、医学生らしくなっていく姿を見ることは楽しかった。

平成 24 年 4 月に、私は本学に異動し、本年 1 月より特任教授を拝命した。分子病理学には次の 3 年生 5 名、2 年生 3 名、1 年生 5 名が加わり、すっかり賑やかになった。

そんなある日のこと、ふと気づくと、私は 1 年生の村田さん木村さんたちと、ストレッチャーを押していた。重くて難儀していると、顔見知りの 2 年生が助けてくれた。私は「社会人枠」で入学した医学科 1 年生で、彼女らと打聴診や腹部超音波など、診療手技の実習をしていたのだ。昔取った杵柄のはずが、最新の機器にいささか戸惑っていた。汗だくで勉強や実習に取り組むのは楽しかったが、医科学研究に入る前に目が覚めて、夢だと気づいた。

せっかく特任教授になったのに、振り出しに戻って 1 年生からやり直すとは何ともシュールな夢ではあったが、学生達に何かを教えるためには、立ち止まることなく歩き続け、彼らの声を聞いていこうと心に誓った。

原口竜摩（特任講師）

一昨年の春から、分子病理学教室に配属となった学生 9 名と一緒に研究をできたことを大変うれしく思います。医科学研究スタート当初は、教員の数に比べて学生の数が圧倒的に多く、正直不安がありました。しかし、ピペットの扱い方など実験に関する知識がゼロに近い状態から、指導教員の教えを守りながら実験をし、研究を着実にすすめていく姿を見て、それが杞憂であると気づくのに時間はかかりませんでした。医科学研究の終盤に差し掛かる頃には、ある種の頼もしさすら感じていたことを覚えています。学業と両立させながら、一つの研究テーマをまとめあげるのは非常に大変だったと思います。学生の皆さん、本当におつかれさまでした。

学生研究の研究成果リスト

分子病理学

1. 学会発表

中川みく、牧田憲二、桑原菜都美、原口竜摩、北澤理子、北澤荘平

アガロースビーズ法による微小組織切片からの遺伝子解析法：胃底腺ポリープと異所性胃粘膜のβカテニン変異

日本病理学会 2012年4月（東京）

藤石　琴、永井由紗、渡部貴文、板東健次、原口竜摩、北澤理子、北澤荘平

9 年の経過中に MYD88 遺伝子の変異が生じた悪性リンパ腫の一症例

日本病理学会 2012 年 4 月（東京）

吉田圭祐、薦田宗則、伊藤千尋、原口竜摩、北澤理子、北澤荘平

前立腺原発のびまん性大細胞型 B 細胞リンパ腫 (DLBCL) non-germinal center B cell-like (non-GCB) 亜型の一例

日本病理学会 2012 年 4 月（東京）

牧田憲二、北澤理子、仙波秀峰、中川みく、藤石　琴、 原口竜摩、北澤荘平

バレット食道の「化生-異形成-癌」進行過程における CDX2 発現とそのプロモータメチル化の関係

日本癌学会総会 2012 年 9 月（札幌）

永井由紗、北澤理子、中川みく、薦田宗則、近藤武史、原口竜摩、北澤荘平

多系統萎縮症 (Multiple-System Atrophy)の一剖検例

第 58 回日本病理学会 秋期特別総会 2012 年 11 月（名古屋）

薦田宗則、北澤理子、牧田憲二、吉田圭佑、竹治みゆき、曽我美子、倉田美恵、原口竜摩、北澤荘平

糖尿病に合併した難治性肺高血圧症の一剖検例

第 58 回日本病理学会 秋期特別総会 2012 年 11 月（名古屋）

2. 論文発表

Nagai Y, Kitazawa R, Nakagawa M, Komoda M, Kondo T, Haraguchi R,

Kitazawa S.
Multiple-system atrophy in long-term professional painter: a case report.
Case Reports in Pathology, 2012, 2012:613180. doi: 10.1155/2012/613180. Epub 2012 Jun 19 (5 pages).

Ito C, Kitazawa R, Makita K, Watanabe T, Toda A, Haraguchi R, Tanaka S, Kitazawa S.
Scrotal cutaneous verruciform xanthoma with monocyte chemoattractant protein-1 immunohistochemical study: a case report.
Journal of Medical Case Reports, 2012, 31;6(1):260. doi: 10.1186/1752-1947-6-260 (4 pages).

Makita K, Kitazawa R, Fujiishi K, Nakagawa M, Haraguchi R, Kitazawa S.
Cdx2 expression and its promoter methylation during metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus.
World Journal of Gastroenterology, 2013 Jan 28;19(4):536-41. doi: 10.3748/ wjg. v19. i4. 536.

Nakagawa M, Kitazawa R, Kuwahara N, Yoshida K, Haraguchi R, Kitazawa S.
Efficient genetic analysis of microdissected samples by agarose-bead method: alterations of beta-catenin gene in fundic gland polyp and heterotopic gastric mucosa of duodenum.
Acta Histochemica et cytochemica, 2013, in press.

Komoda M, Kitazawa R, Makita K, Yoshida K, Takeji M, Soga Y, Kurata M, Haraguchi R, Kitazawa S.
Pulmonary hypertension associated with diffuse deposition of pentosidine in pulmonary arterioles.
Diabetes Research and Clinical Practice, in press, doi.org/10.1016/j.diabres. 2013.01.019

Kuwahara N, Kitazawa R, Fujiishi K, Nagai Y, Haraguchi R, Kitazawa S.
Gastric adenocarcinoma arising in gastritis cystica profunda presenting with

selective loss of KCNE2 expression.
World Journal of Gastroenterology, in press.

北澤荘平, 近藤武史, 中川みく, 藤石　琴, 原口竜摩, 北澤理子.
『技術講座』（病理） 病理標本を用いた DNA シークエンスの方法と実際.
検査と技術 40 巻 1 号 24-29. 2012.

3. 学内発表会発表リスト
伊藤千尋、牧田憲治、渡部貴文、原口竜摩、北澤理子、北澤荘平
皮膚疣状黄色腫の免疫組織化学的検討
第 10 回医科学研究発表会

永井由紗、北澤理子、中川みく、薦田宗則、原口竜摩、北澤荘平
①多系統萎縮症(Multiple-System Atrophy)剖検例の解析
②破骨細胞分化因子受容体(RANK)splicing variant の解析
第 10 回医科学研究発表会

中川みく、牧田憲治、桑原奈都美、北澤理子、原口竜摩、北澤荘平
アガロースビーズ法による微小組織切片からの遺伝子解析法：
胃底腺ポリープと異所性胃粘膜の β カテニン変異
第 10 回医科学研究発表会

薦田宗則、牧田憲二、吉田圭佑、北澤理子、竹治みゆき、曽我美子、倉田美恵、
糖尿病合併症としての難治性肺高血圧症発症メカニズムの解析
原口竜摩、北澤荘平
第 10 回医科学研究発表会

藤石琴、永井由紗、渡部貴文、坂東健次、原口竜摩、北澤理子、北澤荘平
9 年の経過中に MYD88 遺伝子の変異が生じた悪性リンパ腫の一症例
第 10 回医科学研究発表会

牧田憲二、中川みく、藤石　琴、北澤理子、原口竜馬、北澤荘平
バレット食道の「化生-異形成-癌」進行過程における CDX2 発現と遺伝子 5’側

上流のメチル化との関係
第 10 回医科学研究発表会

Hindawi Publishing Corporation
Case Reports in Pathology
Volume 2012, Article ID 613180, 5 pages
doi:10.1155/2012/613180

Case Report

Multiple-System Atrophy in Long-Term Professional Painter: A Case Report

Yusa Nagai,[1] Riko Kitazawa,[1] Miku Nakagawa,[1] Munenori Komoda,[1] Takeshi Kondo,[2] Ryuma Haraguchi,[1] and Sohei Kitazawa[1]

[1] *Division of Molecular Pathology, Ehime University Graduate School of Medicine, Shitsukawa, Ehime, Toon City, 791-0295, Japan*
[2] *Department of Legal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*

Correspondence should be addressed to Sohei Kitazawa, kitazawa@m.ehime-u.ac.jp

Received 26 March 2012; Accepted 15 May 2012

Academic Editors: C. A. Palmer, Z. Schaff, and D. Vlachodimitropoulos

Introduction. Multiple system atrophy (MSA) is a rare and severe adult-onset, sporadic, and progressive neurodegenerative disorder. Here, we describe an autopsy case of MSA in a long-term professional painter. Although typical glial cytoplasmic inclusion (GCI) was not observed in a routine histological examination, strong α-synuclein immunostaining in the nucleus confirmed the diagnosis of MSA. *Case Presentation*. A 48-year-old Japanese man with a long occupational history of professional painter was sent to the emergency room, where he died of multiple organ failure. The patient had suffered tremors and inarticulateness at age 28, developed diabetes at 42 and was diagnosed with spinocerebellar degeneration at 46. A histopathological examination showed severe neuronal loss, gliosis, and tissue rarefaction in the paleostriatum, striate body of the substantia nigra, the pons, and the olivary nucleus of the upper medulla oblongata, intermediolateral of the spinal gray matter (sacral region). α-synuclein-positive GCI in oligodendroglia was occurred in the cerebral cortex, the midbrain, the medulla oblongata, and the spinal cord. These findings confirmed the presence of multiple-system atrophy (OPCA+SDS). *Conclusion*. Although the pathogenesis of MSA is still unclear, prolonged, and extensive exposure to organic solvents, together with a hyperglycemic morbidity attributed to diabetes, may have contributed to the onset and clinical course of the present case.

1. Introduction

Multiple system atrophy (MSA) is a rare and severe sporadic progressive neurodegenerative disorder of adult onset (average age at onset: 55–65 years), more frequent in men (1.3 : 1), and includes striatonigral degeneration (SND), Shy-Drager syndrome (SDS), and sporadic olivopontocerebellar atrophy (OPCA). The term MSA, comprising SND, SDS, and OPCA as one entity, was first introduced by Graham and Oppenheimer in 1969 [1]. MSA is now subclassified into two categories: associated, in varying proportions, with parkinsonism that is poorly responsive to levodopa therapy (MSA-P) and cerebellar dysfunction (MSA-C).

Glial cytoplasmic inclusion (GCI), designated as the histopathological hallmark of MSA in 1989 [2, 3], is an aggregated form of undigested α-synuclein characteristically observed in motor systems, the supraspinal autonomic sections, the putamen, the pallidum, and the lateral part of caudate nucleus, marking MSA as one in the category of α-synucleinopathy.

Here, we describe autopsy findings of MSA in a long-term professional painter. Although typical GCI was not observed in a routine histological examination, strong α-synuclein immunostaining in the nucleus confirmed the diagnosis of MSA.

2. Case Presentation

A 48-year-old Japanese man, a professional painter, was admitted to the emergency room. His past history revealed that at age 28, he consulted a general practitioner with the chief complain of tremors and inarticulateness, and followup showed intoxication from organic solvents. At age 42, he was diagnosed with diabetes and received regular outpatient treatment. Mitochondria-related neuropathy was ruled out through careful family history taking and a series of tests.

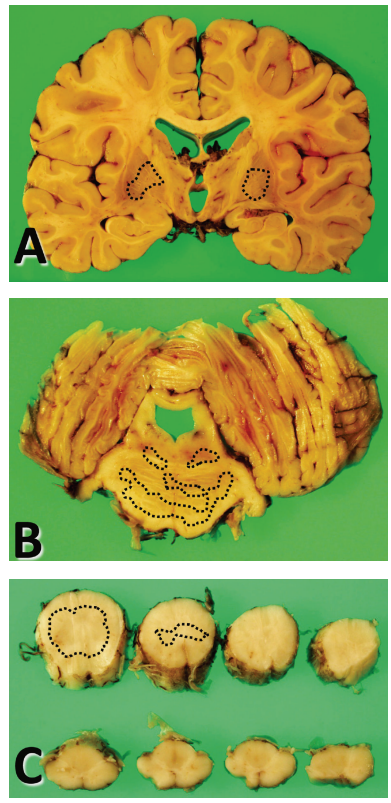


Figure 1: (A–C) Macroscopic findings. Severe atrophy of the paleostriatum together with dilatation of the lateral ventricle is evident in the coronal sections, while the thickness of the cerebral cortex is relatively intact (A). The brainstem, the gray matter of the cerebellar hemisphere (B), and the spinal cord (C) also show marked atrophy.

By then he had started to use a wheelchair because of gait disorders, and complained of amnesia. At 46, the patient was diagnosed with spinocerebellar degeneration through extensive neurological tests. The symptoms gradually progressed to repeated episodes of aspiration pneumonia. The patient was found unconscious with cardiopulmonary arrest attributed to aspiration at supper, and despite attempts at resuscitation, he died of multiple organ failure. The autopsy was done 12 hours thereafter.

Postmortem examination revealed the body of a corpulent man, 167 cm tall, weighing 67.8 kg (BMI 24.8). Traumatic scars were noted in the left temporoparietal area, the precordium, and the right forearm. Marked disuse atrophy was observed in the extremities, especially in the legs. Observed also, besides severe lung congestion (left: 390 g and right: 520 g), were significant amounts of food debris and expectoration in the trachea and bronchi, with rubefaction and hemorrhagic change in the mucous membrane, and reflux esophagitis with numerous erosions in the mucous membrane of the stomach. The clinical diagnosis of repeated aspiration pneumonia was thus confirmed, and the direct cause of death was evidently due to suffocation by misswallowing. Furthermore, fatty liver (1815 g), pancreatic swelling (105 g), a moderate degree of aortic atherosclerosis, and marked accumulation of visceral fat reflected the long-standing history of type 2 diabetes mellitus. A neurogenic bladder with an expanded and trabeculated wall was also noted.

2.1. General Neuropathological Findings. Gross examination of the brain (1300 g) revealed a highly atrophic brainstem, cerebellum, and spinal cord (Figures 1(B) and 1(C)), while the thickness of the cerebral cortex was almost intact (Figure 1(A)). Severe atrophy of the paleostriatum together with dilatation of the lateral ventricle was observed in coronal sections (Figure 1(A)). Additionally, the cerebellar hemisphere demonstrated fading in the gray matter (Figure 1(B)). On the other hand, no depigmentation of the substantia nigra or of the locus coeruleus was observed in sections of the brainstem (Figures 1(B) and 1(C)).

A histopathological examination showed severe neuronal loss, gliosis, and tissue rarefaction in various areas, predominantly in the paleostriatum, the striate body of the substantia nigra, the pons, and the olivary nucleus of the upper medulla oblongata, intermediolateral of the spinal gray matter (sacral region) (Figure 2), all of which suggested multiple system atrophy (OPCA+SDS).

The expression and localization of α-synuclein in the cerebral cortex, the midbrain, the medulla oblongata, and the spinal cord was determined by immunohistochemical analysis of the relevant formalin-fixed and paraffin-embedded specimens. Sections (4-μm thick) were deparaffinized in

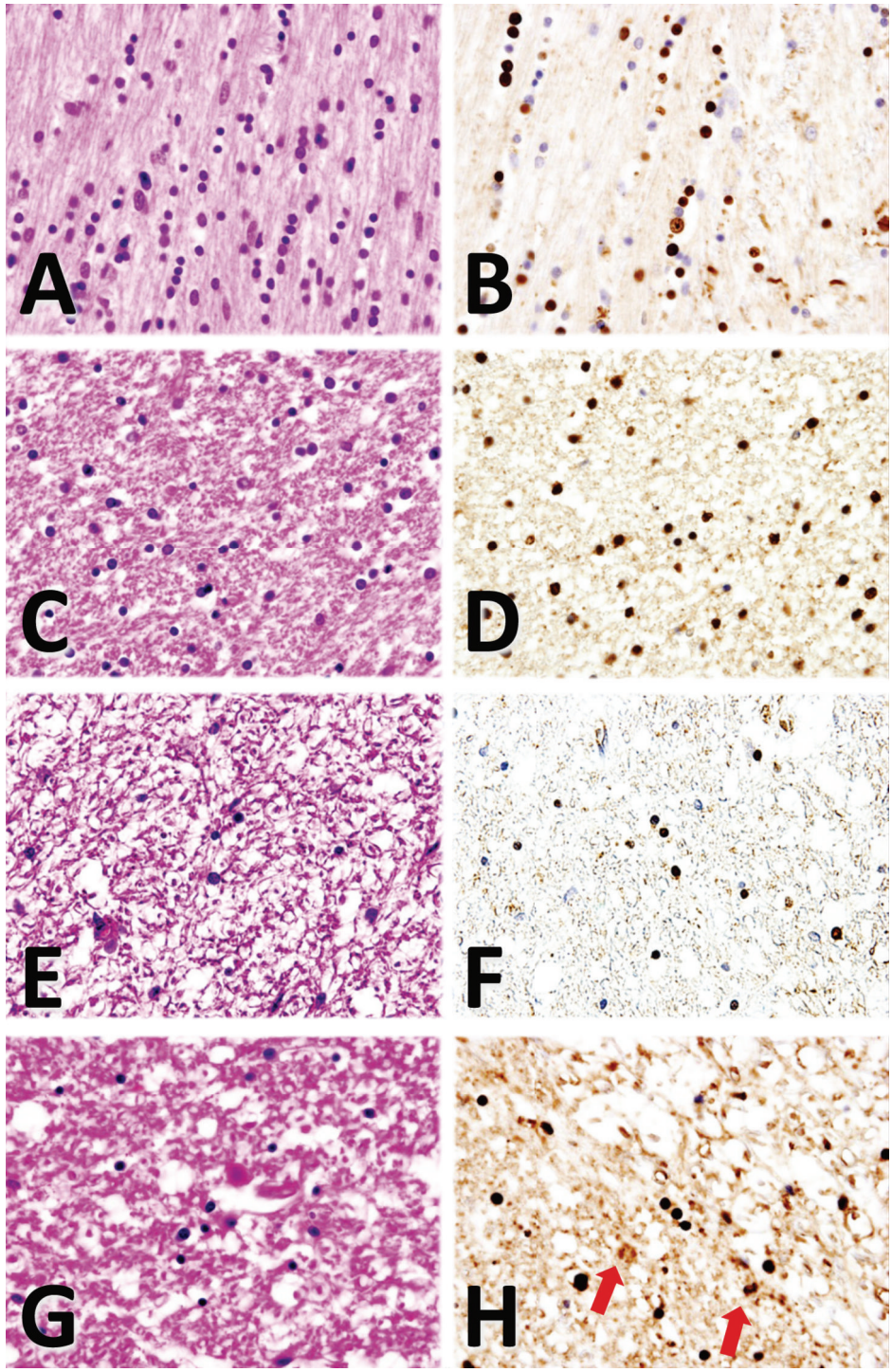


Figure 2: (A–H) Histological and immunohistochemical findings. Interfascicular oligodendroglia with halos in the cytoplasm and astrocytes are observed along with nerve fibers (A, HE staining). α-synuclein staining reveals numerous α-synuclein-positive glial cytoplasmic inclusions (GCI) in the oligodendroglia in the cerebral cortex (B). In the midbrain, the oligodendroglia and astrocytes show severe cytogenic edema (C, HE staining). α-synuclein-positive GCI in the oligodendroglia is also seen in the midbrain (D). The medulla oblongata shows extensive neuronal loss with scant oligodendroglial cells (E, HE staining), where α-synuclein is positive in the cytoplasma (F). Neuronal loss and gliosis are observed in the spinal cord (G, HE staining). Some α-synuclein-positive GCI in the oligodendroglia and astrocytes (arrows) are seen in the spinal cord (H).

xylene for 20 min (solvent refreshed at 10 min and 5 min), immersed in absolute ethanol for 10 min (solvent refreshed at 5 min), rehydrated in 90, 70% ethanol (5 min each), and finally placed in distilled water for 15 min (solvent refreshed every 5 min). The samples were then inactivated in 1 mM EDTA (pH 8.0) plus distilled water in a microwave oven for 15 min (high temperature for 5 min and low for 10 min) and cooled to approximately 35°C for 1 h. After liquid block treatment, the samples were immersed in phosphate buffered saline (PBS) for 15 min (solvent refreshed every 5 min). To observe the localization of α-synuclein, epitope specific rabbit anti-α-synuclein (spring bioscience, CA) was used as the primary antibody. After adding blocking buffer in a moisture chamber and incubating at room temperature for 1 h, the samples were washed 3 times for 5 min each in PBS, incubated again for 1 h with the secondary antibody (SC-2004, santa cruz biotechnology, inc., CA), diluted at 1 : 200 with PBS, and then immersed in PBS for 15 min (solvent refreshed every 5 min). The samples were then incubated with 3,3′-diaminobenzidine, tetrahydrochloride (DAB) for 15 min, washed in PBS, counterstained with hematoxylin, and observed and photographed under a microscope. The anti-α-synuclein antibody immunostained the glial and neuronal inclusions extensively, especially the cytoplasmic and nuclear inclusions in the oligodendroglia. α-synuclein-positive GCI in the oligodendroglia was occurred in the cerebral cortex, the midbrain, the medulla oblongata, and the spinal cord (Figures 2(B), 2(D), 2(F), and 2(H)). While gliosis and tissue rarefaction were observed in the midbrain, those in the locus coeruleus were relatively mild in the sections of the midbrain of the cerebellum level (data not shown).

3. Discussion

Patients with atypical parkinsonism are significantly more exposed to environmental toxins than are controls, and demonstrate a higher risk of disease onset associated with occupational exposure to organic solvents, plastic monomers and additives, pesticides, and metals [4]. Moreover, symptoms and neurological diseases are observed at a higher frequency in first relatives of MSA patients than in controls (23% in MSA cases versus. 10% in controls) [4]. Epidemiological studies in the French West Indies in 1999, on the other hand, implied an association between atypical parkinsonism and high consumption of tropical plants [5]. The European Study on Atypical parkinsonism in 2001 shows a significantly high risk of MSA in subjects with occupational exposure to various toxins, but a significantly lower risk among smokers, a factor often associated with a decreased risk of Parkinson's disease [6]. A more recent case-control study in the Aquitaine, France in 2004, shows a history of farming as significantly more frequent in MSA patients than in controls; it did not, however, reveal an association between occupational exposure to pesticides and MSA [7]. To date, on the other hand, no familial MSA case with a definite genetic background has been reported, nor has the genetic predisposition to MSA been established. Therefore, although exposure to environmental toxic substances may increase the risk of MSA, its pathogenesis is still unclear, and is simply defined as one of α-synucleinopathy characterized by the presence of GCI.

In the present case, the apparent risk factors for neurological manifestations were heavy exposure to organic solvents. Interestingly, an in vitro experiment has revealed that α-synuclein, originally in an unfolded form, is rapidly folded with an enhanced propensity to fibrillate in organic solvents [8]. Furthermore, by measuring hydrodynamic radii and adding glucose to solvents causes thorough collapse of α-synuclein [9]. The prolonged and extensive exposure to organic solvents together with the hyperglycemic condition attributed to diabetes may have contributed to the onset and clinical course of the present case.

4. Conclusion

Because most MSA cases lack a clear history of exposure to organic solvents with concurrent diabetes, some unknown genetic factors could define susceptibility to the disease. Accumulation of data from particular cases like the present one would be conducive to revealing clues in future studies.

Consent

Written informed consent was obtained from the patient's family for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Y. Nagai and S. Kitazawa were involved in whole process. R. Kitazawa and T. Kondo analyzed the autopsy case. M. Nakagawa and M. Komoda assisted clinical aspect. R. Haraguchi was involved in HE staining and immunostaining. All authors read and approved the final version of the paper.

Acknowledgment

The authors thank Ms. Yuki Takaoka for technical assistance.

References

[1] J. G. Graham and D. R. Oppenheimer, "Orthostatic hypotension and nicotine sensitivity in a case of multiple system atrophy," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 32, no. 1, pp. 28–34, 1969.

[2] M. I. Papp, J. E. Kahn, and P. L. Lantos, "Glial cytoplasmic inclusions in the CNS of patients with multiple system atrophy (striatonigral degeneration, olivopontocerebellar atrophy and Shy-Drager syndrome)," *Journal of the Neurological Sciences*, vol. 94, no. 1-3, pp. 79–100, 1989.

[3] T. Ikeda, H. Mashimoto, K. Iwasaki, I. Shimokawa, and T. Matsuo, "A sequential ultrastructural and histoautoradiographic

study of early neoplastic lesions in ethylnitrosourea-induced rat glioma," *Acta Pathologica Japonica*, vol. 39, no. 8, pp. 487–495, 1989.

[4] L. E. Nee, M. R. Gomez, J. Dambrosia, S. Bale, R. Eldridge, and R. J. Polinsky, "Environmental- occupational risk factors and familial associations in multiple system atrophy: a preliminary investigation," *Clinical Autonomic Research*, vol. 1, no. 1, pp. 9–13, 1991.

[5] D. Caparros-Lefebvre and A. Elbaz, "Possible relation of atypical parkinsonism in the French West Indies with consumption of tropical plants: a case-control study," *The Lancet*, vol. 354, no. 9175, pp. 281–286, 1999.

[6] N. Vanacore, V. Bonifati, G. Fabbrini et al., "Epidemiology of multiple system atrophy," *Neurological Sciences*, vol. 22, no. 1, pp. 97–99, 2001.

[7] V. Chrysostome, F. Tison, F. Yekhlef, C. Sourgen, I. Baldi, and J. F. Dartigues, "Epidemiology of multiple system atrophy: a prevalence and pilot risk factor study in Aquitaine, France," *Neuroepidemiology*, vol. 23, no. 4, pp. 201–208, 2004.

[8] L. A. Munishkina, C. Phelan, V. N. Uversky, and A. L. Fink, "Conformational behavior and aggregation of α-synuclein in organic solvents: modeling the effects of membranes," *Biochemistry*, vol. 42, no. 9, pp. 2720–2730, 2003.

[9] A. S. Morar, A. Olteanu, G. B. Young, and G. J. Pielak, "Solvent-induced collapse of α-synuclein and acid-denatured cytochrome c," *Protein Science*, vol. 10, no. 11, pp. 2195–2199, 2001.

JOURNAL OF MEDICAL CASE REPORTS

CASE REPORT Open Access

Scrotal cutaneous verruciform xanthoma with monocyte chemoattractant protein-1 immunohistochemical study: a case report

Chihiro Ito[1], Riko Kitazawac[1], Kenji Makita[1], Takafumi Watanabe[1], Akihiro Toda[2], Ryuma Haraguchi[1], Shinji Tanaka[2] and Sohei Kitazawa[1*]

Abstract

Introduction: Verruciform xanthoma is a rare, benign lesion characterized by hyperkeratosis and aggregates of foam cell macrophages. Here, we describe a case of verruciform xanthoma on the scrotum, in which the immunohistochemical localization of monocyte chemoattractant protein-1, a chemokine of the C-C or beta family that has been shown to induce the recruitment of monocytes for injured tissue, was analyzed to determine which cells release chemoattractants for macrophages.

Case presentation: A 75-year-old Japanese man with a well-defined nodule on the left scrotum was admitted to the hospital. An excision biopsy revealed epidermal papillary proliferation with parakeratosis, hyperkeratosis, and infiltration of foam cell macrophages, whereby a pathological diagnosis of benign cutaneous verruciform xanthoma was made. Immunohistochemically, monocyte chemoattractant protein-1 was observed predominantly on cytokeratin AE1/AE3-positive differentiating keratinocytes in the prickle cell layer. However, while infiltrating macrophages were densely stained for monocyte chemoattractant protein-1, keratinocytes in the basal and parabasal layers were almost negative.

Conclusions: We demonstrated that keratinocyte-derived monocyte chemoattractant protein-1 plays an important role in the establishment of particular histological features of verruciform xanthoma. However, in the present case, unlike in previous reports, monocyte chemoattractant protein-1 immunostaining in keratinocytes in the basal and parabasal layers was not prominent. We speculate that in the active phase of verruciform xanthoma, when continuous stimuli that release monocyte chemoattractant protein-1 from keratinocytes to the surrounding stromal area are present, the apparent immunostaining of monocyte chemoattractant protein-1 can be underestimated because of the void created by accelerated keratinocyte release from the cytoplasmic fraction.

Introduction

Verruciform xanthoma (VX) is an uncommon, benign lesion first reported in the oral cavity in 1971 [1]. A survey of 282 cases of VX involving different mucocutaneous sites has established the lesion as a distinct clinicopathologic entity [2]. It occurs mainly in oral mucosa and occasionally at extra-oral sites, including those on the penis [3], the scrotum [4], and the vulva [5]. Clinically, the lesion is painless, asymptomatic, slow growing (up to 2cm in size), and slightly elevated with a yellowish, reddish, or grayish rough and granular surface [6], mimicking conventional papilloma, verrucous carcinoma and squamous cell carcinoma [7]. Histologically, VX is characterized by papillomatosis, parakeratosis, and accumulation of foam cell macrophages [1,8,9].

This study describes a case of VX on the scrotum and analyzes the immunohistochemical localization of the major macrophage chemotactic factor, monocyte chemoattractant protein-1 (MCP-1), to elucidate the particular tumor-macrophage interaction in VX.

Case presentation

A 75-year-old Japanese man was admitted to our hospital with a gradually growing cutaneous polypoid mass

* Correspondence: kitazawa@m.ehime-u.ac.jp
[1]Division of Molecular Pathology, Ehime University Graduate School of Medicine, Shitsukawa, Toon City, Ehime 791-0295, Japan
Full list of author information is available at the end of the article

that had appeared on the skin of the left scrotum approximately one year before. The tumor measured 13mm in diameter with relatively well-defined whitish-yellow outlines (Figure 1a). Histological examination of an excisional biopsy revealed epidermal papillary proliferation with parakeratosis, hyperkeratosis, and neutrophil infiltration (Figures 1b and 2a). Although mitotic figures were noted in the basal layer (Figure 2a, arrows), cellular atypia was not prominent. Besides abundant plasma cells in the upper dermis, numerous foamy macrophages infiltrated the dermal papillae, forming a characteristic clear zone beneath the basement membrane (Figure 2a, asterisks). Histopathologically, the tumor was diagnosed as a benign cutaneous verruciform xanthoma with negative lateral and deep surgical margins.

To determine which cells release chemoattractants for macrophages, formalin-fixed and paraffin-embedded sections were stained for cytokeratin (AE1/AE3, M3515; Dako, Carpinteria, CA, USA; 1:200), CD68 (M0876; Dako; 1:100) and MCP-1 (DA103; BD Biosciences, San Diego, CA, USA; 1:40, 1:200, 1:800). After microwave heat-induced epitope retrieval, endogenous peroxidase activity was blocked with hydrogen peroxide (H_2O_2) in methanol. Indirect immunohistochemistry with the use of horseradish peroxidase conjugated anti-mouse rabbit antibody revealed that cytokeratin AE1/AE3 was strongly positive in differentiating epidermal keratinocytes, and weakly positive in keratinocytes in the basal and parabasal layers (Figure 2b). However, strong CD68 staining was observed almost exclusively in foamy cells infiltrating beneath the basal cells (Figure 2c). The parts densely stained for MCP-1 were observed in the differentiating cytokeratin AE1/AE3-positive keratinocytes. Clusters of the infiltrating macrophages also stained positive for MCP-1 (Figure 2d).

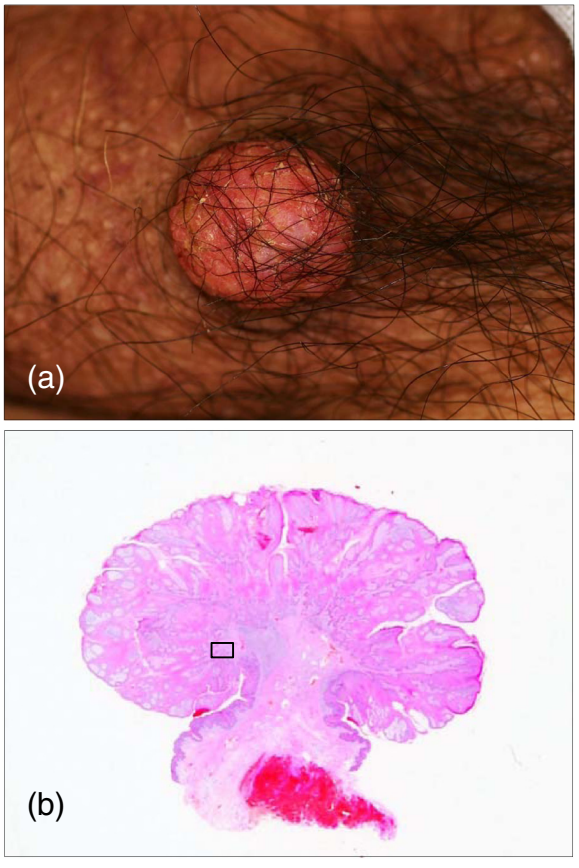


Figure 1 (a) Pedunculated, rubbery soft, red nodule (13mm in diameter) with a mulberry-like surface on the scrotum. (b) The lesion consists of pseudocarcinomatous acanthotic epidermis and stroma with dilated vessels under low magnification (hematoxylin and eosin stain). High magnification of the boxed area in Figure 2(a).

Discussion

Previous studies revealed a possible pathogenesis of VX that included the local release of lipid by damaged keratinocytes through inflammation [10]. Under the assumption that MCP-1 produced by the proliferating keratinocytes, especially by those with close contact with foamy macrophages, plays some roles in developing this particular histological feature, we investigated the immunohistochemical localization of MCP-1. In the present case, however, MCP-1 expression was observed in keratinocytes in the papilloma lesion, but its localization was observed predominantly in differentiating keratinocytes in the prickle cell layer.

MCP-1, produced by many types of cells, is a chemokine of the C-C or beta family that has been shown to induce the recruitment of monocytes for injured tissue; its excessive production by keratinocytes has been implicated in psoriasis and other inflammatory skin diseases; transgenic mice that express murine MCP-1 in the basal layer of epidermis do not, however, exhibit spontaneous cutaneous inflammation or any other discernible skin pathology, but show hypersensitivity responses to elicited inflammation in the skin by the recruitment of dendritic and Langerhans cells [11]. One of the apparent pathophysiological roles of MCP-1 in the skin is, therefore, chemotaxis of immunomodulators to the skin, and the overexpression of MCP-1 *per se* may be a requisite but not sufficient condition for causing VX.

A previous immunohistochemical study on a series of VX cases has revealed that MCP-1 localizes in the basal layer of the epidermis [12]. To explain an aspect of such immunohistochemical differences, we formulated two hypotheses: firstly, scarring beneath the basal layer prevented basal cells from releasing MCP-1, as reported for keloid-derived fibroblasts [13], and secondly, accelerated release of MCP-1 exhausted significant amounts of MCP-1 from the cytoplasm of keratinocytes in the basal and parabasal layers. It is well known that the epidermis

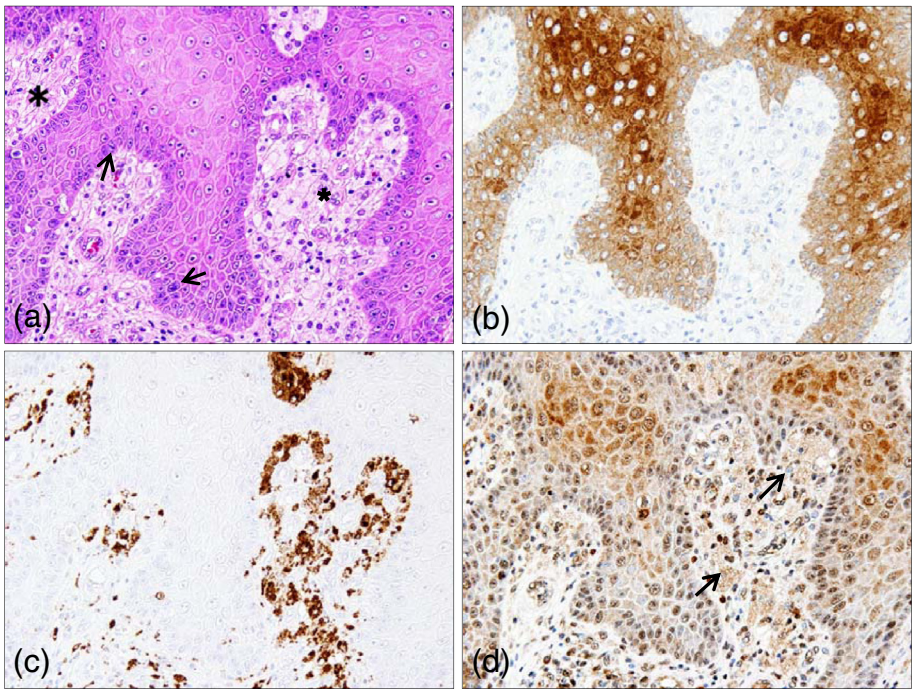


Figure 2 Immunohistochemical evaluation of chemoattractants for macrophages. (a) Hematoxylin and eosin staining shows verruciform xanthoma consisting of hyperkeratosis, a few mitotic figures (arrows) and infiltrating foamy cells (asterisks, ×200). **(b)** Cytokeratin AE1/AE3 showing positive immunostaining in the epithelial cells of the verruciform xanthoma (×200). **(c)** CD68 showing positive cytoplasmic immunostaining exclusively in the majority of foam cells infiltrating beneath the basal cells (×200). **(d)** The parts densely stained for monocyte chemoattractant protein-1 (MCP-1) are observed in the differentiating keratinocytes over the basal and parabasal layers. Clusters of the infiltrating macrophages are also stained positive, albeit less intensely, for MCP-1 (arrows, ×200).

shows a reparative phenotype when overlying a scar or the sclerotic dermis of lichen sclerosus [13,14]. This stromal-keratinocyte interaction is believed to account for the change of keratin AE1 expression from from basal keratinocytes in normal skin to spinous keratinocytes in scars and lichen sclerosus. Thus, our finding of MCP-1 expression in spinous keratinocytes, rather than the basal layer, may be the consequence of an altered dermal/stromal phenotype. It is likely not coincidence that both lichen sclerosus and verruciform xanthoma also show dermal lymphedema; the latter is thought to be the consequence of the former [15]. Additionally, when continuous stimuli to release MCP-1 from keratinocytes to the surrounding stromal area is present, apparent immunostaining of MCP-1 can be underestimated. Also, MCP-1 immunohistochemically localizes in infiltrating or aggregating macrophages themselves [16]. Thus, macrophages recruited by MCP-1 may sustain themselves in both paracrine and autocrine ways.

Conclusions

Although other factors associated with MCP-1 that characterize particular histopathological features of VX have not yet been established, we speculate that MCP-1 expression in proliferating and differentiating keratinocytes may have a supplemental role in the establishment of VX.

Consent

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TI and SK were involved in the whole process. RK, ST and AT analyzed the clinical case. KM and TW assisted with clinical aspects. RH was involved in hematoxylin and eosin staining and immunostaining. All authors read and approved the final version of the manuscript.

Acknowledgements

We thank Ms Yuki Takaoka for technical assistance.

Author details

[1]Division of Molecular Pathology, Ehime University Graduate School of Medicine, Shitsukawa, Toon City, Ehime 791-0295, Japan. [2]Department of Plastic Surgery, Ishikawa Hospital, Kamibun-cho 732-1, Shikoku-chuo City, Ehime 799-0121, Japan.

Received: 7 March 2012 Accepted: 8 June 2012
Published: 31 August 2012

References

1. Shafer W: **Verruciformxanthoma.** *Oral Surg* 1971, **31:**784–789.
2. Philipsen HP, Reichart PA, Takata T, Ogawa I: **Verruciform xanthoma - biological profile of 282 oral lesions based on a literature survey with nine new cases from Japan.** *Oral Oncol* 2003, **39:**325–336.
3. Kraemer BB, Schmidt WA, Foucar E, Rosen T: **Verruciform xanthoma of the penis.** *Arch Dermatol* 1981, **117:**516–518.
4. Kimura S: **Verruciform xanthoma of the scrotum.** *Arch Dermatol* 1984, **120:**1378–1379.
5. Santa Cruz DJ, Martin AS: **Verruciform xanthoma of the vulva. Report of two cases.** *Am J Clin Pathol* 1979, **71:**224–228.
6. Sklavounou A, Laskaris G, Angelopoulos A: **Verruciform xanthoma of the oral mucosa.** *Dermatologica* 1982, **164:**41–46.
7. Toida M, Koizumi H: **Verruciform xanthoma involving the lip: a case report.** *J Oral MaxillofacSurg* 1993, **51:**432–434.
8. Ji-an H, Li Y, Li S: **Verruciform xanthoma of oral cavity: clinicopathological study relating pathogenesis.** *APMIS* 2005, **113:**629–634.
9. Oliveira PT, Jaeger RG, Cabral LAG, Carvalho YR, Costa ALL, Jaeger MMM: **Verruciform xanthoma of the oral mucosa. Report of four cases and a review of the literature.** *Oral Oncol* 2001, **37:**326–331.
10. Cumberland L, Dana A, Resh B, Fitzpatrick J, Goldenberg G: **Verruciform xanthoma in the setting of cutaneous trauma and chronic inflammation: report of a patient and a brief review of the literature.** *J Cutan Pathol* 2010, **37:**895–900.
11. Nakamura K, Williams IR, Kupper TS: **Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin.** *J Invest Dermatol* 1995, **105:**635–643.
12. Ide F, Obara K, Yamada H, Mishima K, Saito I, Kusama K: **Cellular basis of verruciform xanthoma: immunohistochemical and ultrastructural characterization.** *Oral Dis* 2008, **14:**150–157.
13. Fa-Lai Y, Shen H-D, Tai H-Y: **Decreased production of MCP-1 and MMP-2 by keloid-derived fibroblasts.** *Burns* 2009, **35:**348–351.
14. Carlson JA, Carlson GD, Murphy M, Rohwedder A: **Lichen sclerosus exhibiting histologic signs of lymphedema: an essential factor in the pathogenesis of verruciform xanthoma.** *Arch Dermatol* 2012, **148:**260–262.
15. Lu S, Rohwedder A, Murphy M, Carlson JA: **Verruciform xanthoma: localized lymphedema (elephantiasis) is an essential pathogenic factor.** *J Cutan Pathol* 2012, **39:**391–394.
16. Muraoka A, Suehiro I, Fujii M, Ueno H, Hayashi S, Shimizu K, Kitazawa R, Kitazawa S, Murakami K: **Type IIa early gastric cancer with proliferation of xanthoma cells.** *J Gastroenterol* 1998, **33:**326–329.

doi:10.1186/1752-1947-6-260
Cite this article as: Ito *et al.*: Scrotal cutaneous verruciform xanthoma with monocyte chemoattractant protein-1 immunohistochemical study: a case report. *Journal of Medical Case Reports* 2012 **6**:260.

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i4.536

World J Gastroenterol 2013 January 28; 19(4): 536-541
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Cdx2 expression and its promoter methylation during metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus

Kenji Makita, Riko Kitazawa, Shuho Semba, Koto Fujiishi, Miku Nakagawa, Ryuma Haraguchi, Sohei Kitazawa

Kenji Makita, Riko Kitazawa, Koto Fujiishi, Miku Nakagawa, Ryuma Haraguchi, Sohei Kitazawa, Division of Molecular Pathology, Ehime University Graduate School of Medicine, Toon 791-0295, Japan
Shuho Semba, Division of Pathology, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
Author contributions: Makita K, Nakagawa M and Fujiishi K performed the majority of experiments and wrote the manuscript; Semba S and Haraguchi R provided vital reagents and analytical tools; Kitazawa S and Kitazawa R designed the study and were also involved in editing the manuscript.
Supported by Grant-in Aid from Ministry of Education, Sports and Culture (GP Program for Basic Science), Japan
Correspondence to: Dr. Sohei Kitazawa, Division of Molecular Pathology, Ehime University Graduate School of Medicine, Shitsukawa, Toon City, Ehime 791-0295, Japan. kitazawa@m.ehime-u.ac.jp
Telephone: +81-89-9605265 Fax: +81-89-9605267
Received: September 1, 2012 Revised: December 4, 2012
Accepted: December 22, 2012
Published online: January 28, 2013

Abstract

AIM: To examine how the expression of caudal type homebox transcription factor 2 (Cdx2) is regulated in the development of malignancy in Barrett's esophagus.

METHODS: Cdx2, mucin (MUC) series (MUC2, MUC5AC and MUC6), p53 and E-cadherin expression in Barrett's esophagus and adenocarcinoma specimens were examined by immunostaining. Isolated clusters of cells from (1) MUC2 and Cdx2-positive intestinal metaplastic mucosa; (2) MUC5AC and MUC6-positive, and MUC2 and Cdx2-negative high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC); and (3) MUC5AC, MUC6 and Cdx2-positive poorly-differentiated invasive adenocarcinoma (PDA) were analyzed by methylation-specific polymerase chain reaction using sets of primers for detecting methylation status of the *Cdx2* gene.

RESULTS: Most of the non-neoplastic Barrett's esophageal mucosa showing intestinal-type metaplasia with or without low-grade dysplasia was positive for E-cadherin, MUC series and Cdx2, but negative for p53. A portion of the low-grade to HD was positive for E-cadherin, MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. The definite IMC area was strongly positive for MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. Methylation of the Cdx2 promoter was not observed in intestinal metaplasia, while hypermethylation of part of its promoter was observed in hot dipped and IMC. Hypermethylation of a large fraction of the Cdx2 promoter was observed in PDA.

CONCLUSION: Cdx2 expression is restored irrespective of the methylation status of its promoter. Apparent positive immunohistochemical results can be a molecular mark for gene silencing memory.

Key words: Barretti's esophagus; Caudal type homebox transcription factor 2; Intestinal metaplasia; Promoter hypermethylation

Makita K, Kitazawa R, Semba S, Fujiishi K, Nakagawa M, Haraguchi R, Kitazawa S. Cdx2 expression and its promoter methylation during metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus. *World J Gastroenterol* 2013; 19(4): 536-541 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i4/536.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i4.536

INTRODUCTION

Barrett's esophagus, first described in 1950 and refined in 1957, is a condition whereby the distal esophageal squamous epithelium is replaced by metaplastic columnar epithelium[1]. Three types of morphologically distinct

metaplastic columnar epithelia are recognized in Barrett's esophagus: gastric-fundic, gastric-cardiac (junctional type), and intestinal (specialized type) metaplasia[2]. Reflecting a finding that patients with intestinal-type epithelium are at increased risk of developing adenocarcinoma, the American College of Gastroenterology has recently proposed a restricted definition of Barrett's esophagus: "a change in the esophageal epithelium of any length that can be recognized at endoscopy and is confirmed to have intestinal metaplasia at biopsy"[3]. Although a recent cohort study has demonstrated that the frequency of cancer development in Barrett's esophagus is not related to the presence of intestinal metaplasia, metaplastic columnar epithelium, *per se*, is generally accepted as a precancerous process predisposed to develop discrete neoplastic lesions such as the gastric or foveolar type, the adenomatous or intestinal type, hybrid type dysplasia, and intramucosal [high-grade dysplasia (HD) or intramucosal adenocarcinoma (IMC)] and invasive cancers[4]. Cancers derived from Barrett's esophagus are histopathologically classified into two major categories: gastric and intestinal[5]. Since most Barrett-related IMC cases are either gastric or intestinal with distinct phenotypic stability during progression, two separate (gastric and intestinal) pathways of carcinogenesis have been proposed[5]. Importantly, during the progression of the intestinal pathway, a gradual decrease in transcription factor caudal type homebox transcription factor 2 (Cdx2, a caudal-related homeobox gene essential for skeletal and intestinal development has been noted, suggesting its tumor suppressor role in Barrett's esophagus[5].

We encountered a case of invasive esophageal adenocarcinoma developing into intestinal-type dysplasia and IMC, and examined Cdx2 expression and its promoter methylation status in close histopatho- logical relation to the progression stages with the use of microdissection and methylation-specific polymerase chain reaction (MSP).

MATERIALS AND METHODS

Patient

An 81-year-old Japanese man was admitted to our hospital complaining of heartburn especially after eating sweet fare. The patient had undergone stomach surgery (distal partial gastrectomy) due to gastric ulcer nearly forty years earlier. Because of gastric regurgitation, he had undergone endoscopic examination of the upper digestive tract, which revealed severe reflux esophagitis with widespread Barrett's esophagus. A biopsy was taken from irregularly elevated lesions inside the Barrett's esophagus, and a histological examination confirmed esophageal adenocarcinoma in the lesions. An esophagectomy was carried out, and the right hemicolon was rebuilt. The patient has been free of recurrence for two years since the operation.

Immunohistochemistry

The specimens of Barrett's esophagus were subjected to immunohistochemistry using diaminobenzidine as the chromogen. Deparaffinized sections of formalin-fixed tissue were stained with mucin (MUC) series (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), p53 (Lab Vision, Kalamazoo, United States) and E-cadherin (Dako, Denmark) antibody diluted at 1:100 after heat-induced antigen retrieval and with Cdx2 (Dako, Denmark) antibody diluted at 1:50. Anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody for p53 and anti-mouse IgG was used for MUC series, Cdx2 and E-cadherin.

Agarose-bead mediated template preparation

Paraffin-embedded samples were deparaffinized in xylene and subjected to microdissection under light microscopic observation (Leica Microsystems, LMD7000) with the aid of both E-cadherin immunostaining and Cdx2 immunostaining. The microdissected samples were liquefied in low-melting agarose (3.2%) at 1:1, and agarose beads were made by chilling on ice. Beads were treated with proteinase K, followed by bisulfite conversion, as previously described[6].

Polymerase chain reaction amplification and sequencing

Bead fragments were analyzed by MSP using sets of primers for accessing the methylation status of the *Cdx2* gene. The promoter region of the human *Cdx2* genomic sequence (GenBank accession no. AL591024) was searched for CpG islands with an online search engine (www.ebi.ac.uk/emboss/cpgplot). One of the CpG islands (AL591024 nt 28391-28683) was further analyzed for methylation status by MSP. In the first-step polymerase chain reaction (PCR) amplification, a 183-bp amplicon containing 71-bp CpG sites, was amplified with two primers, (forward) 5'-GCCAAGGGGCCTAGGGCTGGA-3', and (reverse) 5'-GTTCACCTCCTAATACAAGCCTTTG-3' (Table 1), under the following conditions: 98°C 2 min, 30 cycles (98 ℃ 10 s, 50 ℃ 15 s, 68 ℃ 39 s). The primers used for second-step PCR were, (forward) 5'-GGAGCTGCCCCGACAGGAGCG-3', and (reverse) 5'-CGCGCCCAGCTCGGn TTTCAGCAA-3' (Table 1), under the following conditions: 98 ℃ 2 min, 25 cycles (98 ℃ 10 s, 60 ℃ 15 s, 68 ℃ 30 s). The PCR mixture contained Mighty AMP® DNA polymerase (Takara, Tokyo, Japan) and bead fragments in a final volume of 25 μL. The PCR products were electrophoresed in a 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

Ethics

Written informed consent was obtained from this patient, and this study was reviewed and approved by the local ethics committee at Ehime University.

RESULTS

Pathological findings

Grossly, a superficial spreading IMC surrounded by low-

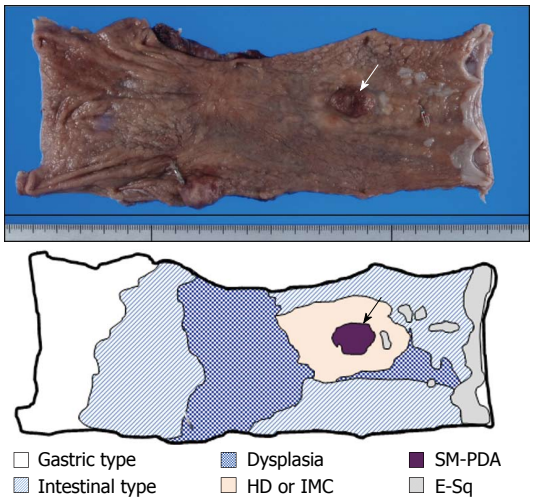


Figure 1 Macroscopic findings of excised esophagus. Surgical specimen shows the presence of superficial spreading carcinoma, extending between 30 mm from the oral and 105 mm from the anal surgical margins. The superficial spreading region is mostly composed of high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC), surrounded by dysplastic change (E-sq low-grade dysplasia). Inside this superficial spreading region, one observable elevated nodule (arrow) is composed of solid and submucosal invasive poorly-differentiated invasive adenocarcinoma (SM-PDA) with lymphatic invasion. The background non-neoplastic esophageal mucosa is extensively replaced by glandular mucosa with and without intestinal metaplasia.

Table 1 Primer sequences used in polymerase chain-based assays, product size and annealing temperature

	Primer sequence	Size (bp)	Temp (℃)
First PCR	(F)5′-GTTAAGGGGTTTAGGGTTGGA	183	60
Nested PCR	(R)5′-CAAAAACTTATATTAAAAAATAAAC		
Methylated	(F) 5′-GGAGTTGTTTCGATAGGAGCGC	71	60
	(R) 5′-TTACTAAAACCGAACTAAACGCG		
Unmethylated	(F) 5′-GGAGTTGTTTTGATAGGAGTGT	71	60
	(R) 5′-TTACTAAAACCAAACTAAACACA		

Temp: Temperature; PCR: Polymerase chain reaction; R: Reverse; F: Forward.

Table 2 Summary of immunohistochemical findings

	G-type	I-type (IM)	HD or IMC	PDA
MUC2	-	+	-	-
MUC5AC	+	-	++	+
MUC6	+	-	++	+
p53	-	-	++	++
E-cadherin	++	++	++	+
Cdx2	-	+	-	+

G-type: Gastric metaplasia; IM: Intestinal metaplasia; HD or IMC: High-grade dysplasia or intramucosal adenocarcinoma; PDA: Poorly-differentiated invasive adenocarcinoma; MUC: Mucin.

grade dysplasia and intestinal-type metaplasia extended between 30 mm from the oral and 105 mm from the anal surgical margins (Figure 1). One elevated nodule was noted inside this superficial spreading region (Figure 1). Microscopically, the background non-neoplastic esophageal mucosa was replaced, very extensively, by gastric foveolar type mucosa with (Figure 1) and without intestinal metaplasia (Figure 1). The superficial spreading IMC region was mostly composed of definite well-differentiated tubular adenocarcinoma or HD, surrounded by dysplastic change (low-grade dysplasia). The oral elevated nodular ridge was a solid, poorly-differentiated, invasive adenocarcinoma with lymphatic invasion, but no venous invasion or metastasis within the esophageal mucosa was observed. Immunohistochemically, the original esophageal squamous epithelium was positive for E-cadherin, but negative for all the MUC series (MUC2, MUC5AC and MUC6), Cdx2 and p53. Most of the non-neoplastic Barrett's esophageal mucosa showing intestinal-type metaplasia with or without low-grade dysplasia was positive for E-cadherin, MUC2, MUC5AC, MUC6 and Cdx2, but negative for p53. A portion of the low-grade to high-grade dysplasia was positive for E-cadherin, MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. The definite IMC area was strongly positive for MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. Figure 2 shows the transitional area between the intestinal metaplasia with low-grade dysplasia and the definite IMC area. A portion of the poorly-differentiated adenocarcinoma was positive for MUC5AC, MUC6, Cdx2 and p53, but negative for MUC2 (Figure 3). Table 2 shows a summary of the immunohistochemical findings.

Microdissection and MSP of the Cdx2 promoter

MSP revealed no methylation in Cdx2-positive Barrett's mucosa with intestinal metaplasia (Figure 4, intestinal type). Microdissected samples from the Cdx2-negative IMC area showed that a fraction of the cells was hypermethylated (Figure 4, IMC). Although Cdx2 expression was found by immunohistochemical analysis, samples from the poorly-differentiated invasive (PDA) area showed a hypermethylation pattern (Figure 4, submucosal invasive PDA).

DISCUSSION

Cdx2 is an intestine-specific transcription factor expressed in cells constituting the mucosal epithelium from the duodenum to the rectum[7]. While Cdx2 is negative for the normal foveolar mucosa of the stomach and the squamous epithelium in the esophagus stemming from the foregut, its heterotopic expression in Barrett's esophagus is observed especially in cases with intestinal-type metaplasia[8]. Among most of the terminal differentiation-specific transcription factors, Cdx2 is known to play a tumor suppressor role in cancer progression in the distal colon, a role, which in adults, is functionally and geographically distinct from the homeotic role of Cdx2 during gut development[9]. In our present case, and in confirming its tumor suppressor role, Cdx2 expression diminished during the progression from intestinal-type metaplasia to distinct IMC. Mirroring Cdx2 expression at the protein level by immunohistochemistry, the hypermethylation of the *Cdx2* gene promoter was revealed (Figure 4, IMC). Since primers used for MSP are set to amplify the *Cdx2* gene promoter with hypermethylation,

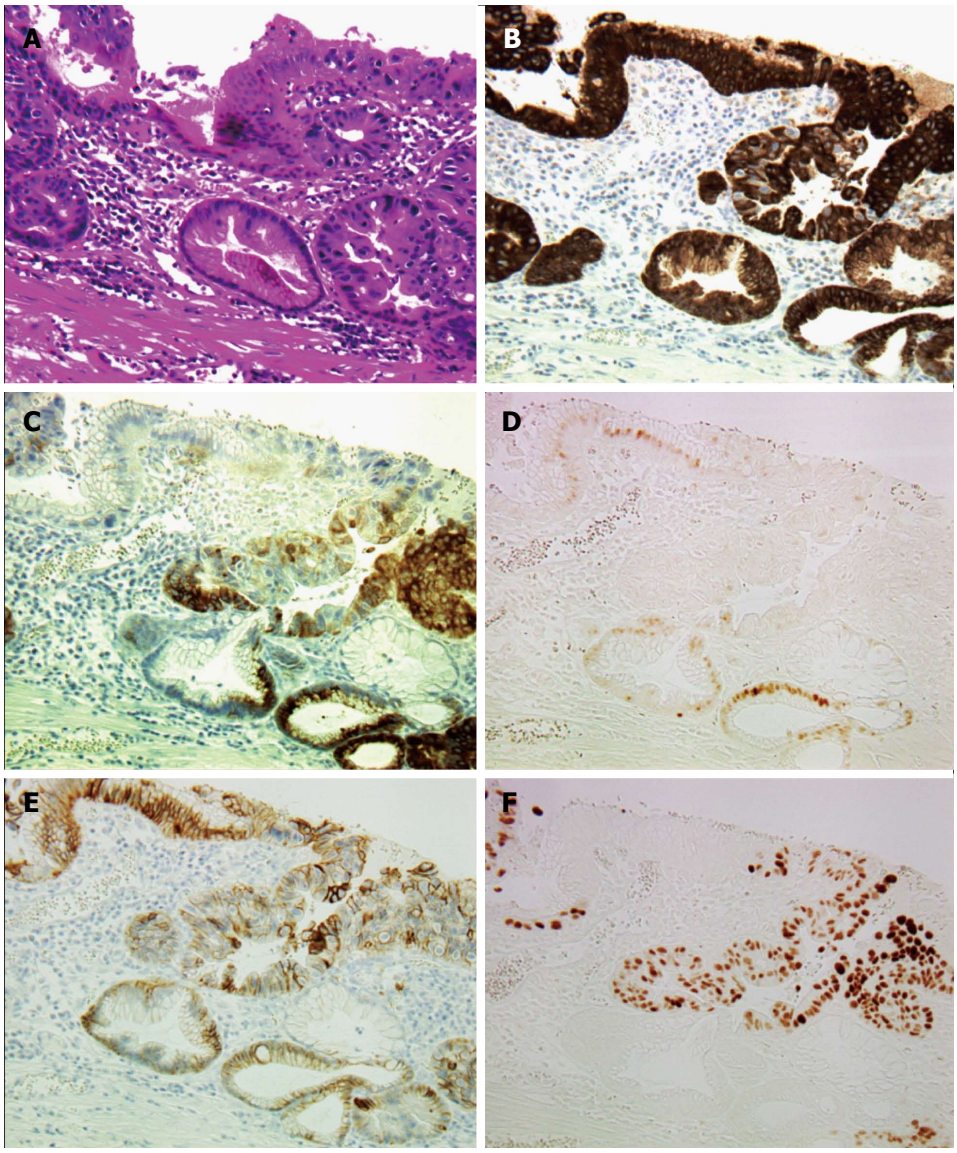


Figure 2 Histological findings of transitional area between intestinal metaplasia and high-grade dysplasia or intramucosal adenocarcinoma (× 200). A: Hematoxylin and eosin staining of transitional area. Intestinal metaplasia (IM) stretches from the upper left to the lower right corner; B: Mucin (MUC) 5AC immunostaining. Strong MUC5AC expression is observed in both IM and high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC) areas; C: MUC6 immunostaining. MUC6 expression is observed mostly in parts of the HD or IMC areas; D: Caudal type homebox transcription factor 2 (Cdx2) immunostaining. Cdx2 expression is observed only in the nuclei of the cells in the IM area; E: E-cad immunostaining. E-cad expression is observed on the membranes of cells in both IM and HD or IMC areas; F: p53 immunostaining. Strong p53 expression is observed in the nuclei of the cells in the HD or IMC area.

i.e., when all CpGs are methylated, a large fraction of the cells may acquire partial or scatter-type CpG methylation and, therefore, the *Cdx2* gene promoter may have been underestimated in our MSP. In support of our current study, Khor *et al*[5] also demonstrated the gradual down-regulation of Cdx2 expression during progression in adenomatous dysplasia, at least in the intestinal pathway of the Barrett esophageal cancers. These data suggest that Cdx2 also plays a tumor-suppressor role in the metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus. In our present case, irrespective of the hypermethylation status of the *Cdx2* gene promoter, Cdx2 expression was restored in PDA as analyzed by immunohistochemistry (Figure 3F). To achieve final gene-silencing, chromatin condensation followed by modifications of histone proteins are essential[10], we therefore hypothesize that epigenetic alterations other than demethylation may lead to *Cdx2* gene reactivation during the progression phase. Indeed, our previous study showed that hypermethylation of the *E-cadherin* gene promoter and MeCP2, a methyl-CpG binding domain protein, synergistically silenced gene expression in colorectal cancers[6]. Therefore, it is evident that hypermethylation of the gene promoter, *per se*, is essential for establishing gene silencing, but not sufficient for blocking gene expression. Since in our present case, Cdx2 reactivation did not correlate with differentiated intestinal phenotype, but was observed in invasive or aggressive phenotypes, the tumor suppressive effect of Cdx2 on these invasive cancer cells might be lost. These somewhat complicated epigenetical events may partly explain the dispersion of Cdx2 expression. Therefore, when characterizing cancer cells by immunophe-

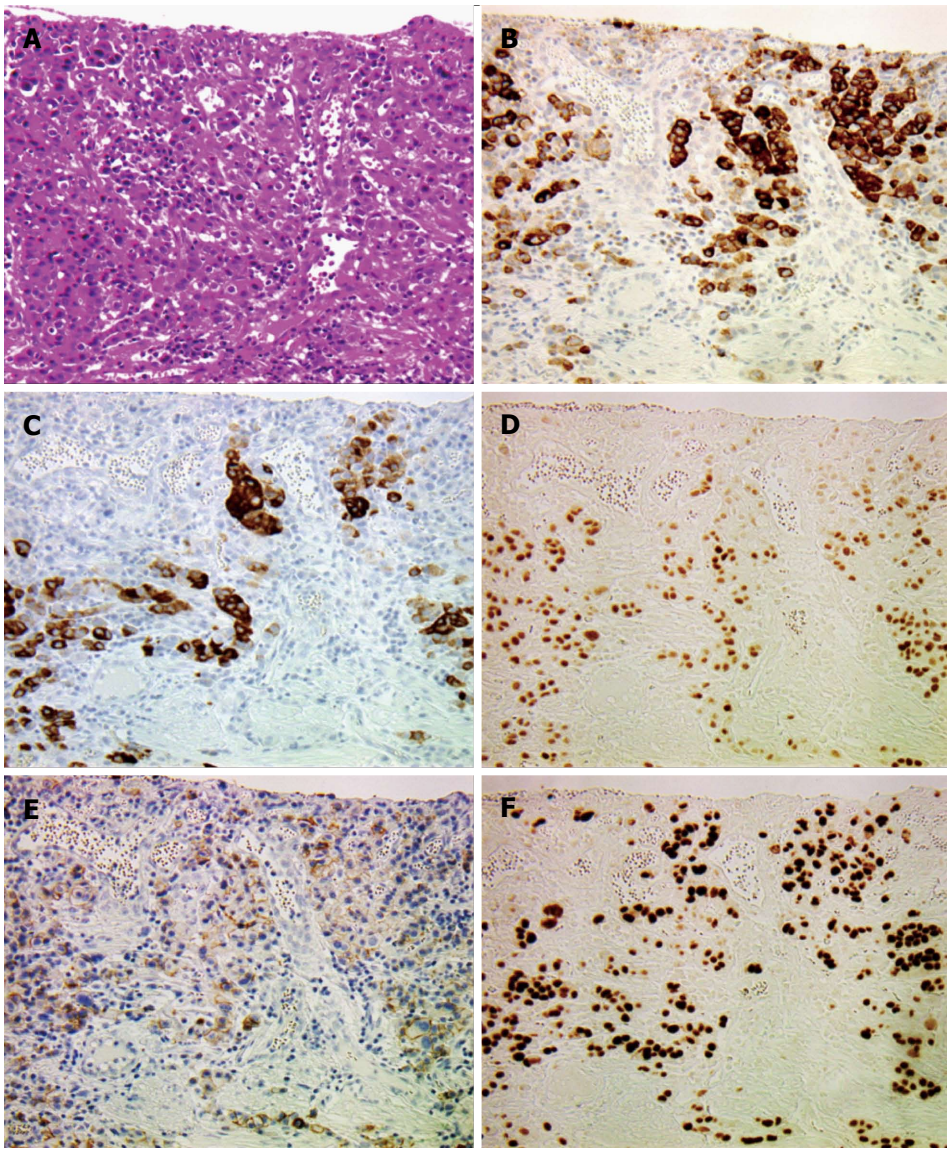


Figure 3 Histological findings of diffuse poorly-differentiated invasive adenocarcinoma (× 200). A: Hematoxylin and eosin staining of poorly-differentiated invasive adenocarcinoma (PDA). Cancer cells are diffusely scattered with prominent stromal reaction; B: Mucin (MUC) 5AC; C: MUC6; D: Caudal type homebox transcription factor 2 expression is positive in the PDA area; E: E-cad expression is markedly reduced in the PDA area; F: Strong p53 expression is observed in the nuclei of the cells in the PDA area.

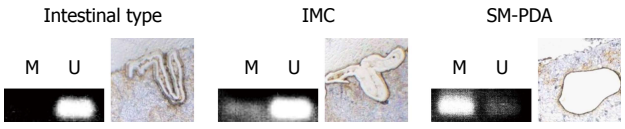


Figure 4 Detection of methylated cytosine by methylation-specific polymerase chain reaction analysis of caudal type homebox transcription factor 2 CpG-island region. Tissue samples were stained with E-cadherin and caudal type homebox transcription factor 2 (Cdx2) to assist cell identification. Cells, either isolated from intestinal metaplasia, intramucosal adenocarcinoma (IMC) or submucosal invasive poorly-differentiated adenocarcinoma (SM-PDA) by laser-assisted microdissection, were subjected to bisulfite conversion and subsequent methylation-specific polymerase chain reaction (MSP). MSP products using primers that specifically amplify only unmethylated DNA are indicated by visible polymerase chain reaction products in line unmethylated pattern (U), while visible polymerase chain reaction products in line methylated pattern (M) indicate those amplified by primers specific for methylated DNA. In intestinal-type metaplasia sections, MSP shows a U pattern, while MSP shows U patterns with partial M patterns in high-grade dysplasia or IMC sections. MSP shows a mostly M pattern in PDA where strong Cdx2 expression is observed (Figure 3D).

notyping, any apparent positive immunohistochemical results should be interpreted carefully with the help of the hypermethylation status as a molecular mark for gene silencing memory[10,11].

ACKNOWLEDGMENTS

We thank Ms. Yuki Takaoka for technical assistance.

COMMENTS

Background

Barrett's esophagus, a pathological condition in which the esophageal squamous epithelium is replaced by metaplastic columnar mucosa, is known to predispose to the development of dysplasia and subsequent cancers.

Research frontiers

Caudal type homebox transcription factor 2 (Cdx2) has recently been shown to play a tumor-suppressor role in the 'metaplasia-dysplasia-carcinoma sequence'

in Barrett's esophagus.

Innovations and breakthroughs

Recent reports have evaluated the phenotypic stability and role of Cdx2 in the neoplastic progression of different types of dysplasias. This suggests that non-intestinalized columnar metaplasia may be an unstable intermediate state at risk for neoplastic progression.

Applications

When characterizing cancer cells by immunophenotyping, any apparent positive immunohistochemical results should be interpreted carefully with the help of the hypermethylation status as a molecular mark for gene silencing memory.

Peer review

The authors examined the expression of Cdx2 and its methylation in Barrett metaplasia and esophageal adenocarcinoma. It revealed that irrespective of the hypermethylation status of the *Cdx2* gene promoter, Cdx2 expression was restored in poorly-differentiated invasive adenocarcinoma. The results are interesting and when characterizing cancer cells by immunophenotyping, any apparent positive immunohistochemical result should be interpreted carefully.

REFERENCES

1 **Bani-Hani KE**, Bani-Hani BK. Columnar-lined esophagus: time to drop the eponym of "Barrett": Historical review. *J Gastroenterol Hepatol* 2008; **23**: 707-715 [PMID: 18410605 DOI: 10.1111/j.1440-1746.2008.05386.x]

2 **Phillips RW**, Frierson HF, Moskaluk CA. Cdx2 as a marker of epithelial intestinal differentiation in the esophagus. *Am J Surg Pathol* 2003; **27**: 1442-1447 [PMID: 14576477 DOI: 10.1097/00000478-200311000-00006]

3 **Montgomery E**. Refining diagnostic criteria for high-grade dysplasia in Barrett esophagus. *Am J Clin Pathol* 2009; **132**: 7-9 [PMID: 19864227 DOI: 10.1309/AJCPPCJC71IFRVVG]

4 **Kelty CJ**, Gough MD, Van Wyk Q, Stephenson TJ, Ackroyd R. Barrett's oesophagus: intestinal metaplasia is not essential for cancer risk. *Scand J Gastroenterol* 2007; **42**: 1271-1274 [PMID: 17852872 DOI: 10.1080/00365520701420735]

5 **Khor TS**, Alfaro EE, Ooi EM, Li Y, Srivastava A, Fujita H, Park Y, Kumarasinghe MP, Lauwers GY. Divergent expression of MUC5AC, MUC6, MUC2, CD10, and CDX-2 in dysplasia and intramucosal adenocarcinomas with intestinal and foveolar morphology: is this evidence of distinct gastric and intestinal pathways to carcinogenesis in Barrett Esophagus? *Am J Surg Pathol* 2012; **36**: 331-342 [PMID: 22261707 DOI: 10.1097/PAS.0b013e31823d08d6]

6 **Darwanto A**, Kitazawa R, Maeda S, Kitazawa S. MeCP2 and promoter methylation cooperatively regulate E-cadherin gene expression in colorectal carcinoma. *Cancer Sci* 2003; **94**: 442-447 [PMID: 12824891 DOI: 10.1111/j.1349-7006.2003.tb01462.x]

7 **Werling RW**, Yaziji H, Bacchi CE, Gown AM. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol* 2003; **27**: 303-310 [PMID: 12604886 DOI: 10.1097/00000478-200303000-00003]

8 **Jaffee IM**, Rahmani M, Singhal MG, Younes M. Expression of the intestinal transcription factor Heaton in carcinoid tumors is a marker of midgut origin. *Arch Pathol Lab Med* 2006; **130**: 1522-1526 [PMID: 17090195 DOI: 10.1043/1543-2165]

9 **Bonhomme C**, Duluc I, Martin E, Chawengsaksophak K, Chenard MP, Kedinger M, Beck F, Freund JN, Domon-Dell C. The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. *Gut* 2003; **52**: 1465-1471 [PMID: 12970140 DOI: 10.1136/gut.52.10.1465]

10 **Raynal NJ**, Si J, Taby RF, Gharibyan V, Ahmed S, Jelinek J, Estécio MR, Issa JP. DNA methylation does not stably lock gene expression but instead serves as a molecular mark for gene silencing memory. *Cancer Res* 2012; **72**: 1170-1181 [PMID: 22219169 DOI: 10.1158/0008-5472.CAN-11-3248]

11 **Liu T**, Zhang X, So CK, Wang S, Wang P, Yan L, Myers R, Chen Z, Patterson AP, Yang CS, Chen X. Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. *Carcinogenesis* 2007; **28**: 488-496 [PMID: 16990345 DOI: 10.1093/carcin/bgl176]

P- Reviewers Pei ZH, Smith MDC **S- Editor** Gou SX
L- Editor Webster JR **E- Editor** Xiong L

JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Advance Publication

Acta Histochem. Cytochem. XX (X): XXX–XXX, XXXX
doi:10.1267/ahc.12022

Efficient Genetic Analysis of Microdissected Samples by Agarose-Bead Method: Alterations of β-Catenin Gene in Fundic Gland Polyp and Heterotopic Gastric Mucosa of Duodenum

Miku Nakagawa[1], Riko Kitazawa[1], Natsumi Kuwahara[1], Keisuke Yoshida[1], Ryuma Haraguchi[1] and Sohei Kitazawa[1]

[1]*Division of Molecular Pathology, Ehime University Graduate School of Medicine, Shitsukawa, Toon City, Ehime 791–0295, Japan*

Received June 7, 2012; accepted November 14, 2012; published online Month XX, 2013

Molecular genetic analyses of archival formalin-fixed, paraffin-embedded (FFPE) pathological specimens taken at biopsy or autopsy, are occasionally compromised because the DNA molecules therein are inevitably degraded. Furthermore, since these tissue samples comprise various cell types, the analyses based on mixtures of such heterogeneous populations often fail to reflect the nature of the affected cells. In the present study, to elucidate the contribution of β-catenin gene mutation to the fundic gland polyp and the heterotopic gastric mucosa in the duodenum, we successfully introduced an agarose-bead mediated technique as an effectual tool for retrospective morphology-oriented genetic analyses. Microdissected samples were embedded in low-melting agarose, and directly treated with proteinase K. A fragment of the agarose-bead was used as a template for polymerase chain reaction to analyze β-catenin mutation. Of the six cases of heterotopic gastric mucosa in the duodenum associated with fundic gland polyps, one showed a common 1-bp missense mutation at codon 37 shared by both the fundic gland polyp and the heterotopic gastric mucosa. Alternatively, a 1-bp silent mutation at codon 33 and missense mutation at codon 32 were identified only in the heterotopic gastric mucosa. Agarose-bead mediated technique shows superior sensitivity to the previously described techniques and is an effectual tool for retrospective morphology-oriented genetic analyses using a large number of archival pathological samples stored for long periods in the pathology laboratory.

Key words:

I. Introduction

With the introduction of the polymerase chain reaction (PCR) technique, genetic alterations as minute as the point mutation of a specific gene can be detected from routine formalin-fixed, paraffin-embedded (FFPE) pathological specimens. Inevitably, however, the process, starting with tissue samples like pathological specimens taken at biopsy or autopsy, involves various cell types including infiltrating inflammatory and reactive stromal cells. Therefore, molecular genetic analyses of such mixed, heterogeneous cells sometimes fail to reflect the nature of the affected population. To overcome this drawback, the selection of target cells by microdissection is crucial to assessing the molecular pathogenesis of disease, especially when *in situ* evaluation is difficult. To date, however, although PCR has been sensitive enough to amplify target DNA from a single cell, the effectual genetic analysis of FFPE microdissected samples is often circumscribed.

The fundic gland polyp origination from gastric mucosa is the most common gastric polyp, and since it often occurs, besides being sporadic, in association with familial adenomatous polyposis (FAP), it is thought to carry an abnormality to the Wnt signaling pathway [1, 9]. Frequent

Correspondence to: Sohei Kitazawa, Division of Molecular Pathology, Ehime University Graduate School of Medicine, Shitsukawa, Toon City, Ehime 791–0295, Japan. E-mail: kitazawa@m.ehime-u.ac.jp

somatic mutations of the β-catenin gene, one of the main components of the Wnt pathway, have recently been reported in sporadic fundic gland polyps [1, 9]. Furthermore, because patients with fundic gland polyp often have heterotopic gastric mucosa of the duodenum, we speculated that heterotopic gastric mucosa of the duodenum might carry the same genetic alterations as fundic gland polyp.

In this study we used a modified agarose-bead mediated technique for the genetic analysis of tissue from FFPE samples that had previously withstood assessment by routine DNA extraction procedures.

II. Materials and Methods

Samples

Routinely formalin-fixed (10%) and paraffin-embedded (FFPE) tissue samples were obtained from two patients manifesting both fundic gland polyps in the stomach and heterotopic gastric mucosa in the duodenum, and from four patients with only heterotopic gastric mucosa (Table 1). The samples archived in Ishikawa Hospital (Shikoku-chuo, Ehime) and the Division of Molecular Pathology, Ehime University (Toon, Ehime) were used in this study. All of the present cases, previously analyzed by commercially available DNA extraction kits and conventional DNA extraction protocols, were not informative by the PCR method. Written informed consent was obtained from all patients, and this study was reviewed and approved by the local ethics committee at Ehime University.

Immunohistochemistry

The specimens of fundic gland polyps in the stomach and the heterotopic gastric mucosa in the duodenum were subjected to immunohistochemistry using diaminobenzidine as the chromogen. Deparaffinized sections of formalin-fixed tissue were stained with β-catenin antibody (rabbit monoclonal antibody; Cell Signaling Technology®) diluted at 1:200 after heat-induced antigen retrieval. HRP-conjugated anti-rabbit IgG was used as the secondary antibody (DAKO K4003). Non-immunized rabbit serum was used as negative controls.

Agarose-bead mediated template preparation

Paraffin-embedded samples from hematoxylin and eosin-stained sections were deparaffinized in xylene and subjected to microdissection under a light microscope (Leica Microsystems LMD7000). Typical areas consisting of hyperplastic fundic glands were selectively dissected from surrounding normal-appearing gastric and duodenal mucosa (Fig. 1A). The microdissected samples were suspended in 5 μl of 1×TE and then mixed with pre-warmed and liquefied low-melting agarose (3.2%) at 1:1. A total of 10 μl agarose beads containing 1×TE and tissue fragments was formed in pre-chilled 250 μl mineral oil and then incubated at 50°C overnight in 1000 μl of 200 μg/ml proteinase K, 10 mM Tris-HCl (pH 8.0) and 25 mM ethylene diamine tetraacetic acid (EDTA). Bead fragments were washed in 1×TE, sliced into several pieces, and then used directly as a template for PCR amplification. Figure 1B illustrates the agarose-bead mediated technique.

PCR amplification and sequencing

Bead fragments were analyzed by nested PCR using sets of primers encompassing exon 3 of the β-catenin gene. The primers for the first-step PCR amplification were BCAT-F-1 (5'-AGTCACTGGCAGCAACAGTC-3') and BCAT-R-1 (5'-CTCTTCCTCAGGATTGCCTT-3'). The PCR condition was as follows: 30 cycles at 98°C for 10 sec, 55°C for 15 sec, and 68°C for 30 sec. The PCR mixture contained Mighty AMP® DNA polymerase (Takara, Tokyo, Japan) and bead fragments in a final volume of 25 μl. The primers used for second-step PCR were BCAT-F-2 (5'-GGCAGCAACAGTCTTACCTG-3') and BCAT-R-2 (5'-CTCAGGATTGCCTTTACCAC-3'). The second-step PCR condition was as follows: 25 cycles at 98°C for 10 sec, 60°C for 15 sec, and 68°C for 30 sec. The PCR mixture contained Mighty AMP® DNA polymerase (Takara, Tokyo, Japan) and bead fragments in a final volume of 50 μl. The PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. The products were cloned into the pMD20-T vector (Takara, Japan), transformed into *Escherichia coli* DH10B competent cells, and identified by blue white screening. Recombinant plasmids were purified

Table 1. *Summary of cases*

Case No.	Lesion(s)	Genetic alteration	Resulting AA* alteration
Case 1	FGP*/HGM*	c.96C>A and c.109T>C/c.109T>C	p.D32E and p.S37P/p.S37P
Case 2	FGP/HGM	None/c.96C>A	None/p.D32E
Case 3	HGM	c.99T>C	p.S33S
Case 4	HGM	None	None
Case 5	HGM	None	None
Case 6	HGM	None	None

* FGP: fundic gland polyp; HGM: heterotopic gastric mucosa; AA: amino acid.
* c: complementary DNA.
* C>A: from C to A.
* p: protein.

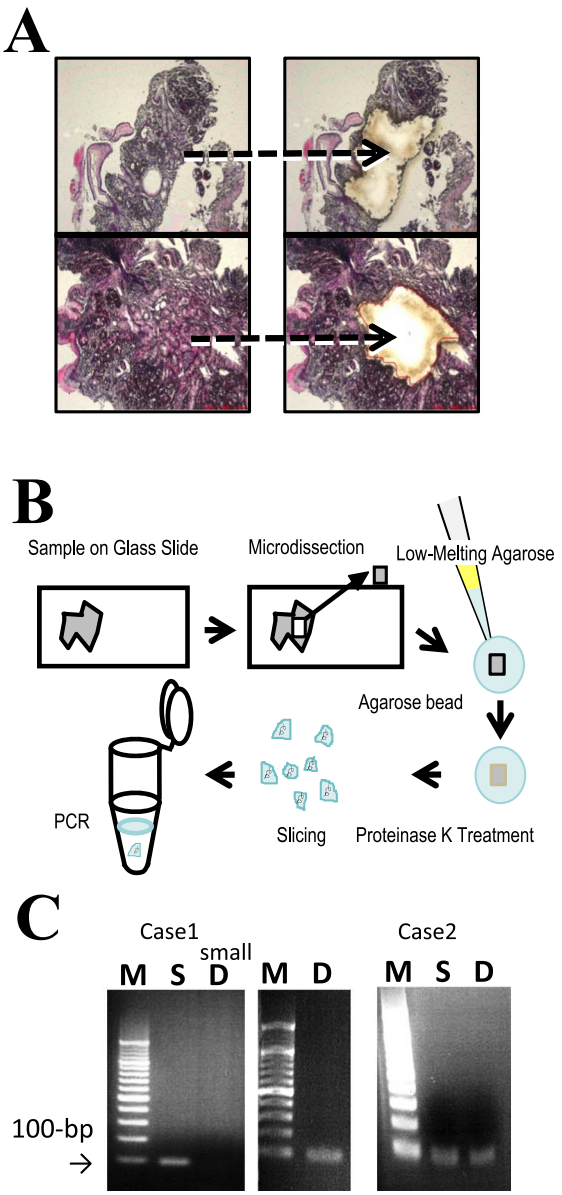


Fig. 1. Microdissection, agarose-bead mediated preparation for PCR template and PCR amplification. **A)** Sections of the fundic gland polyp and the heterotopic gastric mucosa were selected strictly and subjected to microdissection under a light microscope. **B)** Schema of procedures for agarose-bead mediated preparation for PCR template. Microdissected samples were embedded in low-melting agarose. Beads were then formed by chilling in ice-cold water. The beads were treated with proteinase K, heat inactivated and subjected to PCR using a portion of the bead fragment as a template. **C)** PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Lane M: a molecular weight marker of 100-bp ladder. The PCR products obtained using sets of primers encompassing exon 3 of the β-catenin gene were visualized as 100-bp DNA bands. While the five cells microdissected from duodenum (**D**) of case 1 does not show clear PCR product (case 1: **D**, small), the rest of the samples containing at least 20 cells showed positive PCR amplification.

using a NucleoSpin® Plasmid kit (Macherey-Nagel, place), and sequenced using ABI PRISM® on an ABI 310 Genetic Analyzer.

III. Results

Specimens from two of the six patients showed both typical histopathological features of fundic gland polyps and heterotopic gastric mucosa; the other four cases showed only heterotopic gastric mucosa. Dilated glands lined by oxyntic epithelium were noted in two cases of fundic gland polyps of the stomach (Figs. 2A and 3A). In all six cases of heterotopic gastric mucosa of the duodenum, the oxyntic epithelium was typically nondysplastic, and the junction between the gastric-type and the duodenal surface epithelium was apparent (Figs. 2C and 3C). As in the normal fundic gland, immunohistochemistry of β-catenin in the fundic gland polyp and in the heterotopic gastric mucosa (Figs. 2B and 3B) showed predominantly membranous staining in the epithelial cells lining the dilated fundic glands. Although much less extensive, cytoplasmic staining of β-catenin was also observed (Figs. 2B and 3B). In the heterotopic gastric mucosa of the duodenum, β-catenin immunostaining was observed on the membrane, and only weak cytoplasmic staining of β-catenin was noted cytoplasm (Figs. 2D and 3D), similar to that of the fundic gland polyp. All cases of heterotopic gastric mucosa, either associated with fundic gland polyp or sporadic, showed similar β-catenin immunostaining. Irrespective of the presence or absence of the β-catenin gene mutation, there were no significant differences in β-catenin immunostaining of the normal fundic gland, the fundic gland polyp, and the heterotopic gastric mucosa.

Sections of fundic gland polyps and heterotopic gastric mucosa were selected strictly and subjected to microdissection under a light microscope (Fig. 1A). Agarose beads were formed and all PCR procedures were successfully carried out using a portion of the bead fragment as a template (Fig. 1B). PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Lane M shows a molecular weight marker of 100-bp ladder. The PCR products obtained using sets of primers encompassing exon 3 of the β-catenin gene were visualized as 100-bp DNA bands. While five cells microdissected from duodenum (D) of case 1 did not show clear PCR product (case 1: D, small), the rest of the samples containing at least 20 cells showed positive PCR amplification.

In case 1, two 1-bp missense mutations at codon 32 (GAC to GAA resulting in the replacement of aspartic acid by glutamic acid), and codon 37 (TCT to CCT resulting in the replacement of serine by proline) were identified in the fundic gland polyp of the stomach. The same 1-bp missense mutation at codon 37 was identified in the heterotopic gastric mucosa (Table 1 and Fig. 4, case 1). In case 2, a 1-bp missense mutation at codon 32, was identified in the heterotopic gastric mucosa, while no mutation was observed

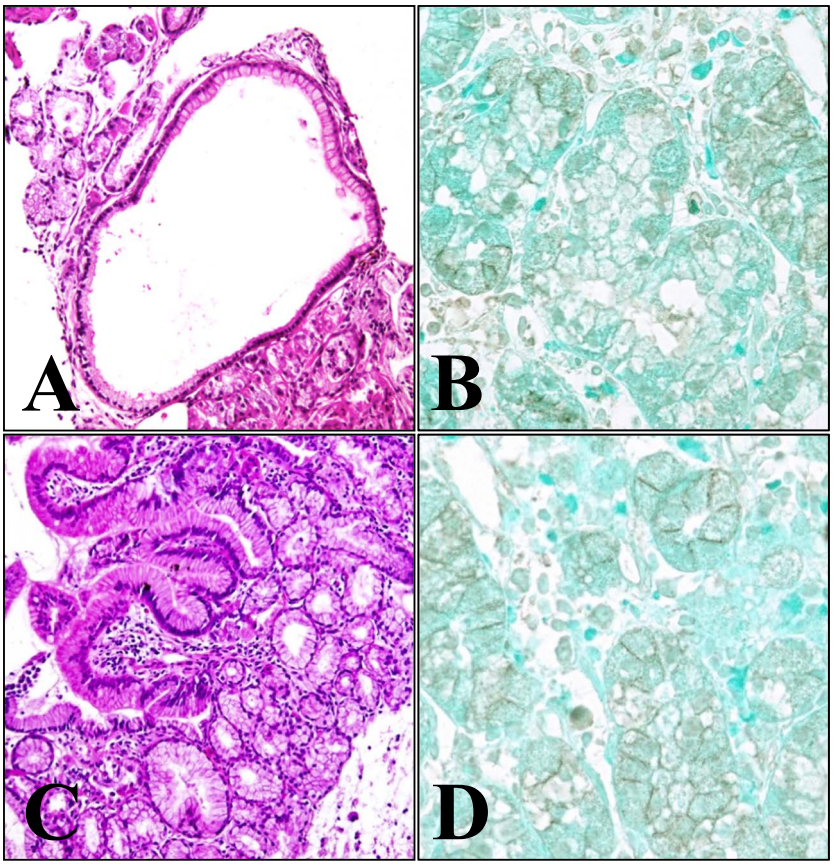


Fig. 2. Histopathological features and immunohistochemical staining for β-catenin of case 1. Dilated glands lined by oxyntic epithelium are noted in the fundic gland polyp of the stomach (**A**, HE, ×100). The diffuse membranous and partly cytoplasmic distribution of β-catenin are noted (**B**, ×400). In the heterotopic gastric mucosa of the duodenum, the oxyntic epithelium is typically nondysplastic, and the junction between the gastric-type and duodenal surface epithelium is apparent (**C**, HE, ×100). β-catenin immunostaining shows mainly membranous staining (**D**, ×400).

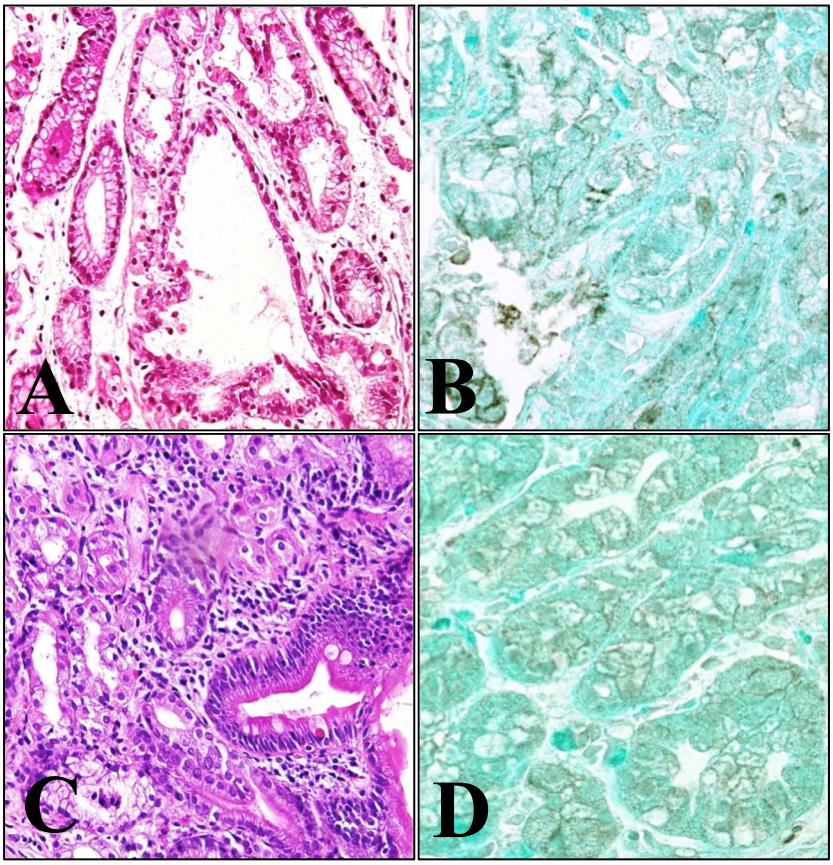


Fig. 3. Histopathological features and immunohistochemical staining for β-catenin of case 2. As in case 1, dilated glands lined by oxyntic epithelium are also observed in the fundic gland polyp of the stomach (**A**, HE, ×100). β-catenin in the fundic gland polyp showed predominantly membranous staining in the epithelial cells lining the dilated fundic glands. Small fractions of cytoplasmic staining and nuclear accumulation of β-catenin are also seen (**B**, ×400). In the heterotopic gastric mucosa of the duodenum, gastric-type glands are covered by duodenal mucosa (**C**, HE, ×100). Membranous β-catenin immunostaining is observed (**D**, ×400).

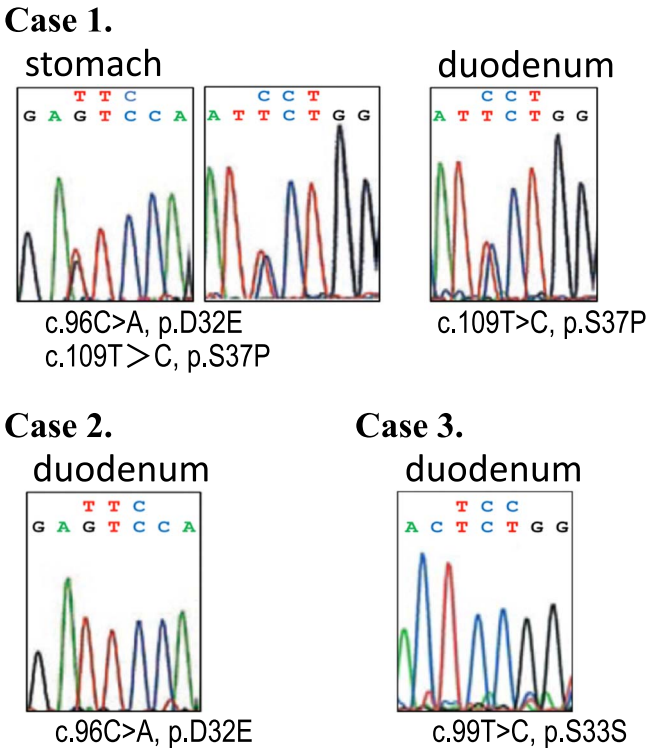


Fig. 4. β-catenin mutations in fundic gland polyp and heterotopic gastric mucosa in duodenum. In case 1, two 1-bp missense mutations at codon 32 (GAC to GAA resulting in the replacement of aspartic acid by glutamic acid), and codon 37 (TCT to CCT resulting in the replacement of serine by proline) were identified in the fundic gland polyp of the stomach. The same 1-bp missense mutation at codon 37 was identified in the heterotopic gastric mucosa (case 1). In case 2, a 1-bp missense mutation at codon 32, resulting in the replacement of aspartic acid by glutamic acid, was identified in the heterotopic gastric mucosa, while no mutation was observed in the fundic gland polyp of the stomach (case 2). In case 3, a 1-bp silent mutation at codon 33 was identified in the heterotopic gastric mucosa. In cases 1 and 2, DNA sequences were read in an antisense direction.

in the fundic gland polyp of the stomach (Table 1 and Fig. 4, case 2). In case 3, a 1-bp silent mutation at codon 33 was identified in the heterotopic gastric mucosa (Table 1 and Fig. 4, case 3). The other three cases of heterotopic gastric mucosa in the duodenum showed no DNA alterations in the portion of the β-catenin gene examined (Table 1).

IV. Discussion

Alterations of the β-catenin gene have been reported in various malignant and boundary-malignant neoplasms including colorectal carcinoma, hepatocellular carcinoma, endometrial adenocarcinoma, and desmoid tumors [5, 7, 9]. β-catenin gene alterations at phosphorylation sites protect the β-catenin from ubiquitination, resulting in the nuclear accumulation of β-catenin. Moreover, somatic β-catenin gene alterations have been found in the fundic gland polyp, a relatively common benign lesion in the stomach [9]. The frequent association of the fundic gland polyp with heterotopic gastric mucosa in the duodenum prompted us to investigate, with the use of FFPE samples, whether or not heterotopic gastric mucosa in the duodenum, either sporadic or the fundic gland polyp associated type, is attributable to alterations of the β-catenin gene.

The use of archival FFPE pathological specimens for molecular biological analysis has become increasingly important. Tissues stored in paraffin blocks can be potential sources of DNA for retrospective genetic analysis. Nonetheless, formalin-fixation causes various alterations of nucleic acids, including degradation attributed to endogenous nuclease and crosslinking between nucleic acids and proteins [2, 4]. Moreover, because pathological specimens inevitably comprise heterogeneous populations of cells, objective cell populations must be separated by microdissection. Several methods for DNA extraction from paraffin-embedded tissues have been introduced. Several commercial kits for rapid DNA extraction without DNA-cleaning steps, and a more conventional DNA-preparation protocol with DNA-cleaning are now widely used for handling FFPE samples [3, 6]. Yet, these commercial and conventional methods, sometimes fail to assess minute or microdissected FFPE samples mainly because of sample loss during the DNA extraction process. Indeed, all our microdissected samples from FFPE specimens were noninformative by our assays with commercial kits (DNA Isolater PS-Rapid Reagent®, Wako, Japan, Takara DEXPAT®, Takara, Japan) and conventional techniques. To minimize this DNA loss, we introduced an ‘en bloc’ treatment during proteinase K treatment, cleaning and buffer exchange processes with the use of the agarose-bead method.

In our current study of the six cases of heterotopic gastric mucosa in the duodenum associated with fundic gland polyps, one showed a common 1-bp missense mutation at codon 37 shared by both the fundic gland polyp and the heterotopic gastric mucosa. Alternatively, a 1-bp silent mutation at codon 33 and missense mutation at codon 32 were identified only in the heterotopic gastric mucosa. Since silent mutation sometimes causes alterations in protein expression levels by affecting translational and/or protein-folding efficiency, the genetic alteration can be pathogenic [8]. The other cases, however, showed no DNA alterations in either the fundic gland polyp or the heterotopic gastric mucosa in the duodenum. Besides β-catenin, because the Wnt signaling pathway consists of many components such as APC, GSK-3β and Axin, alteration of these genes may be involved in these cases. The controversy that genetic alterations seen in heterotopic gastric mucosa in the duodenum may derive from the same tissue progenitor cells bearing the same genetic alteration as that of fundic gland polyps, or may simply be an incidental event by exogenous carcinogens, suggests that the heterotopic gastric mucosa in the duodenum may be a heterogeneous population. Further studies are needed to resolve the controversy.

In conclusion, the agarose-bead mediated technique is an effectual tool for retrospective morphology-oriented genetic analyses using a large number of archival pathological samples stored for long periods in the pathology laboratory.

V. Acknowledgments

We wish to thank Ms Yuki Takaoka for technical assistance.

VI. References

1. Abraham, S. C., Giardiello, F. M., Hamilton, S. R., Nobukawa, B. and Wu, T. (2001) Sporadic fundic gland polyps: common gastric polyps arising through activating mutations in the β-catenin gene. *Am. J. Pathol.* 158; 1005–1010.
2. Fujimoto, J., Hirano, T., Ichikawa, N., Okamoto, E., Takeuchi, M., Ueki, T. and Yamamoto, H. (1998) Adverse effect of prolonged formalin fixation on DNA histograms in paraffin-embedded tissue. *Acta Histochem. Cytochem.* 31; 171–175.
3. Huijsmans, C. J., Damen, J., Hermans, M. H., Linden, J. C. and Savelkoul, P. H. (2010) Comparative analysis of four methods to extract DNA from paraffin-embedded tissues: effect on downstream molecular applications. *BMC Res. Notes* 3; 239.
4. Kitazawa, S., Kitazawa, R. and Maeda, S. (2000) Identification of methylated cytosine from archival formalin-fixed paraffin-embedded specimens. *Lab. Invest.* 80; 275–276.
5. Koch, A., Albrecht, S., Denkhaus, D., Leuschner, I., Pietsch, T. and Schweinitz, D. V. (1999) Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the β-catenin gene. *Cancer Res.* 59; 269–273.
6. Kullmann, F., Bocker, T., Ruschoff, J. and Scholmerich, J. (1998) A comparison of methods for DNA extraction from paraffin-embedded tissue for microsatellite instability analysis by polymerase chain reaction. *Acta Biotechnol.* 1; 77–83.
7. Lazar, A. J. F., Bolshakov, S., Habeeb, S., Hajibashi, S., Lev, D., Lopez-Terrada, D., Mayordomo-Aranda, E., Pollock, R. E., Tuvin, D. and Warneke, C. L. (2008) Specific mutations in the β-catenin gene (CTNNB1) correlate with local recurrence in sporadic desmoid tumors. *Am. J. Pathol.* 173; 1518–1527.
8. Salzman, D. W. and Weidhaas, J. B. (2011) miRNAs in the spotlight: making 'silent' mutations speak up. *Nat. Med.* 17; 934–935.
9. Sekine, S., Hirohashi, S., Nakanishi, Y., Sakamoto, M., Shibata, T., Shimoda, T. and Yamauchi, Y. (2002) β-catenin mutations in sporadic fundic gland polyps. *Virchows Arch.* 440; 381–386.

Contents available at Sciverse ScienceDirect
Diabetes Research and Clinical Practice
journal homepage: www.elsevier.com/locate/diabres

Pulmonary hypertension associated with diffuse deposition of pentosidine in pulmonary arterioles

Munenori Komoda[a], Riko Kitazawa[a], Kenji Makita[a], Keisuke Yoshida[a], Miyuki Takeji[b], Yoshiko Soga[b], Mie Kurata[c], Ryuma Haraguchi[a], Sohei Kitazawa[a,*]

[a] Division of Molecular Pathology, Ehime University Graduate School of Medicine, Shitsukawa 454, Toon City, Ehime 791-0295, Japan
[b] Diagnostic Pathology, Ehime University Graduate School of Medicine, Shitsukawa 454, Toon City, Ehime 791-0295, Japan
[c] Pathogenomics, Ehime University Graduate School of Medicine, Shitsukawa 454, Toon City, Ehime 791-0295, Japan

ARTICLE INFO

Article history:
Received 16 November 2012
Accepted 21 January 2013

Keywords:
Pulmonary hyprtension
Diabetes
Pentosidine
Autopsy

ABSTRACT

Diabetes induces advanced glycation end products (AGEs) that per se are not only a major cause of oxidative stress but also reduce the plasticity of connective tissue by pathological collagen cross-linking. We describe a case of severe pulmonary hypertension manifesting as a major diabetic complication. Impaired pulmonary arteriolar plasticity attributed to pentosidine, together with increased circulation volume by hyperosmotic pressure and reduction in myocardial compliance by multiple patchy fibrosis, may contribute to the clinical manifestation of severe pulmonary hypertension.

1. Introduction

A number of diabetic complications are currently considered to be related to cellular damage by oxidative stress. Under diabetic conditions, oxidative stress is induced by various mechanisms including the formation of advanced glycation end products (AGEs), increased polyol pathway flux, activation of protein kinase C isoforms, glucose autoxidation, and mitochondrial overproduction of superoxide [1–3]. Among these, AGEs comprise reactive and cross-linking molecules that are formed from the non-enzymatic glycation of reducing sugars and proteins, lipids, and nucleic acids [1,2]. AGEs per se are not only a major cause of oxidative stress but they also reduce the plasticity of bone structure by inducing "unfavorable" pathological collagen cross-linking [4]. Here, we describe a case of severe pulmonary hypertension manifesting as a major diabetic complication with evidence of pentosidine (an AGE) inducing cross-links between arginine and lysine residues in collagen, predominantly and diffusely localized in pulmonary arterioles.

2. Case presentation

A 66-year-old Japanese woman presenting with shortness of breath and dyspnea on exertion was referred to our hospital. The patient had previously been diagnosed with diabetes at age 60, and despite a dietary regimen and oral medication, had developed renal failure and was started on hemodialysis at the age of 62. She developed angina pectoris and was treated by coronary artery intervention just before the referral. At admission a series of examinations including an echocardiogram and a cardiac catheter test revealed the presence of severe pulmonary hypertension. Strict management of the dialysis and optimal treatment of the hypertension were, however, ineffective. Finally, the patient developed severe dyspnea, dizziness and nausea associated with pulmonary hypertension and died of cardiac arrest. Autopsy was carried out 2 h after her death. Grossly, the body was that of a slightly overweight woman with increased pleural effusion (left 200 ml, right 300 ml); heart was enlarged (weight 466 g) and

* *Corresponding author*. Tel.: +81 960 5265; fax: +81 960 5267.
E-mail address: kitazawa@m.ehime-u.ac.jp (S. Kitazawa).
0168-8227/$ – see front matter
http://dx.doi.org/10.1016/j.diabres.2013.01.019

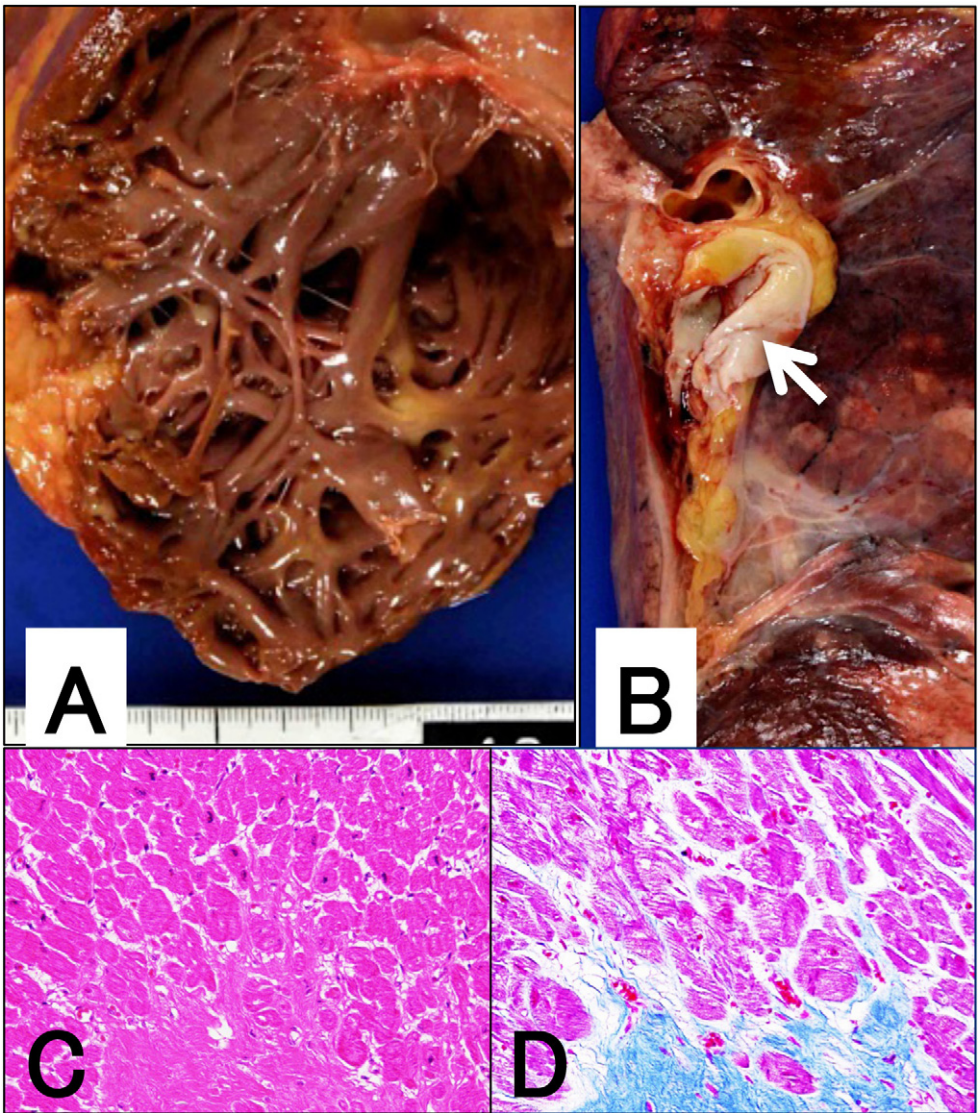


Fig. 1 – The heart, enlarged and weighing 466 g, shows marked trabeculation of the right ventricle (A). The hilum of the left lung (320 g) shows a dilated pulmonary artery (B, white arrow). Microscopically the heart illustrates hypertrophy of myocardial cells with multiple interstitial patchy fibrosis (HE, C x200; Azan-Mallory, D x200).

showed right ventricular trabeculation (Fig. 1A). The lungs were congested (left 320 g, right 439 g) with dilated pulmonary arteries (Fig. 1B). Reflecting the longstanding diabetic condition, major arteries showed severe atherosclerotic change, and the surface of the kidney displayed a fine granular appearance, reflecting nodular diabetic glomerulosclerosis. Microscopically, the heart demonstrated hypertrophy of myocardial cells with prominent dark nuclei as well as mild and diffuse interstitial fibrosis (Fig. 1C, HE and 1D, Azan-Mallory). Arterioles in the lung showed circular loosening or degeneration of the connective tissue from the medial to the adventitial area (Fig. 2A x200, HE and 2B, elastica-Masson x200). Markers of oxidative stress such as AGEs and pentosidine (an AGE), 8-hydroxydeoxyguanosine (8-OHdG), and the receptor for AGE (RAGE) were assessed by immunohistochemistry with the use of specific antibodies: anti-AGE monoclonal antibody and anti-pentosidine monoclonal antibody (Transgenic Co, Kumamoto, Japan), RAGE polyclonal antibody (Santa Cruz Biotechnology, Inc, CA U.S.A.), and anti-8-OHdG monoclonal antibody (Institute for the Control of Aging, Nikken SEIL Co., Ltd, Shizuoka, Japan). Both AGEs (Fig. 2C x200) and pentosidine (Fig. 2D x200) were strongly positive around the medial to the adventitial area of the arterioles. Strong nuclear staining of 8-OhdG was also noted (Fig. 2E x200). Alveolar epithelial cells were positive for RAGE (Fig. 2F x200).

3. Discussion

Chronic and persistent hyperglycemia induces AGEs by a non-enzymatic glycation process, which then induces cellular damage, especially of endothelial cells. Many of the various diabetic complications are now attributed to the conse-

Please cite this article in press as: Munenori K, et al. Pulmonary hypertension associated with diffuse deposition of pentosidine in pulmonary arterioles. Diabetes Res Clin Pract (2013), http://dx.doi.org/10.1016/j.diabres.2013.01.019

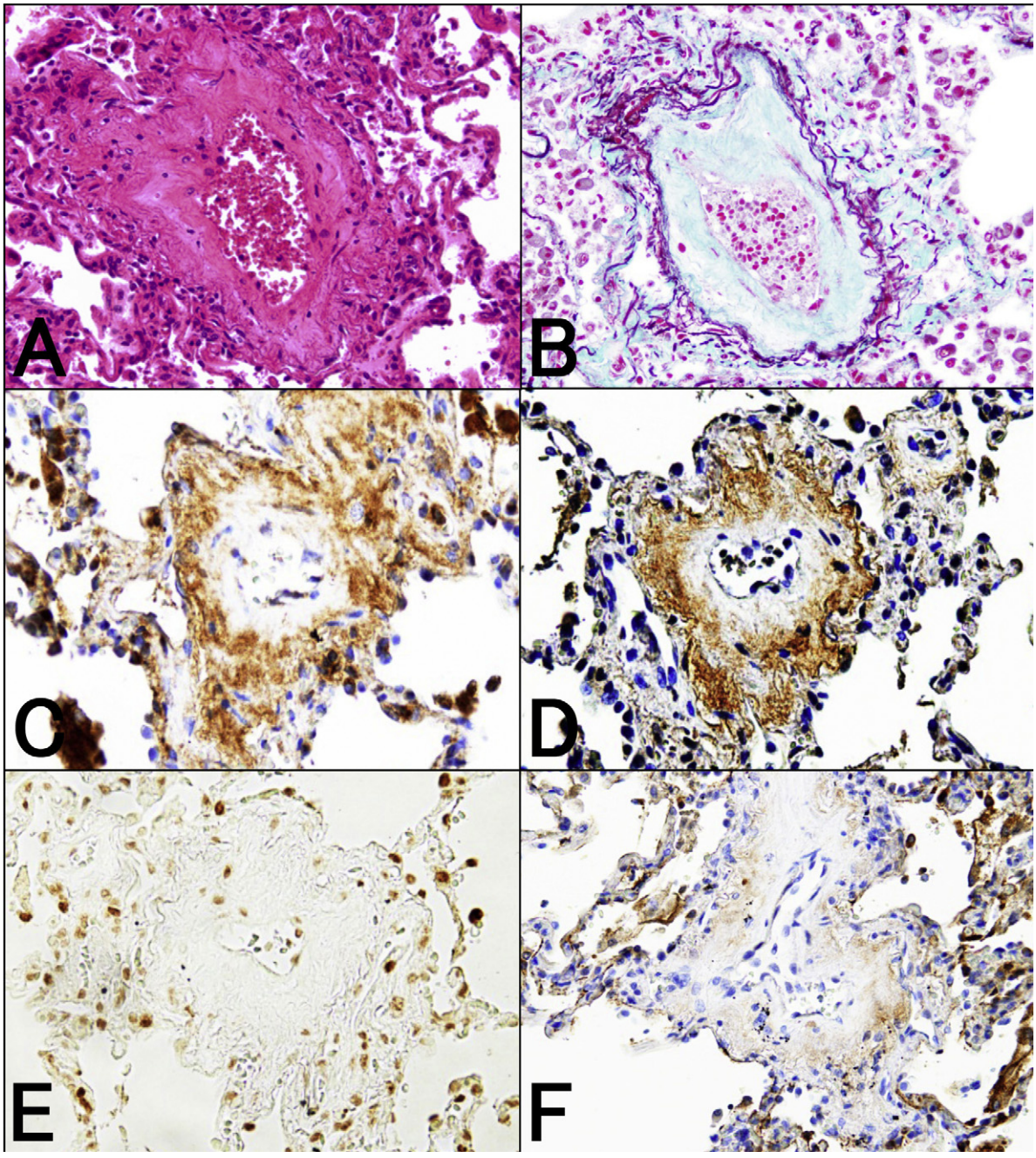


Fig. 2 – Arterioles in the lung show circulatory degeneration (A, HE and B, elastica-Masson) of the connective tissue from the medial to the adventitial area. By immunohistochemistry, advanced glycation end products (AGEs) are strongly positive around the degenerated area of arterioles (C). Pentosidine, a marker of pathological cross-linking of collagen is also strongly positive in the same area (D). Nuclear accumulation of 8-hydroxydeoxyguanosine (8-OHdG) is seen among endothelial and alveolar cells (E). Expression of receptor for AGEs (RAGE) is limited to alveolar cells (F).

quences of oxidative stress by AGEs. Moreover, direct cross-linking of collagen by AGEs, especially by pentosidine, induces pathological cross-linking, resulting in myocardial stiffening [5] and reduced bone strength [6]. In our current case, we have demonstrated for the first time deposition of pentosidine on the collagenous part of pulmonary arterioles, indicating that pulmonary arterioles are also a target of pathological cross-linking by AGEs. We therefore speculate that (1) increased circulation volume induced through the status of hyperosmotic pressure by hyperglycemia, (2) reduction in myocardial compliance by multiple patchy fibrosis in the heart, and (3) impaired pulmonary arteriolar plasticity attributed to pathological cross-linking of collagen by AGEs and all contributed to the clinical manifestation of severe pulmonary hypertension.

Pulmonary hypertension is a syndrome that exhibits a complex pathophysiology that cannot be explained by a single

Please cite this article in press as: Munenori K, et al. Pulmonary hypertension associated with diffuse deposition of pentosidine in pulmonary arterioles. Diabetes Res Clin Pract (2013), http://dx.doi.org/10.1016/j.diabres.2013.01.019

factor [7,8]. Genetic alterations, such as bone morphogenetic protein receptor type 2 (BMPR2) and voltage-gated potassium channels, underlie many heritable and sporadic cases of pulmonary hypertension [9,10]. Furthermore, loss of NO bioavailability and the subsequent endothelial dysfunction is an additional component of pulmonary hypertension [10]. Clinically significant pulmonary hypertension as a complication in diabetic patients is, however, very rare and mostly overlooked [11]. On the other hand, elevated glycated hemoglobin A1c (HbA1c), an independent predictor of long-term prognosis, is frequently observed in pulmonary hypertension [12]. Collectively, collagen fibers in pulmonary arterioles may escape AGEs-induced pathological cross-linking either by a high clearance of AGEs or because of their lower susceptibility to insult than that of other tissues. However in conjunction with other etiological background factors for pulmonary hypertension, pathological cross-linking by AGEs may accelerate disease progression and render it clinically overt.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work is supported in part by Grant-in-Aid from GP Program, Ministry of Education, Sports and Culture, Japan. We appreciate Ms. Yuki Takaoka for technical assistance.

REFERENCES

[1] Noyski J, Zakliczyski M, Konecka-Mrwka D, Przybylski R, Zembala M, Zieliska T, et al. Advanced glycation end-products in myocardium-supported vessels: effects of heart failure and diabetes mellitus. J Heart Lung Transplant 2011;30(5):558–64.
[2] Boudina S, Abel D. Diabetic cadiomyopathy revisited. Circulation 2007;115:3213–23.
[3] Sumandea MP, Steinberg SF. Redox signaling and cardiac sarcomeres. J Biol Chem 2011;286(12): 9921–7.
[4] Saito M, Fujii K, Mori Y, Marumo K. Role of collagen enzymatic and glycation induced cross-links as a determinant of bone quality in spontaneously diabetic WBN/Kob rats. Osteoporos Int 2006;17: 1514–23.
[5] Falcão-Pires I, Palladini G, Gonçalves N, van der Velden J, Moreira-Gonçalves D, Miranda-Silva D, et al. Distinct mechanisms for diastolic dysfunction in diabetes mellitus and chronic pressure-overload. Basic Res Cardiol 2011;106(5):801–14.
[6] Goto S, Fujii H, Kono K, Nakai K, Hamada Y, Yamato H, et al. Carvedilol ameliorates low-turnover bone disease in non-obese Type 2 diabeteas. Am J Nephrol 2011;34: 281–90.
[7] Oygar DD, Zekican G. Pulmonary hypertension in dialysis patients. Ren Fail 2012;34(7):840–4.
[8] Pugh ME, Robbins IM, Rice TW, West J, Newman JH, Hemnes AR. Unrecognized glucose intolerance is common in pulmonary arterial hypertension. J Heart Lung Transplant 2011;30(8):904–11.
[9] Majka S, Hagen M, Blackwell T, Harral J, Johnson JA, Gendron R, et al. Physiologic and molecular consequences of endothelial Bmpr2 mutation. Respir Res 2011; 12:84.
[10] Bonnet S, Archer SL. Potassium channel diversity in the pulmonary arteries and pulmonary veins: implications for regulation of the pulmonary vasculature in health and during pulmonary hypertension. Pharmacol Ther 2007;115(1):56–69.
[11] Fouty B. Diabetes and the pulmonary circulation. Am J Physiol Lung Cell Mol Physiol 2008;295:L725–6.
[12] Belly MJ, Tiede H, Morty RE, Schulz R, Voswinckel R, Tanislav C, et al. HbA1c in pulmonary arterial hypertention: a marker of prognostic relevance? J Heart Lung Transplant 2012;31:1109–14.

Please cite this article in press as: Munenori K, et al. Pulmonary hypertension associated with diffuse deposition of pentosidine in pulmonary arterioles. Diabetes Res Clin Pract (2013), http://dx.doi.org/10.1016/j.diabres.2013.01.019

World Journal of Gastroenterology, in press

Gastric adenocarcinoma arising in gastritis cystica profunda presenting with selective loss of KCNE2 expression

Natsumi Kuwahara[1], Riko Kitazawa[1], Koto Fujiishi[1], Yusa Nagai[1], Ryuma Haraguchi[1], Sohei Kitazawa[1*]

[1]Division of Molecular Pathology, Ehime University, Graduate School of Medicine, Shitsukawa 454, Toon City, Ehime 791-0295, Japan

*Corresponding author

Abstract

Gastritis cystica profunda (GCP) is a rare condition caused by ectopic entrapment of gastric glands, probably secondary to the disruption of muscularis mucosae. GCP is often associated with gastric adenocarcinoma, and loss of the KCNE2b subunit from potassium channel complexes is considered a common primary target molecule leads to both GCP and malignancy. In this study, we, for the first time, analyzed the expression of KCNE2 in surgically excised tissue from human gastric cancer associated with GCP and confirmed that reduced KCNE2 expression correlates with disease formation.

Introduction

Gastritis cystica profunda (GCP) is a rare condition with nonspecific symptoms and radiographic images, making its diagnosis difficult without definitive surgical resection [1]. Clinically, GCP can be misdiagnosed as gastric lymphoma, stromal tumors, gastric cancer, or Menetrier disease. Histopathologically, GCP shows disruption of the integrity of muscularis mucosa that leads to cystically dilated submucosal glands with superficial inflammation in the lamina propria [1, 2]. Since the majority of GCP cases are seen secondary to prolonged chronic inflammation, ischemia, gastric surgery and suturing material, an injury of the muscularis mucosae is assumed to trigger the ectopic entrapment of gastric glands in the submucosa, the muscularis mucosae or serosa and to lead to GCP [1, 2]. Moreover, GCP is often associated with gastric adenocarcinoma indicates that it can lead to a secondary malignancy [2]. Indeed, experiments have shown that animals predisposed to Helicobacter infection develop not only secondary GCP but also subsequent gastric carcinoma [3]. This close association between GCP and malignancy has been interpreted as concurrent sharing of causative factors common to both disease conditions [1, 3]. Recently, with the use of the Kcne2 (also known as MiRP1) deficient mouse model, loss of the KCNE2b subunit from potassium channel complexes is considered a common primary target molecule that gives rise to both GCP and malignancy [4].

Here, the expression of KCNE2 in surgically excised tissue from human gastric cancer associated with GCP is, for the first time, analyzed to confirm that its reduced level correlates with disease formation.

Case Report

A 63-year-old man was admitted to the hospital with the chief complain of abdominal pain. The patient's past medical and family history was unremarkable. An endoscopic and other examinations revealed the presence of multiple erosive lesions, and a biopsy demonstrated only severe superficial gastritis with erosion. Under the diagnosis of benign erosive gastritis, the patient was treated with medication and followed up monthly. After one year, however, a follow-up endoscopic examination revealed an irregularly-shaped ulcerating lesion, and the pathological diagnosis of adenocarcinoma was made through biopsy analysis. The patient underwent distal partial gastrectomy, and pathological examination of the excised stomach (Fig. 1, left) revealed the presence of ectopic cystic mucosa with intestinal metaplasia, especially in the oral side (Fig. 1, right, dotted area). Overlaying the ectopic cystic mucosa, a well- to moderately-differentiated tubular adenocarcinoma was observed in the superficial proper mucosal layer with part of a poorly- differentiated component infiltrating the submucosal layer in the body of the stomach (Fig. 1, right, hatched area). The pathological diagnosis of gastric adenocarcinoma arising in gastritis cystica profunda was made.

Immunohistochemistry

Since targeted deletion of Kcne2 in mice causes gastric lesions resembling gastritis cystica profunda and gastric neoplasia [4], we examined the expression of KCNE2 in the current case immunohistochemically. Also, since signaling through the estrogen receptor (ER) modulates KCNE2 expression [5], we additionally examined the expression of ER. To determine the localization of KCNE2 and ER, rabbit polyclonal

anti-KCNE2 antibody (ABCAM, Cambridge, UK) and rabbit monoclonal anti-ER (EST) antibody (EPIOTOMICS, USA) were used as primary antibodies. Tissue sections were deparaffinized in xylene for 20 min (solvent refreshed at 10 min and 5 min) and immersed in absolute ethanol for 10 min (solvent refreshed at 5 min), then rehydrated in 90 and 70% ethanol (5 min each), and finally placed in distilled water for 15 min (solvent refreshed every 5 min). The samples were inactivated in 1 mM EDTA (pH8.0) plus distilled water in a microwave for 15 min (high heat for 5 min and low heat for 10 min), and then cooled to approximately 25˚C over 1 h. After being undergoing liquid block treatment, the samples were immersed in PBS for 15 min (solvent refreshed every 5 min). After adding blocking buffer to the moisture chamber and incubating the samples with primary antibodies at room temperature for 1 h, they were washed 3 times for 5 min each in PBS, incubated in the moisture chamber at room temperature for 30 min with the secondary antibody diluted at 1:200 with PBS, immersed in PBS for 15 min (solvent refreshed every 5 min), then incubated with DAB for 15 min and washed in PBS.

Immunohistochemical analyses revealed that KCNE2 was universally and strongly expressed on the surface of the cells at the bottom of the cryptic glands, while its expression was diminished in the cystic or dilated lesions (Fig. 2A, B and C). ER expression was observed in both non-cystic and cystic glands (Fig. 2D). In adenocarcinoma, KCNE2 expression was significantly reduced compared with the surrounding non-cancerous gastric mucosa with intestinal metaplasia (Fig. 3A, B and C), while ER expression was observed in both cancerous and non-cancerous glands (Fig. 3D).

Discussion

In this study the expression status of KCNE2 in surgically excised gastric adenocarcinoma coexisting with GCP was examined in light of that the Kcne2-deficient mouse model develops both GCP and gastric cancer. Since KCNE2 expression is modulated by ERa [5], and since ERa expression status per se is related to carcinogenesis and progression stages of gastric cancer [6], we additionally examined the immunohistochemical expression of ERa in in the excised tissue.

KCNE2 has originally been identified as a potassium channel protein, and in the stomach, it is expressed mainly in the cytoplasm of parietal cells [5, 7, 8]. Reduction of Kcne2 in experimental animal models results in profoundly reduced proton secretion, abnormal parietal cell morphology, achlorhydria, hypergastrinemia [5, 8], and striking gastric glandular hyperplasia arising from an increase in the number of non-acid-secretory cells [4]. Functionally, Kcne2 also exerts anti-proliferative effects on gastric cancer cells by down-regulating Cyclin D1 and restricting cell growth [5, 8]. Indeed, reflecting anti-proliferative or tumor-suppressor functions of Kcne2, long-term observation of Kcne2 -/- mice has revealed that reduced Kcne2 expression causes diffuse hyperplasia in gastric mucosa, resulting in a pathologic condition similar to gastric cancer associated with GCP [4].

We examined here, for the first time, the expression of KCNE2 in surgically excised stomach tissue demonstrating both GCP and adenocarcinoma. While KCNE2 expression in both GCP and adenocarcinoma areas was diminished, that in surrounding non-neoplastic and non-cystic cells was clearly maintained, as determined by immunohistochemical analysis. These data, albeit from a single case study, suggest that selective loss of KCNE2 expression is related to the development and clinical manifestation of GCP with subsequent occurrence of cancer. Furthermore, because selective loss of KCNE2 expression is seen at the level of a single cystic gland and cancer cell nest unit, silencing KCNE2 expression may occur at the level of a single tissue progenitor cell. Although the precise molecular mechanism of such selective loss of KCNE2 expression is largely unknown, it is at least not by the loss of estrogen receptor signaling. Further genetical and epigenetical studies based on a cumulative case study are needed to elucidate the role of KCNE2 expression in the development of GCP.

Acknowledgements

We appreciate Ms. Yuki Takaoka for technical assistance. This work is supported in part by Grant-in-Aid from Ministry of Education, Sports and Culture (GP Program for Basic Science), Japan

References

1. Fonde EC, et al., Gastritis cystica profunda, Am J Gastroenterol. 81(6) (1986) 459-464
2. Mitomi H, et al., Imunohistochemical analysis of a case of gastritis cystica profunda associated with carcinoma development, Scand J gastroenterol. 33 (1998) 1226-1229
3. Wang TC, et al., Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer, Gastroenterology 118 (2000) 36-47
4. Roepke TK, et al., Targeted deletion of Kcne2 causes gastritis cystica profunda and gastric neoplasia, *PLoS ONE*. 5 (2010) e11451
5. Kundu P, et al., Hormonal regulation of cardiac KCNE2 gene expression, Molecular and Cellular Endocrinology 292 (2008) 50-62
6. Hogan AM, et al., Estrogen and gastrointestinal malignancy, Mol Cell Endocrinol. 307 (2009) 19-24
7. Pan Y, et al., KCNE2, a down-regulated gene identified by in silico analysis, suppressed proliferation of gastric cancer cells, *Cancer Letters* 246 (2007) 129-138
8. Lee MP, et al., Targeted disruption of the Kvlqt1 gene causes deafness and gastric hyperplasia in mice, J Clin. Invest. 106 (2000) 1447-1455

Figure and figure legends

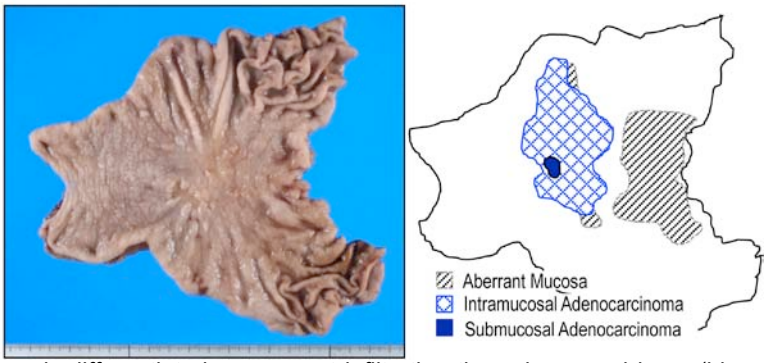


Figure 1. Macroscopic finding and distribution of ectopic cystic lesion and cancer in surgically excised stomach
Surgically excised stomach (left) and schematic distribution of the lesions (right). Ectopic cystic mucosa is located mainly on the oral side and partly in the body part overlapped with a cancer lesion (shaded area). A well- to moderately-differentiated tubular adenocarcinoma in the intramucosal layer (checked area) and part of a poorly-differentiated component infiltrating the submucosal layer (blue area) are located mainly in the body part of the stomach.

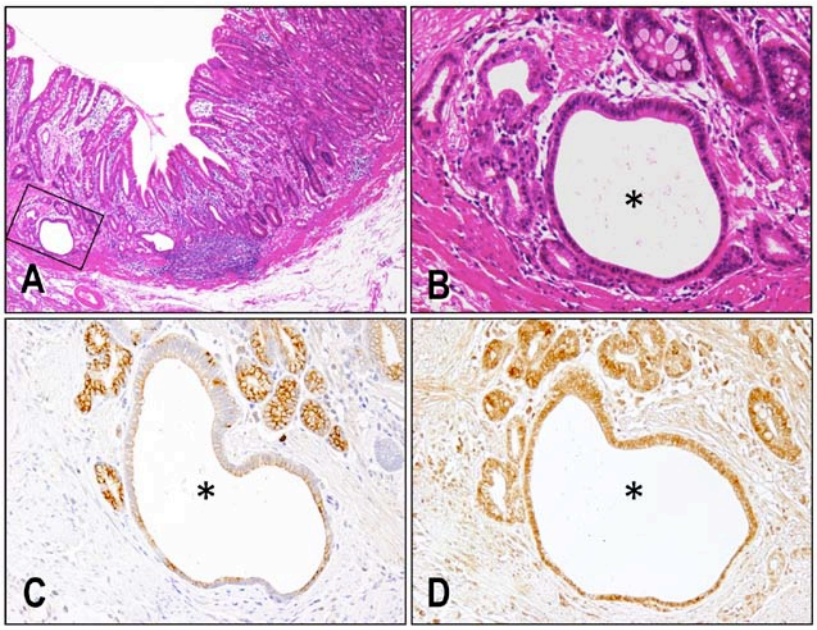


Figure 2. Expression of KCNE2 and ER in non-neoplastic cystic lesion by immunohistochemistry
(A) Low power magnification of a transitional area from the non-neoplastic mucosal layer with intramucosal cystic lesions (left) to the intramucosal adenocarcinoma (right, H.E., X40). (B) High power magnification (squared area in figure A) around the intramucosal cystic lesion (asterisk, H.E., X200). (C) KCNE2 immunostaining of a serial section of (B) shows that KCNE2 is almost negative in the dilated cystic gland (asterisk), while the surrounding non-cystic glands are positive (X200). (D) Estrogen receptor (ER) immunostaining of serial sections of (B) and (C) show that ER is equally expressed in both cystic (asterisk) and non-cystic glands (X200).

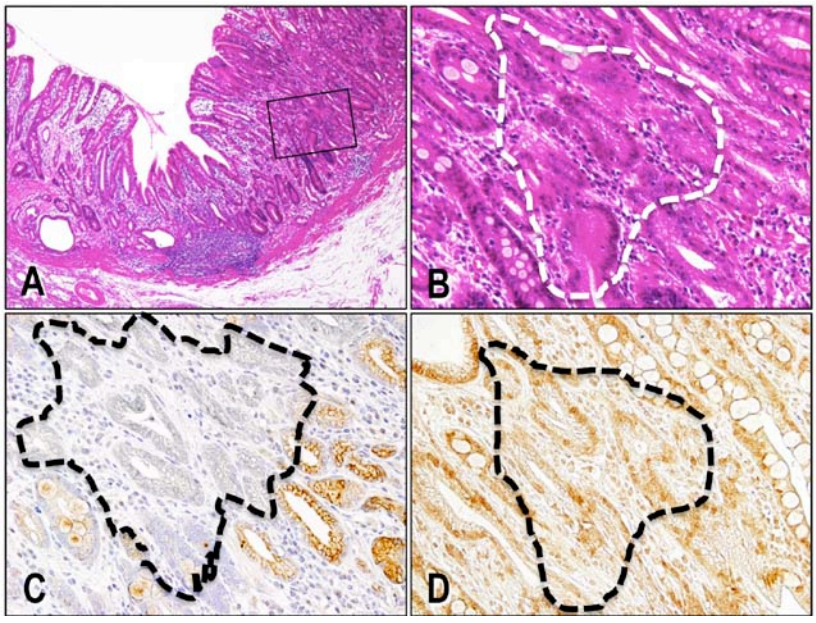


Figure 3. Expression of KCNE2 and ER in adenocarcinoma area
(A) Low power magnification of a transitional area from the non-neoplastic mucosal layer with intramucosal cystic lesions (left) to the intramucosal adenocarcinoma (right, H.E., X40). (B) High power magnification (squared area in figure A) around the intramucosal adenocarcinoma (circled by white-hatched line, H.E., X200). (C) KCNE2 immunostaining of a serial section of (B) shows that KCNE2 expression is almost negative in adenocarcinoma (circled by black-hatched line), while the surrounding non-neoplastic glands are positive (X200). (D) Estrogen receptor (ER) immunostaining of serial sections of (B) and (C) show that ER is equally expressed in both cancerous (circled by black-hatched line) and non-neoplastic glands (X200).

学生研究員活動報告書　2012 年度

伊藤　千尋 Ito Chihiro

初級学生研究員　　医学科 4 年
指導教員　北澤荘平　　所属研究室：分子病理学

自己紹介：松山市出身、出身校は松山南高等学校です。大学へ入学してから空手を始め、最近黒帯をいただきました。力や技だけでなく､精神的にももっと強くなりたいと思っている今日この頃です。

分子病理学教室では、三回生の医科学研究Ⅰの履修時からお世話になっています。元々顕微鏡で組織を見るのが好きだったのと、北澤先生の講義で病理学が臨床と基礎をバランスよく学べる学問であることを知って興味を持ったのがきっかけです。

約一年半に渡って、実験の基本的な手技はもちろん、研究にあたって他の機関の方に協力をお願いする方法、論文の書き方、見やすいスライドの作り方など、心構えからテクニックに至るまで多くのことを学ぶことができました。また、病理解剖の見学で病変部分を実際に見て触れるなど、病理学ならではの貴重な経験もさせていただきました。

現在四回生の医科学研究Ⅱ、初級研究員と引き続き分子病理学教室に所属しています。研究を通じてこれまで学んできたことを今後に活かせるよう、精進して参りたいと思います。

研究業績

1. 論文等

Scrotal cutaneous verruciform xanthoma with MCP-1 immunohistochemical study: a case report

Chihiro Ito, Riko Kitazawa, Kenji Makita, Takafumi Watanabe, Akihiro Toda, Ryuma Haraguchi, Shinji Tanaka and Sohei Kitazawa (Journal of Medical Case Reports, 2012, 31;6(1):260, 4 pages)

2. 学会発表（筆頭演者なく、共同発表のみ）

前立腺原発のびまん性大細胞型 B 細胞リンパ腫 (DLBCL) non-germinal center B cell-like (non-GCB) 亜型の一例　（ポスター）

吉田圭祐、薦田宗則、伊藤千尋、原口竜摩、北澤理子、北澤荘平

第 **101 回**日本病理学会 2012 年 4 月（東京）

3. 医科学研究発表会発表演題

皮膚疣状黄色腫の免疫組織化学的検討　（口演）

伊藤千尋、牧田憲治、渡部貴文、原口竜摩、北澤理子、北澤荘平

第 10 回医科学研究発表会

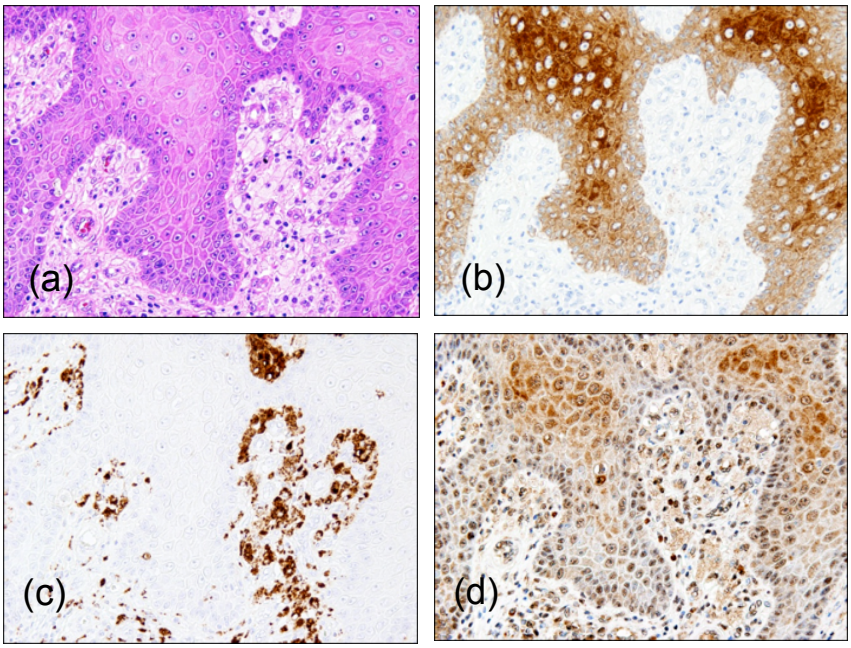
研究概要

皮膚疣状黄色腫の免疫組織化学的検討

【はじめに】疣状黄色腫は、組織学的には乳頭腫症、不全角化症、泡沫マクロファージの集積によって特徴づけられている稀な良性腫瘍である。口腔粘膜に生じる症例が多いが、時に陰茎、陰嚢、外陰などを含む口腔外の症例も報告されている。本研究では陰嚢疣状黄色腫の症例を示し、疣状黄色腫と泡沫マクロファージの間に特殊な相互関係があることを明らかにするため、主要なマクロファージ走化因子である単球走化性活性因子（MCP-1）の免疫組織化学的な局在を中心に分析した。

【症例】70 台後半男性。約 1 年前より徐々に増大する左陰嚢腫瘤が出現し、近医を受診した。腫瘍は黄白色で直径 13mm、比較的境界明瞭であった。良性皮膚腫瘍の診断にて、局所的な切除を受けた。病理組織化学的な検査で、本腫瘍は表皮の乳頭腫様増殖を示す一方、真皮表層部に帯状にマクロファージの集簇が認められ、良性皮膚疣状黄色腫と診断された。側方・深部共に切除断端陰性であった。本例に特徴的なマクロファージの集簇に着目し、マクロファージ走化性因子の検出を試みた。ホルマリン固定されパラフィンに包埋された切片を用いて免疫組織学的にサイトケラチン（AE1/AE3）、CD68、MCP-1 抗体を用いて検索した。サイトケラチン AE1/AE3 は腫瘍全体に陽性であったが、分化した表皮ケラチノサイトで特に強陽性を示す一方、基底層や傍基底層のケラチノサイトでは弱陽性であった。一方、浸潤した泡沫細胞は CD68 に強陽性を示し、これらの細胞がマクロファージ由来で有ることが確認された。マクロファージの代表的走化因子である MCP-1 は、AE1/AE3 強陽性の分化したケラチノサイトに一致して陽性を示した。また、一部の集簇したマクロファージ自身にも MCP-1 の染色性が認められた。

【まとめと考察】腫瘍性に増殖したケラチノサイトにマクロファージ走化因子 MCP-1 が陽性で有り、腫瘍由来の MCP-1 がマクロファージを真皮表層に遊走させることにより、疣状黄色腫の組織学的特徴の成立に重要な役割を果たすということが確認された。また、瘢痕組織の影響でケラチノサイトの AE1/AE3 発現が低下し、リンパ浮腫が生じることが知られている。本例において、瘢痕部に近接する基底層や傍基底層において AE1/AE3 発現が低下しており、リンパ浮腫が集簇したマクロファージの二次的な泡沫化に寄与した可能性がある。



組織標本
(a)　HE 染色(×200)
(b)　AE1/AE3 染色(×200)
(c)　CD68 染色(×200)
(d)　MCP-1 染色(×200)

学生研究員活動報告書　2012 年度

桑原　奈都美 Kuwahara Natsumi

初級学生研究員　　**医学科 4 年**
指導教員　北澤荘平　　**所属研究室：分子病理学**

自己紹介：山口県出身、出身高校は山口高等学校です。趣味は音楽の演奏と鑑賞、読書です。特技はバイオリンで、地元のオーケストラに所属し演奏活動を行っています。

分子病理学教室には、三年次の医科学研究 I の履修時より所属しています。

教室に所属して初めて先生から教わったことは、研究の進め方や論文の書き方でした。高校生の時までは、自分のテーマを持ちそれについて継続して研究する経験はほとんどありませんでしたので、難しそうな実験なんてできるのだろうか、英語の論文なんてわたしに書けるのだろうかと漠然とした不安を覚えたことを覚えています。しかしながら、それから先生方のご指導や、同じ教室に所属する友人たちの助けを借りながらも試行錯誤を重ねることで、どうにか現在まで成果を一本の論文としてまとめることができました。自分で実験を行い、結果の解釈をし、論文を書くという一連の過程を通して、研究の難しさ、そして面白さを僅かながら知ることができたように感じます。

四年生となった現在、授業の一環として教室に通う期間は既に終わっていますが、今も初級研究員として分子病理学教室に引き続き所属させていただいています。今後は、研究テーマを更に発展させ深めていくことは勿論、自分の目標を実現させるため、将来を見据えた研究活動を行っていきたいと考えています。

研究業績

1. 論文等

Gastric adenocarcinoma arising in gastritis cysticaprofunda presenting with selective loss of KCNE2 expression
NatsumiKuwahara, RikoKitazawa, Koto fujiishi, Yusa Nagai, RyumaHaraguchi, Sohei Kitazawa, World Journal of Gastroenterology, in press.

Efficient genetic analysis of microdissected samples by agarose-bead method: alterations of beta-catenin gene in fundic gland polyp and heterotopic gastric mucosa of duodenum
Miku Nakagawa, Riko Kitazawa, NatsumiKuwahara, Keisuke Yoshida, RyumaHaraguchi, Sohei Kitazawa, ActaHistochemica et cytochemica, 2013, in press.

2. 学会発表

アガロースビーズ法による微小組織切片からの遺伝子解析法：胃底腺ポリープと異所性胃粘膜のβカテニン変異
中川みく、牧田憲二、桑原奈都美、原口竜摩、北澤理子、北澤荘平
日本病理学会 2012 年 4 月（東京）

3. 医科学研究発表会発表演題

トランスレーショナルリーサーチによる、深部嚢胞胃炎、胃癌手術例における KCNE2 の発現消失の解析 (予定)

研究概要

深部嚢胞胃炎における KCNE2 の発現消失について

深部嚢胞胃炎について

・稀な疾患であるが、特異的な症状や単純写真の所見が見られないため、外科的切除を行わないと確定診断が難しい

・組織病理学的には、粘膜筋板の統合性の崩壊がみられ、それにより粘膜下腺の嚢胞状拡張と粘膜固有層表面の炎症がおこる

・多くの深部嚢胞胃炎の症例が慢性炎症、虚血、胃の手術後に発生していたため、粘膜筋板の損傷が原因であると考えられていた

・続発性の悪性腫瘍として、胃の腺癌との関係があると考えられている

・近年、KCNE2 のベータサブユニットが深部嚢胞胃炎と悪性腫瘍両方を起こすターゲット分子として注目されている

KCNE2 について

・ナトリウムチャネルタンパクであり、正常では胃粘膜にある胃腺の細胞質に発現している

・消化管の正常な成長調節には必要なタンパクであり、胃癌の細胞増生を抑制するはたらきがある。

・サイクリン D1 の発現を調節的に抑制することで、細胞増生を抑制する効果がある

・実験動物において、KCNE2 の発現を抑制したモデルでは H^+の分泌が大きく低下し、胃の壁細胞の異型や胃酸欠乏、高ガストリン血症を起こす

・KCNE2 の発現は、エストロゲンレセプター(ER)によって調節されている

実験結果と今後

胃の深部嚢胞癌と診断された患者より切除された組織のうち、嚢胞部分と腫瘍部分のそれぞれに免疫組織染色を行い、KCNE2 と ER の局在を調査した。

免疫組織染色の結果、KCNE2 は腺底部細胞の表面には強く発現していたが、一方で嚢胞部では発現が低下していた(図 A,B,C)。ER の発現は、嚢胞部と非嚢胞部の両方でみることができた(図 D)。腺癌部分では、周囲の腸上皮化成部に比べ KCNE2 の発現が明らかに低下していたが(図 A,B,C)、ER は腺癌部と非癌部の両方で発現がみられた(図 D)。

以上の結果より、KCNE2 の選択的な発現低下は深部嚢胞胃炎とそれに引き続く癌の発生に関係があることが推測できる。さらに、KCNE2 の選択的発現低下が単一の嚢胞や腫瘍部分で見られることから、KCNE2 のサイレンシングがある一つの前駆細胞に対して起こっていることが考えられる。この選択的発現低下が起こる正確なメカニズムは大部分が未だ不明であるが、ER のシグナル低下によるものではないことが実験により明らかになった。

今後は、深部嚢胞胃炎の発生に対して KCNE2 が果たす役割を明らかにするため、症例研究に基づきジェネティクスとエピジェネティクス両面での調査を行う必要がある。

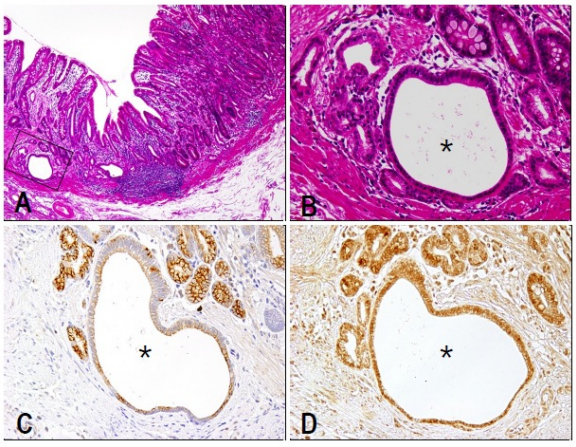


図 1

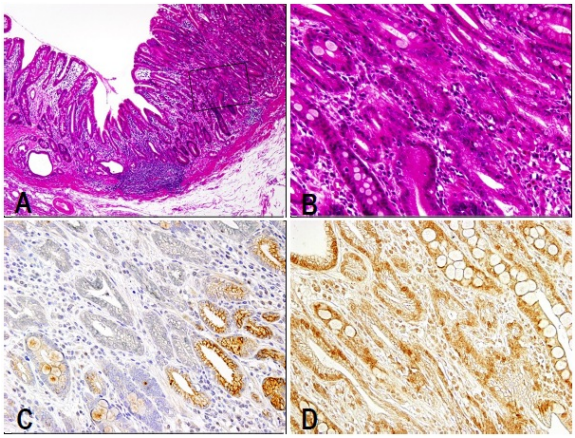


図 2

学生研究員活動報告書　2012 年度

薦田　宗則 Komoda munenori

初級学生研究員　　**医学科 4 年**
指導教員　北澤荘平　　**所属研究室：分子病理学**

自己紹介：兵庫県出身、出身高校は御影高等学校です。部活動は合気道です。

分子病理学教室には、三年次の医科学研究 I から所属しています。教室に所属する前は病理や研究というものがどういうものかわからないまま始まりましたが、北澤先生はじめ教室に所属されている方々のご指導のもと病理学とはどういうものか、その中で研究や論文作成がどのように進められているのか学ぶことができました。研究や論文作成においてはほとんど手取り足取り先生方に教えてもらい論文作成や発表準備をしていく中で研究内容や論文の本当の意義をより学び深めていくことができ、研究や論文の面白さがわかるようになりました。

四回生になってからは初級研究員になり今後は中級研究員を目指して頑張っていきたいと思っています。その中で自分なりに課題をみつけ向上心を持って学びたいと思います。

研究業績

1. 論文等

Multiple-system atrophy in long-term professional painter: a case report.
Yusa Nagai, Riko Kitazawa, Miku Nakagawa, Munenori Komoda, Takeshi Kondo, Ryuma Haraguchi and Sohei Kitazawa. Case Reports in Pathology, 2012, 2012:613180. doi: 10.1155/2012/613180. Epub 2012 Jun 19 (5 pages).

Pulmonary hypertension associated with diffuse deposition of pentosidine in pulmonary arterioles. *Munenori Komoda, Riko Kitazawa, Kenji Makita, Keisuke Yoshida, Miyuki Takeji, Yoshiko Soga, Mie Kurata, Ryuma Haraguchi, Sohei Kitazawa, Diabetes Research and Clinical Practice, in press (doi.org/10.1016/j.diabres.2013.01.019).*

2. 学会発表
糖尿病に合併した難治性肺高血圧症の一剖検例　（ポスター）
薦田宗則、北澤理子、牧田憲二、吉田圭佑、竹治みゆき、曽我美子、倉田美恵、原口竜摩、北澤荘平
第 58 回日本病理学会 秋期特別総会　2012 年 11 月（名古屋）

3. 医科学研究発表会発表演題
糖尿病に合併した難治性肺高血圧症の一剖検例　（口演）
薦田宗則、牧田憲二、吉田圭佑、北澤理子、竹治みゆき、曽我美子、倉田美恵、原口竜摩、北澤荘平
第 10 回 医科学研究発表会

研究概要

糖尿病に合併した難治性肺高血圧症　一剖検例よりの病態解析

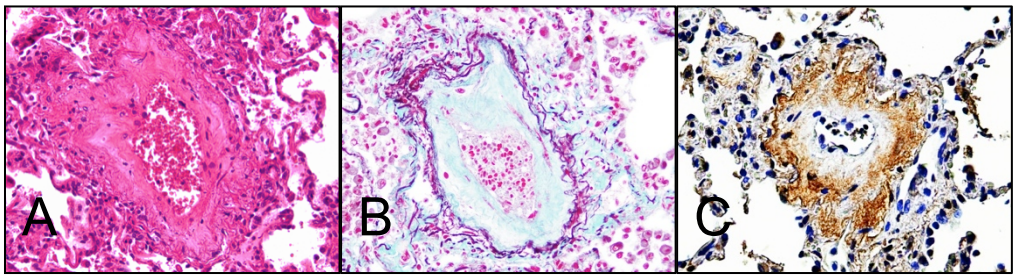
【はじめに】糖尿病では慢性的な高血糖状態により、糖化反応が生じ、種々の advanced glycation end products (AGEs)が組織に沈着することにより種々の合併症が引き起こされる。AGEs による

細胞傷害は、主として酸化的ストレスによるものであり、その結果生じる血管障害が主たる糖尿病合併症の原因となる。一方、AGEs による膠原繊維の異常な架橋形成（いわゆる悪玉架橋）が骨では糖尿病性骨病変の主たる原因とされている。私どもは、糖尿病に高度の肺高血圧症を来した剖検症例を経験し、肺血管病変を中心に解析したので、報告する。

【症例】６０代後半女性、労作時呼吸困難と息切れを主訴として当院を受診し、精査目的で入院となった。患者は６年前に、糖尿病と診断され、栄養管理及び経口血糖降下剤の投薬にて加療されていた。その後、腎機能障害が進行し、４年前に血液透析が開始されていた。入院後、種々の糖尿病合併症に加え、重症の治療抵抗性肺高血圧症が明らかとなった。呼吸困難が進行し、多臓器不全にて死亡した。死後２時間で病理解剖が行われた。

【剖検所見】心臓は４６６ｇと加重しており、左室肥大と右室の肉柱形成、拡張が見られ、心筋細胞の肥大と巣状線維化が見られた。主幹動脈は高度複合型粥状硬化を示し、腎表面は細顆粒状の外観を示していた。肺には、うっ血と肺細動脈平滑筋周囲結合織に同心円状の浮腫と弛緩性変化が見られた。

【免疫組織学染色と考察】免疫組織化学的に AGEs、AGE の一種で悪玉架橋の原因となるペントシジン、酸化的ストレスマーカーである 8-OHdG、AGE の受容体 RAGE を検索した。本例では糖尿病性腎糸球体硬化病変などに AGEs の高度の沈着を認める一方、肺細動脈病変部にも、AGEs、ペントシジンの局在が見られた。ペントシジンは、膠原線維の異常な架橋によって組織の可塑性低下を来すことが知られている。本例における肺高血圧には、高血糖による循環血液量の増加、心筋巣状線維化による心臓の拡張障害に加え、ペントシジンによる肺細動脈周囲の膠原線維硬化による肺動脈拡張障害が複合的に重なった結果、高度の治療抵抗性の肺高血圧症に至ったものと推定する。



血管外膜内側の結合組織に環状に弛緩・膨化が見られる肺細小動脈
A:HE 染色, B: Elastica Masson 染色, C: ペントシジン免疫染色

学生研究員活動報告書　2012 年度

永井　由紗　Nagai Yusa

初級学生研究員　　**医学科 4 年**
指導教員　北澤荘平　　**所属研究室：分子病理学**

自己紹介：松山東高校出身です。今の一番のリフレッシュ法は旅行です。世界遺産や博物館を巡ったり、自然の中をのんびりと歩いたりすることが好きです。日常と違った空間で過ごすことで、次の目標に向かう新たなエネルギーを得ることが、旅の醍醐味だと思っています。

分子病理学教室には、3 年生のときからお世話になっています。北澤先生の授業を受けて、研究の面から、もっと病理学を学んでみたいと思ったことがきっかけです。私は、剖検例の解析と細胞レベルでの解析の両方に関わらせていただきましたが、どちらも、作業の正確さと迅速さの重要性を痛感しました。もともと実験というものにどちらかというと苦手意識をもっていましたが、1 年半を通して、少なからず成長できたと思います。また、実験的作業のみならず、病理解剖の見学、論文作成や学会発表など、最初から最後までの流れを経験し、失敗もくり返すことで、情報を得るだけではなく、発信することの大切さと難しさを学ぶことができました。新たに学んだことや反省点を次に活かし、これからも精進していきたいです。

研究業績：

1. 論文等

Multiple-system atrophy in long-term professional painter: a case report. Yusa Nagai, Riko Kitazawa, Miku Nakagawa, Munenori Komoda, Takeshi Kondo, Ryuma Haragushi and Sohei Kitazawa. Case Reports in Pathology, 2012, 2012:613180. doi: 10.1155/2012/613180. Epub 2012 Jun 19 (5 pages).

Gastric adenocarcinoma arising in gastritis cystica profunda presenting with selective loss of KCNE2 expression. Natsumi Kuwahara, Riko Kitazawa, Koto Fujiishi, Yusa Nagai, Ryuma Haraguchi,Sohei Kitazawa, World Journal of Gastroenterology, in press.

2. 学会発表

多系統萎縮症 (Multiple-System Atrophy)の一剖検例. 永井由紗、北澤理子、中川みく、薦田宗則、近藤武史、原口竜摩、北澤荘平. 第 58 回日本病理学会 秋期特別総会　2012 年 11

月（名古屋）

3. 医科学研究発表会発表演題

永井由紗、北澤理子、中川みく、薦田宗則、原口竜摩、北澤荘平

①多系統萎縮症(Multiple-System Atrophy)剖検例の解析

②破骨細胞分化因子受容体(RANK)splicing variant の解析

第 10 回医科学研究発表会

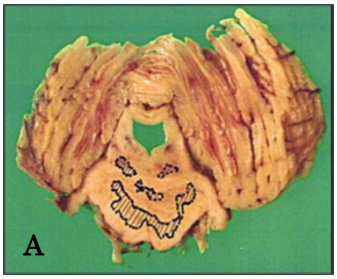
研究概要：

多系統萎縮症(Multiple-System Atrophy)剖検例、および破骨細胞分化因子受容体(RANK)splicing variant の解析

① MSA 剖検症例

[背景]　多系統萎縮症(MSA)は、稀な進行性の神経変性疾患である。病理組織学的には、オリゴデンドログリア内のα-シヌクレイン陽性 glial cytoplasmic inclusion (GCI)が挙げられる。多系統萎縮症(MSA)の病因は未だ不明瞭であるが、環境上の有害物質への暴露は、MSA 発症のリスクを増加させるという報告がある。さらに、生体外での実験において、α-シヌクレインの完全な崩壊が、グルコースの付加により引き起こされることが分かっている。

[症例]　40 代後半の塗装業男性、夕食を喉に詰まらせて倒れているのを発見され、救急搬送された。患者は、20 年前からふらつきと言語不明瞭を呈し、6 年前に糖尿病、2 年前に脊髄小脳変性症と診断された。搬送後、多臓器不全にて死亡、死後 12 時間で病理解剖が行われた。免疫組織化学的に、大脳皮質、中脳、延髄、脊髄のオリゴデンドログリア内にα-シヌクレイン陽性 glial cytoplasmic inclusion (GCI)を証明し、多系統萎縮症(MSA)と診断した。



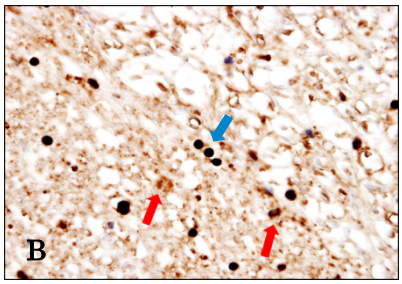


図 A：脳幹の灰白質萎縮

図 B: α-シヌクレイン陽性オリゴデンドログリアとアストロサイト

[結果]　本例では、2 型糖尿病による持続的な高血糖状態と、有機溶媒への過度な暴露とが、α-シヌクレイン凝集を促進し、MSA の発病および進展に寄与した可能性がある。

② RANK splicing variant の解析

破骨細胞の最終分化には破骨細胞分化因子 RANKL と、受容体 RANK との結合を要する。受容体 RANK は、前破骨細胞から成熟破骨細胞に発現する。マウス RANK は 625 アミノ酸、10 の Exon からなるが、Exon2a に stop codon を持つ新規の splicing variant (vRANK)を検出した。マウス vRANK は in vitro で、破骨細胞への抑制作用を示した。ヒト白血病細

胞 HL60 から、Exon2a に stop codon を持ち、38 アミノ酸を code する vRANK を検出した。ヒト vRANK は、ビタミン D 存在下に、PMA や TGF-β で発現が増加する。RNA splicing については、68kD の src-associated substrate in mitosis (SAM68)が関与する構造変化が記載されており、現在、si RNA を用いて、HL60 における SAM68 を knock down して、vRANK mRNA 発現に対する効果を検討している。

学生研究員活動報告書　2012 年度

中川　みく Nakagawa Miku

初級学生研究員　　**医学科 4 年**
指導教員　北澤荘平　　**所属研究室：分子病理学**

自己紹介：松山市出身、出身校は新田青雲中等教育学校です。大学 入学してから競技スキーを始めました。長期休暇には新潟や長野でスキーを楽しんでいます！

分子病理学教室には、三回生の医科学研究 I の履修時から所属しています。病理学を授業で習いとても興味が湧いたので、医科学研究 I でも病理学教室に通いたいと思ったのがきっかけです。私の研究は胃底腺ポリープと十二指腸異所性胃粘膜のβカテニン遺伝子変異をテーマとして三回生の夏休みよりスタートしました。先生方には毎日毎日 PCR 法や遺伝子シークエンスの手法などの遺伝子解析のイロハを手取り足取り教えていただきました。一番印象深いエピソードはエレクトロポレーション法で形質転換を行うときに何度も火花を散らしたことです。きちんとキュベットの水分は拭き取って大腸菌と DNA 量の比率も考えているのに何度やっても爆発してしまい、パルスをかけるスイッチを押すのが恐怖だった日々もありました。同じ手順でやっているつもりでも先生のようにうまくいかないことをもどかしく思うこともありましたが、「研究は失敗してなんぼや！」と心の中で自分を励ましながら今まで研究を続けてきました。また、研究をスタートさせるときに北澤先生に実験ノートを作ることの大切さを教えていただき、失敗しても成功してもその日に行ったことをノートにまとめるという作業も実験と同時に毎日行ってきました。実験ノートは私の宝物です。

現在、四回生ですが初級研究員として引き続き分子病理学教室に所属させていただいております。今後は、更に遺伝子解析の症例数を増やしていき、βカテニン遺伝子変異と胃底腺ポリープ・十二指腸異所性胃粘膜の関連性について検討を進めてまいりたいと思います。

研究業績

1. 論文等

Efficient genetic analysis of microdissected samples by agarose-bead method: alterations of beta-catenin gene in fundic gland polyp and heterotopic gastric mucosa of duodenum
Miku Nakagawa, Riko Kitazawa, NatsumiKuwahara, Keisuke Yoshida, RyumaHaraguchi, Sohei Kitazawa, ActaHistochemica et cytochemica, 2013, in press.

Multiple-system atrophy in long-term professional painter: a case report. *Yusa Nagai, Riko Kitazawa, Miku Nakagawa, Munenori Komoda, Takeshi Kondo, Ryuma Haragushi and Sohei Kitazawa. Case Reports in Pathology, 2012, 2012:613180. doi: 10.1155/2012/613180. Epub 2012 Jun 19 (5 pages).*

Barrett esophagus with metaplasia-dysplasia-carcinoma sequence: A case report with molecular epigenetic study. *Kenji Makita, Riko Kitazawa, Koto Fujiishi, Miku Nakagawa, Haraguchi Ryuma, Kitazawa Sohei, World Journal of Gastroenterology, 2013 Jan 28;19(4):536-41. doi: 10.3748/ wjg. v19. i4. 536.*

『技術講座』（病理） 病理標本を用いた DNA シークエンスの方法と実際. 北澤荘平，近藤武史，中川みく，藤石　琴，原口竜摩，北澤理子．検査と技術 40 巻 1 号 24-29．2012.

2, 学会発表

アガロースビーズ法による微小組織切片からの遺伝子解析法：胃底腺ポリープと異所性胃粘膜のβカテニン変異. 中川みく、牧田憲二、桑原菜都美、原口竜摩、北澤理子、北澤荘平. 日本病理学会 2012年4月（東京）

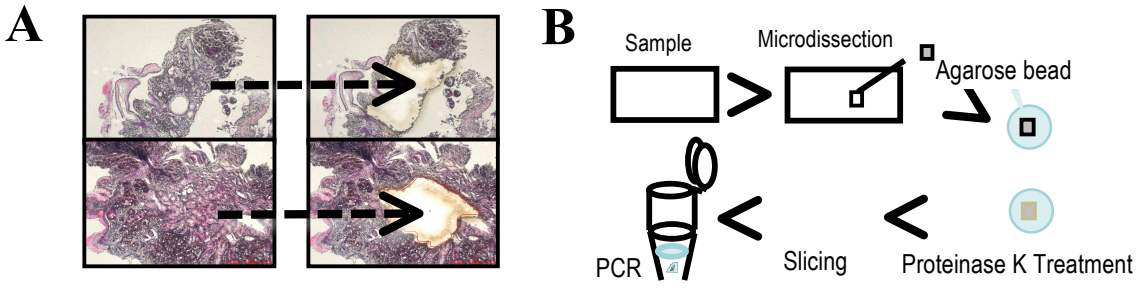
3. 医科学研究発表会発表演題
中川みく、牧田憲治、桑原奈都美、北澤理子、原口竜摩、北澤荘平
アガロースビーズ法による微小組織切片からの遺伝子解析法：
胃底腺ポリープと異所性胃粘膜のβカテニン変異
第10回医科学研究発表会

研究テーマ
アガロースビーズ法による微小組織切片からの遺伝子解析法：胃底腺ポリープと異所性胃粘膜のβカテニン変異

【目的】一般的に患者検体より DNA を抽出する際、病変部のみならず非病変部、炎症細胞、や血管等が混在しており、得られた生化学データは必ずしも疾患本来の性質を正しく反映したものではない。そこで私どもはマイクロダイセクション法を用いて病変部と非病変部を形態学的に区別し、得られた微小組織にアガロースビーズ法を適応することにより、抽出過程での DNA の損失を最小限に抑えた効率の良い遺伝子解析を試みた。胃底腺ポリープと十二指腸異所性胃粘膜におけるβカテニン遺伝子異常の解析を例に紹介する。

【方法】内視鏡的に切除した胃底腺ポリープと十二指腸異所性胃粘膜の共存例 2 例、十二指腸異所性胃粘膜のみの 4 例の計 6 例を対象とした。マイクロダイセクションにて病変部位を選別採取し、低温融解アガロースに封入後、固形化させビーズを作成した。ビーズの状態で proteinase K 処理し、加熱処理後、再度固形化したアガロースビーズを細切し、その一つを鋳型として PCR 法を施行した。その後 Plasmid DNA を抽出し塩基配列を決定した。

【結果と考察】胃底腺ポリープと十二指腸異所性胃粘膜の共存例 2 例中 1 例において、双方がβカテニン遺伝子エクソン 3 のコドン 37 の同一のミスセンス点突然変異(c.109T＞C,p.S37P)を有することが確認された。他の 1 例では十二指腸異所性胃粘膜のみにコドン 32 のミスセンス点突然変異(c.96C＞A,p.D32E)が確認された。十二指腸異所性胃粘膜のみの症例 4 例中 1 例のみにコドン 33 のサイレント点突然変異(c.99T＞C,p.S33S)が確認された。以上、同一患者より得られた胃底腺ポリープと十二指腸異所性胃粘膜において同一の変異を認めたことより、両者は同一の組織幹細胞の変異に由来する病変である可能性が示唆された。また、サイレント点突然変異を含む 3 例で十二指腸異所性胃粘膜のβカテニン遺伝子異常が確認され、Wnt-βカテニン系の異常が十二指腸異所性胃粘膜の病態に深く関わっている可能性が出てきた。今後、症例数を更に増やして検討する必要がある。今回、数年経過したパラフィン包埋検体からマイクロダイセクション法を用いて形態学的に区別される微小細胞集団を選別し、更に、アガロースビーズ法を適応することで抽出過程での DNA の損失を最小限にとどめ、効率よく遺伝子解析を行うことが可能であった。



方法の概略図
図 A の様に胃底腺ポリープ(上)、異所性胃粘膜(下)より病変部を選別採取し、B に示すアガロースビーズ法で DNA を増幅し解析した。

学生研究員活動報告書　2012 年度

藤石　琴 Koto Fujiishi

初級学生研究員　　**医学科 4 年**
指導教員　北澤荘平　　**所属研究室：分子病理学**

自己紹介: 愛媛県松山市出身で、松山西中等教育学校の一期生です。趣味は 10 歳の頃から続けているお琴で、合気道部と邦楽部に所属しています。

分子病理学教室では三年次医科学研究 I の履修時からお世話になっています。授業で病理学を学んだ際に、とても興味深い学問だと感じて興味を持ったことがきっかけでした。先生方には、研究の手技はもちろん、研究を行う際の心構えなどから教えていただきました。先生方に丁寧にご指導いただいて研究を進める中で、研究の意義や奥深さを知ることができました。実験を行って得られた結果を解釈し、新たな知見を他者に発信していこうとする姿勢は、とても大切だと感じています。実験は上手くいかないことも多くあり、とても大変で難しい作業だと感じることもありますが、これまで多くの人々がこのような小さな発見を積み重ねて現在の医学があるのだということを自分自身で実感できたことが、とても良かったと思っています。

現在は、実験から得られた結果を論文にする作業を行っているところです。今後は、さらに研究を進めて掘り下げていきたいと考えています。そして、これからも広い視野を持って研究を続けていきたいです。

研究業績

1. 論文等

Gastric adenocarcinoma arising in gastritis cystica profunda presenting with selective loss of KCNE2 expression.
Natsumi Kuwahara, Riko Kitazawa, Koto Fujiishi, Yusa Nagai, Ryuma Haraguchi,Sohei Kitazawa, World Journal of Gastroenterology, in press.

Barrett esophagus with metaplasia-dysplasia-carcinoma sequence: A case report with molecular epigenetic study.
Kenji Makita, Riko Kitazawa, Koto Fujiishi, Miku Nakagawa, Ryuma Haraguchi, Sohei Kitazawa, World Journal of Gastroenterology, 2013 Jan 28;19(4):536-41. doi: 10.3748/ wjg. v19. i4. 536.

『技術講座』（病理） 病理標本を用いたDNAシークエンスの方法と実際.
北澤荘平, 近藤武史, 中川みく, 藤石　琴, 原口竜摩, 北澤理子.
検査と技術 40巻1号 24-29. 2012

2. 学会発表

9 年の経過中に MYD88 遺伝子の変異が生じた悪性リンパ腫の一症例.
藤石　琴、永井由紗、渡部貴文、板東健次、原口竜摩、北澤理子、北澤荘平.
日本病理学会 2012 年 4 月（東京）

3. 医科学研究発表(平成24年9月21日)

藤石　琴、永井由紗、渡部貴文、板東健次、原口竜摩、北澤理子、北澤荘平

9年の経過中にMYD88遺伝子の変異が生じた悪性リンパ腫の一症例

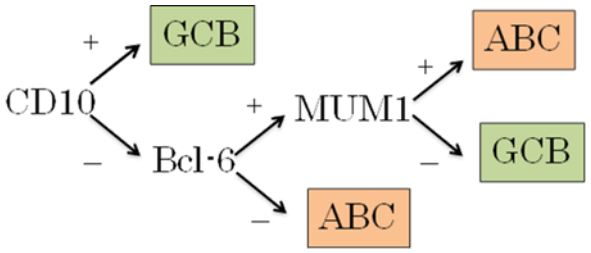
研究概要

9 年の経過中に MYD88 遺伝子の変異が生じた悪性リンパ腫の一症例

【はじめに】びまん性大細胞性 B 細胞リンパ腫(DLBCL, diffuse large B-cell lymphoma)は、大型 B リンパ球がびまん性増殖の像を呈する悪性リンパ腫であり、リンパ腫の中で最も高頻度にみられる。代表的亜型として胚中心 B 細胞由来の GCB 型と活性化 B 細胞由来の ABC 型があり、2004 年に提唱された Hans らによる DLBCL，NOS の細分類によって、免疫組織化学染色の結果から 2 つの亜型を分類することができる。近年、DLBCL の ABC 型では MYD88 の p.L265P が多く見られ、このタイプは治療抵抗性で予後不良であると報告されている。

【症例】60 代男性。40 歳頃に B 型肝炎の既往あり。9 年前に耳下腺腫瘤が発生し、近医にて摘出された。MALT リンパ腫と診断され、放射線・化学療法を受けて寛解した。経過中に B 型肝炎の急性増悪を認め、抗肝炎ウイルス薬の投与を受けていた。1 年前に悪性リンパ腫が再発し、化学療法が施行されたが奏功せず、リンパ腫は全身に広がり死亡した。病理解剖にて、全身諸臓器に浸潤する びまん性大細胞型 B 細胞リンパ腫(DLBCL) と確認された。腫瘍細胞は CD10(−), BCL6(+), MUM1(+)であり、MYD88 遺伝子のミスセンス変異（c.794T>C）も認められ、Hans らによる DLBCL, NOS の細分類に基づいて activated B-cell like (ABC) 型と診断した。一方、9 年前の病理組織では MYD88 遺伝子変異は検出されなかった。また、免疫グロブリン超可変領域の同一性の検討によりモノクローナル性を確認することができた。

【考察】経過中に MYD88 遺伝子変異が新たに加わったと考えられる。本例のように T から C への変異はサイミジンアナログ製剤が関与する報告もある。本症例では、化学療法後に肝炎の再燃をきたした際に抗肝炎ウイルス薬としてラミブジンの投与を受けており、この抗肝炎ウイルス薬により変異が誘発された可能性も考えられる。



Hans CP et al, Blood, 2004 により分類

GCB：germinal center B-cell like
ABC：activated B-cell like(nonGCB)

学生研究員活動報告書　2012 年度

牧田　憲二 Makita Kenji

中級学生研究員　　**医学科 4 年**
指導教員 北澤荘平　　**所属研究室：分子病理学**

自己紹介：鳥取県出身です。出身高校は鳥取西高校です。部活動は陸上をしています。

私は、三年次の医科学研究 I 履修時より分子病理学教室に所属しています。

最初、医科学研究に取り組むに当たり、二つの目標を立てました。それは①基本的な研究手技を身につけること②研究を通して対象疾患に関する知識・理解を深めることです。

①についてですが、これまで、分子病理学教室の先生方によるご指導の下、組織染色や遺伝子解析など多くのことを教えて頂きました。

②については、自分が行った研究結果から論文を作成するに当たり、多くの文献を調べる必要があり、対象疾患に関しての知識・理解が深まりました。しかし、それ以上に、たった数ページの論文を書くことの難しさや、論文がやっと雑誌に認められた時の喜びを体験できたことは、非常に貴重な経験に成ったと思います。

上記以外にも学会発表など本当にたくさんの経験をさせて頂きました。この学んだことを生かし、今後さらに研究をしていきたいと思います。

研究業績

1）論文等

Barrett esophagus with metaplasia-dysplasia-carcinoma sequence: A case report with molecular epigenetic study.
Kenji Makita, Riko Kitazawa, Koto Fujiishi, Miku Nakagawa, Haraguchi Ryuma, Kitazawa Sohei, World Journal of Gastroenterology, 2013 Jan 28;19(4):536-41. doi: 10.3748/ wjg. v19. i4. 536.

Scrotal cutaneous verruciform xanthoma with monocyte chemoattractant protein-1 immunohistochemical study: a case report.
Chihiro Ito, Riko Kitazawa, Kenji Makita, Takafumi Watanabe, Akihiro Toda, Ryuma Haraguchi, Shinji Tanaka and Sohei Kitazawa, Journal of Medical Case Reports, 2012, 2012 Aug 31;6(1):260. doi: 10.1186/1752-1947-6-260 (4 pages).

Pulmonary hypertension associated with diffuse deposition of pentosidine in pulmonary arterioles.
Munenori Komoda, Riko Kitazawa, Kenji Makita, Keisuke Yoshida, Miyuki Takeji, Yoshiko Soga, Mie Kurata, Ryuma Haraguchi, Sohei Kitazawa, Diabetes Research and Clinical Practice, in press (doi.org/10.1016/j.diabres.2013.01.019).

2）学会発表

CDX2 expression and its promoter methylation during metaplasia-dysplasia-carcinoma sequence in Barrett esophagus (ポスター)
牧田憲二、北澤理子、仙波秀峰、中川みく、藤石 琴、原口竜摩、北澤荘平.　日本癌学会総会　2012 年 9 月（札幌）

3）医科学研究発表会発表演題

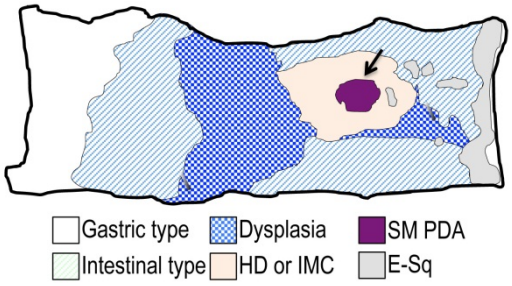
バレット食道の「化生-異形成-癌」進行過程における CDX2 発現とそのプロモータメチル化の関係.
牧田憲二、北澤理子、仙波秀峰、中川みく、藤石 琴、 原口竜摩、北澤荘平
第 10 回医科学研究発表会

研究概要

バレット食道の「化生 - 異形成 - 癌」進行過程における CDX2 発現と遺伝子 5’側上流のメチル化との関係

【はじめに】バレット食道は、反復する逆流性食道炎等で認められ、食道扁平上皮が円柱上皮化生する病的状態である。バレット食道腺癌の発生母地であり、腺上皮化生、異形成がその前癌状態と考えられている。腺上皮化生には、胃固有腺化生および腸上皮化生があり、後者は前者を経由して生じることが知られている。今回、腸上皮化生、異形成、上皮内癌、浸潤癌が認められたバレット食道腺癌の手術例を用いて、上皮の粘膜形質、CDX2 蛋白発現、CDX2 遺伝子 DNA メチル化を各病変部位より得られた微小検体を用いて比較解析したので報告する。

【症例】81 歳男性、胸焼けを主訴に来院。約 40 年前に胃潰瘍にて胃切除術を施行。胃酸逆流症の診断の下に上部食道の内視鏡検査を施行し、極めて広範囲のバレット食道を伴う重度の逆流性食道炎が認められた。不整隆起部分の生検組織より腺癌との病理診断を受け、食道全摘術が行われた。摘出臓器の病理診断では、左図のように胃型(Gatric type)、腸型の化生(Intestinal type, IM)、異形成(Dysplasia)、上皮内癌(HD or IMC)、進行癌(SM PDA)が広範囲に認められた。



【免疫組織化学とメチル化解析】免疫組織化学的に(1) MUC2 と CDX2 陽性の腸型化生粘膜 (IM)、(2) MUC5AC と MUC6 陽性、MUC2 と CDX2 陰性粘膜内癌(HD or IMC)、(3) MUC5AC、MUC6、CDX2 陽性の浸潤性低分化癌 (PDA) に区別された。CDX2 遺伝子上流領域のメチル化状態をマイクロダイゼクションによって得られたサンプルにメチル化特異的 PCR 法を用いて分析した。その結果、IM 部では、DNA のメチル化はなく、CDX2 陰性の IMC 領域では、メチル化が見られた。一方、免疫組織化学的に CDX2 再発現が見られた PDA 領域でもメチル化状態が持続していた。

【まとめと考察】最近、腸上皮化生に関与するホメオボックス遺伝子 CDX2 が、バレット食道の「化生-異形成-癌」進行過程で腫瘍制御因子として働くことが示された。CDX2 遺伝子上流領域のメチル化は CDX2 陽性の IM から CDX2 陰性の HD や IMC に移行する過程で観察され、浸潤癌への移行に際しても維持されていた。ところが CDX2 は浸潤癌でメチル化の存在とは関係なく発現が認められた。このことは、癌の進行過程で、クロマチン構造の変化により、メチル化を乗り越えて CDX2 発現の再活性化があっても、すでに腫瘍抑制因子としての機能を喪失している可能性を示唆するものである。従って、CDX2 発現の臨床病理学的意義の評価に際して、免疫組織学的表現型のみにより解釈することは、腫瘍の性質を正しく反映していない場合があることに注意すべきである。

学生研究員活動報告書　2012 年度

吉田　圭佑 Yoshida Keisuke

初級学生研究員　　**医学科 4 年**

指導教員 北澤荘平　　**所属研究室：分子病理学**

自己紹介：徳島県出身、城南高校出身です。陸上部に所属しています。

分子病理学教室には、三年次の医科学研究 I から所属しています。高校では理科の実験に興味があり、スーパーサイエンスハイスクールという実験を中心に行うクラスに所属していました。授業で実験の面白さに触れ、研究に対する興味をもちました。大学では分子病理学教室に所属し、本格的な研究の一部を体験させて頂きました。はじめは要点が曖昧なまま研究をしていました。しかし何が大切なのかを考え、丁寧な指導を受けるにつれて理解が深まりました。英語の論文や複雑な実験を少しずつ進めていくにつれて研究の難しさや基礎知識の大切さを僅かながら学べたと感じます。現在は 4 回生となり、授業として学ぶのではなく、自主的に学ぶために分子病理学教室に所属させて頂いています。今後は勉学に励み研究と論文の作成を行います。そして医者として、また研究者としての技術と心構えを少しずつ身につけていき、将来の自分の在り方を考え日々頑張ります。

研究業績

1. 論文等

Pulmonary hypertension associated with diffuse deposition of pentosidine in pulmonary arterioles

Munenori Komoda, Riko Kitazawa, Kenji Makita, Keisuke Yoshida, Miyuki Takeji, Yoshiko Soga, Mie Kurata, Ryuma Haraguchi, Sohei Kitazawa, Diabetes Researchand Clinical Practice, in press (doi.org/10.1016/j.diabr es.2013
.01.019)

Efficient genetic analysis of microdissected samples by agarose-bead method: alterations of beta-catenin gene in fundic gland polyp and heterotopic gastric mucosa of duodenum.

Miku Nakagawa, Riko Kitazawa, Natsumi Kuwahara, Keisuke Yoshida, Ryuma Haraguchi,

Sohei Kitazawa, Acta Histochemica et cytochemica, 2013, in press.

2. 学会発表

前立腺原発のびまん性大細胞型Ｂ細胞リンパ腫 non-germinal center B-cell-like (non-GCB)亜型の一例

吉田圭佑、薦田宗則、伊藤千尋、北澤理子、原口竜摩、北澤荘平　日本病理学会総会（東京）　2012 年 5 月

3. 医科学研究発表会発表演題

前立腺原発のびまん性大細胞型Ｂ細胞リンパ腫non-germinal center B-cell-like (non-GCB)亜型の分子病理学的解析

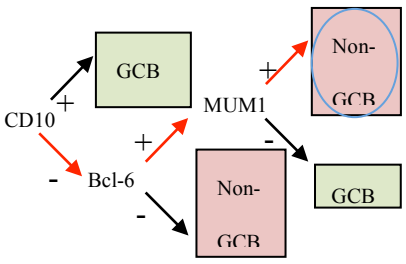
吉田圭佑、薦田宗則、伊藤千尋、北澤理子、原口竜摩、北澤荘平

第10回医科学研究発表会

研究概要

前立腺原発のびまん性大細胞型Ｂ細胞リンパ腫（DLBCL） non-germinal center B-cell-like (non-GCB)亜型の一例

【はじめに】前立腺肥大の原因は、BPH が大部分を占め、悪性腫瘍である前立腺癌も高齢者を中心に近年増加傾向にある。その一方で、非上皮性腫瘍はまれである。私どもは前立腺に原発した悪性リンパ腫の 1 例を経験したので、分子生物学的解析を加え報告する。



Hans CP et al.Blood2004 より

GCB: germinal center B-cell like

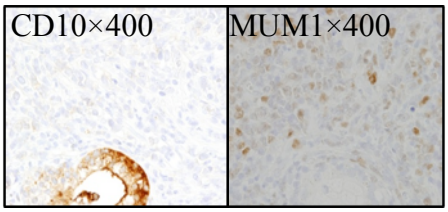
nonGCB: activated B-cell-like(ABC)等

Diffuse Large B-cell Lymphoma (DLBCL)について

DLBCL は、形態的に大型のリンパ腫で、B 細胞形質を持つものの総称であり、ヘテロな疾患群である。DLBCL は Hans 等の検討により、一般的に右図のように免疫組織化学的に GCB 型、non-GCB 型に大別され、この中で、non-GCB 型は、その大部分を占めている

ABC 型とそれ以外の type3 とに分けられている。ABC 型には MYD88 遺伝子に極めて特異的かつ高頻度に遺伝子変異が存在することが 2010 年に報告された。すなわち、免疫組織化学染色で non-GCB 型に分類されるもののうち、MYD88 遺伝子に変異があるものは ABC 型とし、ない場合にはそれ以外の亜型、すなわち type3 として細分類することが出来る。

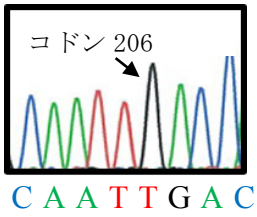
私どもは、前立腺に発生した DLBCL 症例について、分子病理学的な解析を加えて、その細胞形質について検討した。



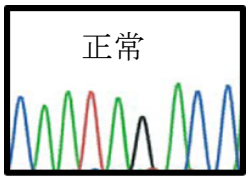
結果とまとめ

免疫組織化学染色では、腫瘍細胞は CD10(－), BCL6(±), MUM1(＋)であり、 non-GCB 亜型と診断した。 遺伝子解析ではコドン 206 にサイレント変異（c.618A>T）が認められた。

本例は免疫学的には non-GCB であり、分子学的には ABC に高頻度で特異的な MYD88 変異がなかったことから、分子病理学的には Type3 であると推定される。



C A A T T G A C



C A A T A G A C

このサイレント変異は MYD88 の活性化を介するリンパ腫の病態形成に関与した可能性があると推測する。サイレント変異では、蛋白質の 1 次構造レベルでは変化は無いが、mRNA の転写効率の違いや蛋白質折りたたみ時の効率が異なるとの報告もあり、この症例に認められたサイレント変異は、MYD88 の蛋白質発現量亢進に関与していた可能性があり、type3 例においても MYD88 の関与する症例がありうると思われる。