投与量など，各臓器別に方法論を確立する必要がある．また，色素法かRI法かについてもその検証は十分になされていない．色素法とRI法でのSNが必ずしも一致しない症例を経験しており，この点をどのように解釈するかが問題の一つである．現時点ではRIで十分であると考えるが，併用がベストであるということには異論はない．しかし，施設の問題やSNとしてのRIのcut-off値の問題もあり，癌治療のあり方をどの臓器でもかえるほどのインパクトがあるかは疑問である．さらに，本法を行うにあたってはlearning curveの問題もあり，臓器に応じたlearning phaseが必要である．これら方法論の解決いかんによっては，そのメリットはきわめて大であり，SNNSを応用することによりある種の癌においては将来的に外科治療のあり方がかわるであろう．まずは現在，本邦においてすすめられている胃癌に対するSNNSの二つのprospective randomized control studyの結果がまたれる．

III. 今後の展望

今後の解決すべきさらなる課題として，標準手技の確立，SNのcriteriaの確立，術中微小転移診断の確立，消化器癌におけるSN conceptについてのエビデンスの確立があげられる．すなわち，今後，消化器外科においても，SNNSが腫瘍外科の分野において一つの潮流となることと思われる．しかし，新たな潮流が本物の流れになるには，科学的根拠を明確に示すとともに，基礎リンパ学，放射線医学，病理学ならびに臨床外科学の治験を駆使して標準的方法論を明確にすることが不可欠である(図3)．

いずれにしても，これからの癌治療の個別化の戦略としては，癌の広がり，stagingの正確な把握と，患者のバックグランド，activityに加えて，癌の生物学的悪性度のもとに判断すべきであり，標準的治療もしくは癌治療の個別化がすすめられるであろう．

おわりに

SNNSについて歴史的展開と種々の命題を論述したが，これら諸問題が解決されたうえで，SNの概念が広く臨床展開されることを期待したい．いずれにしても，センチネルリンパ節の概念はリンパ節転移の有無を検索するうえできわめて高い診断手技であり，乳癌においては癌の外科治療のあり方をかえたが，消化器癌においてもかえるほどのインパクトがある．事実，臨床医にとって，リアルタイムにリンパ節転移状況を把握できる点は最大の魅力である．ひいては癌の個別化治療を確約してくれるツールであることに疑問の余地はない．関係者のさらなる尽力に期待したい．

◆◆◆ 文献 ◆◆◆

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手術手技研究会記事

当番世話人
細川　正夫

指定研究発表

座長：夏越　祥次（鹿児島大学腫瘍制御学・消化器外科学）

主題Ⅱ　私の推奨する手術手技1，2

座長：幕内　博康（東海大学外科）
小川　純一（秋田大学呼吸器外科）

2009年5月16日ロイトン札幌にて行われた第63回手術手技研究会より収録。

指定研究発表
「胃癌センチネルリンパ節生検における リンパ管内の癌細胞検出とその臨床的 意義の検討」

竹内裕也（慶應義塾大学一般・消化器外科）：先生方すでにご存じかと思いますが，センチネルリンパ節というのは腫瘍原発巣からリンパ流を受ける最初のリンパ節のことです（**図122**）。すでに乳癌では術中にセンチネルリンパ節生検を行いまして，転移がなければ他の腋窩郭清を完全に省略するという治療が実地臨床として行われています。では，はたして胃癌に対してはどうなのかということで，sentinel node navigation surgery研究会が最近多施設共同研究を行いました。約400例の胃癌センチネルリンパ節生検の検討でセンチネルリンパ節同定率97.5%，転移検出感度93%，センチネルリンパ節を指標としたリンパ節転移正診率は99%ということで，きわめて良好な結果を示しております。

そこで，近い将来，早期胃癌に対してはセンチネルリンパ節生検をまず行って，術中に転移がないことがわかれば，腹腔鏡下にこのような縮小手術を行うことが期待されています（**図123**）。また，一方ではESDという治療が進歩して，ESD＋センチネルリンパ節生検というような新しい取り組みも期待されております。

そこで問題になってくる点が1つあります。乳癌ではセンチネルリンパ節をpick upで，つまりただセンチネルリンパ節だけを生検しておりますが，はたして胃癌でそれはよいことなのでしょうか（**図124**）。乳癌のようにセンチネルリンパ節だけのpick upでは，たとえば偽陰性症例といって，センチネルリンパ節に転移がないけれど，他のリンパ節に微小転移があったりするようなことも，頻度は少ないけれどあると言われております。また，原発巣とセンチネ

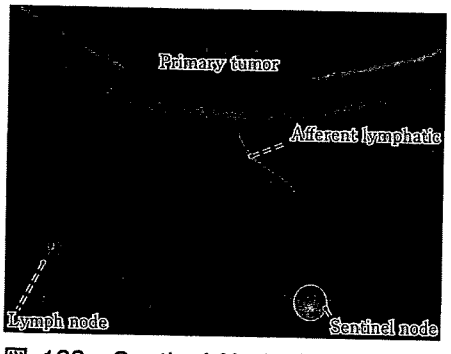

図 122　Sentinel Node Concept

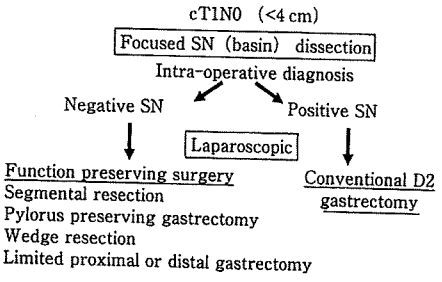

図 123　Current status of SN mapping for gastric cancer

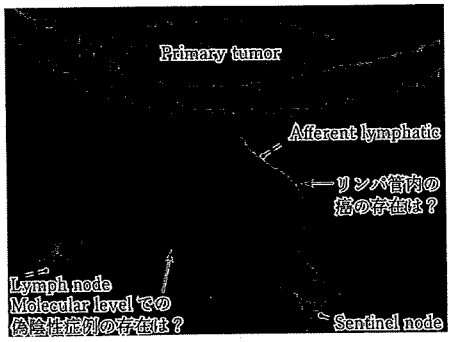

図 124　センチネルリンパ節だけの pick up による転移検索でよいのか

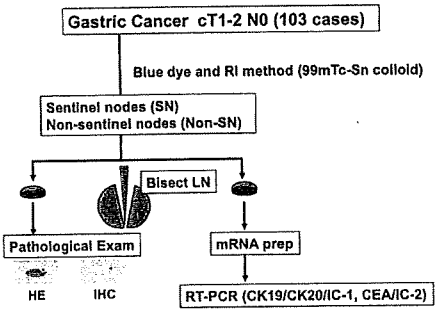

図 126　研究 1　胃癌センチネルリンパ節の微小転移診断

病理学的・分子生物学的手法を用いて①胃癌センチネルリンパ節理論の検証と，②胃癌原発巣からセンチネルリンパ節にいたる一次リンパ管内の癌細胞の存在を探る。

この結果から胃癌センチネルリンパ節生検における pick up 法の可否を検証することを目的とする。

図 125　本研究の主旨

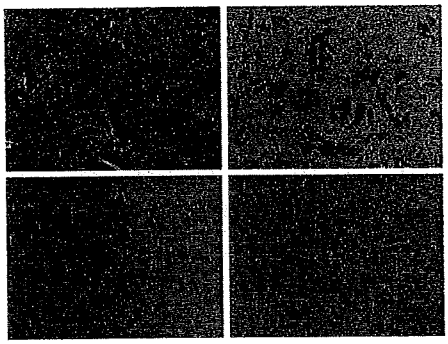

図 127

ルリンパ節を結ぶ一次リンパ管に癌がいてもおかしくないわけです。そのような疑問を持って，この研究を始めることにいたしました。

今回，病理学的，分子生物学的手法を用いて，胃癌センチネルリンパ節理論の検証と，胃癌原発巣からセンチネルリンパ節に至る一次リンパ管内の癌細胞の存在を探ります。この結果から，胃癌センチネルリンパ節生検における pick up 法の可否を検証することを目的としました（図 125）。

そのため，「センチネルリンパ節の微小転移診断」と「一次リンパ管内の癌存在診断」の 2 つの研究を行いました。

まず，胃癌センチネルリンパ節の微小転移診断法の開発とセンチネルリンパ節理論の検証です。当院では色素法と RI 法の両方を用いてセンチネルリンパ節を同定しております。その後，通常郭清も行っておりますので，センチネルリンパ節と非センチネルリンパ節をそれぞれ半割して，一方は病理で普通の HE 染色とサイトケラチンによる免染，それからもう半割したものを，メッセンジャー RNA をとって RT-PCR 法を行っております（図 126）。

図 127 は通常のセンチネルリンパ節の組織像ですが，このようにマッシブに転移があればサイトケラチン染色をしなくてもわかるのですが，たとえばこのような微小なマイクロメタや ITC と言われているようなものであれば，通常の HE 観察ではなかなか診断はむずかしいということになります。

そこで，私どもは Roche Diagnostics 社と共同開発して，術中診断可能な real time RT-PCR 法を開発しました。マーカーとしては CK19, CK20, CEA を用いております。Sensitivity ではメッセンジャー RNA 10 コピーぐらいで検出できることになっております（図 128）。

cT1/T2 N0 103 例の結果です。まず病理組織学的所見と PCR の結果を比較検討しました。病理組織学的に陽性であった 13 例は全例 RT-PCR 陽性でした。一方，病理組織学的に陰性

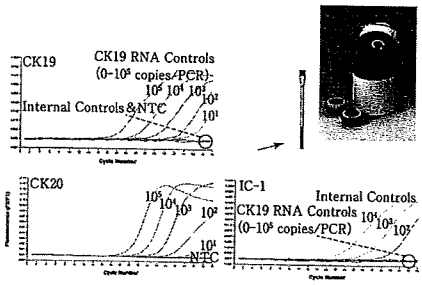

図 128　Real time RT-PCR on LightCycler

SN status	RT-PCR +	RT-PCR -
H&E/IHC +	13 (13%)	0 (0%)
H&E/IHC -	28 (27%)	62 (60%)

Sensitivity 100% (13/13)
31% (28/90) upstaged

図 129　Results (n=103)

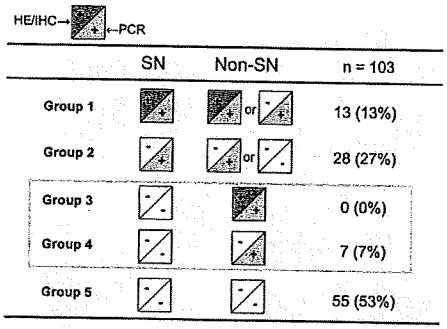

図 130　Results

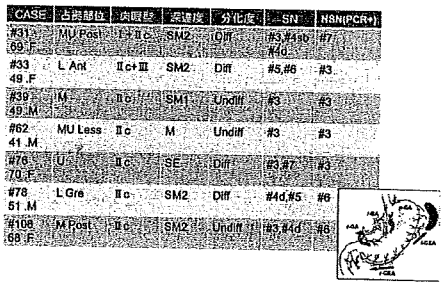

図 131　Molecular level での偽陰性7例
(Kinami et al. Int J Clin Oncol, 2008)

であった90例中28例（約30%）がRT-PCR陽性でした（図129）。

次にセンチネルリンパ節とそれ以外のノンセンチネルリンパ節で，転移状況によってグループ1〜5に分けてみました(図130)。このグループ1，2，5というのがセンチネルリンパ節理論が成り立つグループです。グループ3と4というのはいわゆる偽陰性症例で，すなわちセンチネルリンパ節の転移はないのですが，ノンセンチネルリンパ節に病理組織学的に，あるいはPCRで転移があるというものです。幸い病理組織学的には転移のあるものはなかったのですが，PCRで7例がノンセンチネルリンパ節に転移陽性ということでした。

この7例の内訳を見てみますと，腫瘍が比較的大きなもの，SEの症例，未分化なものなどありましたが，胃のリンパ流域は5領域あると言われていますが，センチネルリンパ節とPCRで陽性だったリンパ節が，7例ともそれぞれ同じリンパ領域に含まれることがわかりました（図131）。

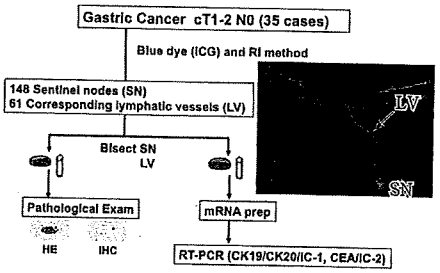

図 132　研究2　一次リンパ管内の癌存在診断

次の研究ですが，一次リンパ管内の癌存在診断です（図132)。cT1/T2N0胃癌で，35例ですが，手術加刀時に術中内視鏡下にICG，色素を注入します。そうするとリンパ管が染め出されて，センチネルリンパ節まで染まってまいります。これをセットに摘出して，リンパ管，センチネルリンパ節，それぞれ半割して，病理学的に，あるいはRTPCRで転移検索を行いました。

図133はリンパ管がよく見えた症例です。たとえば，原発巣がこのあたり（図133矢印）

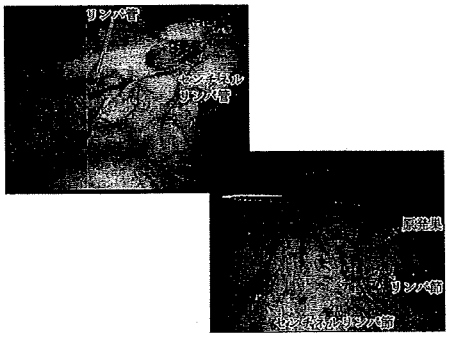

図 133

Parameter	
Gender	
Male/Female	20/15
Median age (yr)	59(27-79)
Tumor location	
Upper/middle/lower	6/26/3
Histologic type	
Differentiated/Undifferentiated	15/20
pT factor	
M/SM/MP/SS	17/13/4/1
ly+/ly-	10/25
v+/v-	5/30

図 135 Clinicopathologic Findings of 35 Patients With cN0 Gastric Cancer

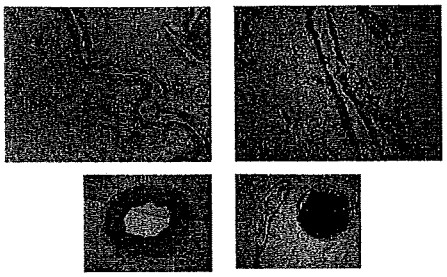

図 134 一次リンパ管

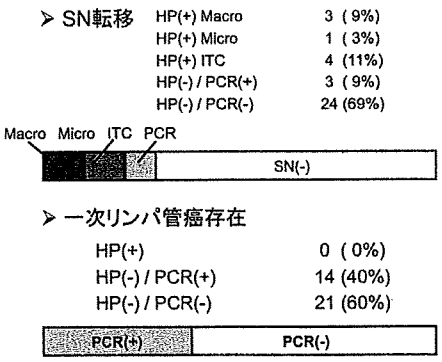

図 136

にあるのですが，リンパ管があってセンチネルリンパ節，ここにも緑色のリンパ管があります（図 133 矢印）。見える症例はあるのですが，見えない症例もあって，全例リンパ管がとれたというわけではありません。病理組織学的に，自分はこれまで太いリンパ管を気にして見たことがなかったのですが，病理の先生に伺うと，壁が比較的薄くて，平滑筋があまりない，蛇行がある，弱々しい弁が随所に見られる，血管ではありませんので，赤血球がないということで，比較的太いリンパ管であれば同定は可能です（図 134）。動脈，静脈との鑑別は容易で，リンパ節のように細胞が詰まっているわけではないので，中の癌細胞の同定は比較的容易ではあるということです。

35 例の内訳をお示しします（図 135）。まずセンチネルリンパ節のほうの結果ですが，病理組織学的にマクロの転移陽性であったものが 3 例（9%）ありました。2 mm 以下のマイクロメタであったのが 1 例（3%），0.2 mm 以下の ITC が 4 例（11%），病理組織学的には検出できなかったのですが，PCR 陽性だったのが 3 例（9%）ありました。残りの 70%がセンチネルリンパ節完全転移陰性ということでした（図 136）。

一方，問題のリンパ管のほうですが，残念ながら，病理組織学的に 1 例も癌細胞を見つけることはできませんでした。一方，PCR で 40%の症例が陽性，60%が陰性でした。

そこで，一次リンパ管の PCR 陽性と既存の病理組織学的背景と何か相関がないかということで調べてみたのですが，いくつか面白いことがわかりました。

まず，センチネルリンパ節転移状況との相関を見てみますと，センチネルリンパ節転移があった 11 例中 10 例が一次リンパ管も PCR 陽性でした（図 137）。これは当たり前といえば当たり前かもしれません。リンパ管の先にセンチネルリンパ節があるわけですから，センチネ

n＝35		一次リンパ管	
		-	+
SN	-	20（83%）	4（17%）
	+	1（9%）	10（91%）

P＜0.0001

図 137　SN転移状況と一次リンパ管内癌存在は有意に相関する

n＝35		一次リンパ管	
		-	+
ly	-	18（72%）	7（28%）
	+	3（30%）	7（70%）

P＝0.05

図 139　原発巣ly因子と一次リンパ管内癌存在は有意に相関する

n＝35		一次リンパ管	
		-	+
原発巣分化度	分化型	12（80%）	3（20%）
	未分化型	9（45%）	11（55%）

P＝0.046

図 138　原発巣分化度と一次リンパ管内癌存在は有意に相関する

CASE	占拠部位	肉眼型	深達度	分化度	ly	v	最大径
59 M	M Less	0 IIc	T1(M)	Diff	0	0	4.0
62 M	M Gre	0 IIc	T1(SM1)	Undiff	0	0	3.8
63 M	M Less	0 IIc	T1(SM2)	Diff	1	0	2.0
46 F	M Gre	0 IIc	T2(MP)	Diff	1	1	2.0

ly0　20例中2例（10%）が一次リンパ管陽性
ly1　4例中2例（50%）が一次リンパ管陽性

P＝0.11

図 140　SN陰性／一次リンパ管陽性4例（17%）

⇒ Molecular levelでもSNコンセプトは成立するが偽陰性例は存在する
⇒偽陰性例におけるPCR陽性リンパ節はSNと同じbasin内に含まれる可能性が高い
⇒ SN転移陽性と一次リンパ管陽性は有意に相関する
⇒未分化型癌やly陽性と一次リンパ管陽性は有意に相関する
⇒ SN陰性例でも一次リンパ管陽性例が約20％存在する可能性がある。とくにly陽性例は注意が必要である。

図 141　まとめ

ルリンパ節に転移があれば，その手前のリンパ管に癌細胞がいてもおかしくはないということです。一方，センチネルリンパ節が転移陰性であった24例中4例（17%）でPCR陽性だったのです。

その他，原発巣の分化度で見てみますと，分化型，未分化型で分けてみますと，未分化型で一次リンパ管PCR陽性の割合が有意に高いということがわかりました（**図138**）。また，原発巣のly因子で見てみますと，原発巣でly陽性だと，PCR陽性の割合が有意に高いことがわかりました（**図139**）。

さきほどのセンチネルリンパ節転移陰性24例中，一次リンパ管が陽性だったのが4例（17％）ありましたが，その内訳です（**図140**）。なにか特徴がないかと調べたのですが，ly陰性であった20例中，一次リンパ管陽性だったのは2例（10%）のみだったのですが，ly陽性例4例中2例が一次リンパ管陽性でした。これは有意差はないのですが，やはりセンチネルリンパ節が転移陰性であったとしても，原発巣のly因子が陽性である場合は，その先の一次リンパ管にも癌がいる可能性が高く注意が必要なことがわかりました。

以上まとめますと，PCRの検討でも胃癌センチネルリンパ節理論は成立しますが，数は少ないのですが偽陰性症例は存在していました。偽陰性例におけるPCR陽性リンパ節はセンチネルリンパ節と同じリンパ流域に含まれる可能性が高いことがわかりました（**図141**）。

センチネルリンパ節転移陽性と一次リンパ管

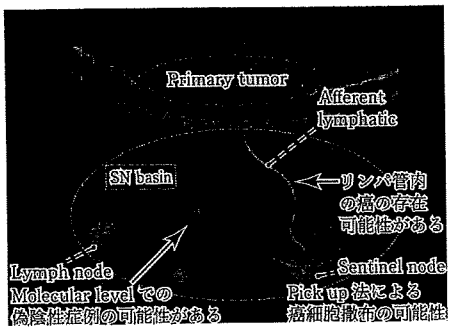

図 142　センチネルリンパ節だけの pick up による転移検索でよいのか

陽性は有意に相関していました。また，未分化型や ly 陽性と一次リンパ管陽性は相関していました。

センチネルリンパ節転移陰性例でも一次リンパ管陽性例が約 2 割存在しておりました。とくに ly 陽性例は注意が必要であると考えられました。

最初のクリニカルクエスチョンである，乳癌のようなセンチネルリンパ節だけの pick up による転移検索でよいのかという疑問に対しては，やはり偽陰性症例の可能性が少数ですがあるということや，リンパ管内の癌の存在の可能性が示唆され，とくにセンチネルリンパ節が転移陰性であっても，一次リンパ管の中に癌がいる可能性があります。もしそのような状況で，pick up 法によるセンチネルリンパ節生検を行った場合，リンパ管断端から癌細胞が撒布される可能性も指摘されます。たとえば乳癌のような悪性度が比較的高くないものであれば，臨床的には意味がないかもしれませんが，ことに消化器癌，胃癌のような場合は，現時点ではセンチネルリンパ節を含むリンパ流域をまず *en bloc* に切除して，バックテーブルでセンチネルリンパ節を同定して転移検索をするのが安全なのではないかというのが私の結論です（**図 142**）。ご清聴ありがとうございました。

夏越祥次（司会）：竹内先生ありがとうございました。私たちも，血中の遊離癌細胞がかなり早い時期から出ているものですから，リンパ管にもおそらくあるのだろうと思っておりましたが，先生にそれを明快に説明していただけました。今後はリンパ管の中の癌細胞が着床していくかどうかということも含めて，またご報告いただきたいと思います。本日はおめでとうございます。ありがとうございました。

主題Ⅱ-1
食道癌（開胸）—開胸食道癌手術におけるクリティカルポイント—〜手術野の展開 / 局所解剖の認識 / 血管・神経の温存〜

梶山美明（順天堂大学上部消化管外科学　教授）：食道癌手術が困難な理由には，この 3 つが主に考えられると思います（**図 143**）。手術野を得ることが困難である。局所解剖の認識が困難である。合併症が多い。これらに対してそれぞれ，手術野の展開，局所解剖の認識，血管神経の温存ということからお話しさせていただいて，10 分のビデオを提示させていただきます。

今回のテーマは「私の推奨する手術手技」ということですが，私自身が皆様に誇れるような手術ではございません。秋山先生，鶴丸先生から教えていただき，私なりに解釈した結果を本日提示させていただきたいと思います。

観念的になりますが，どんな手術も基本手技を着実に積み重ねていくということがコツである。あえて言うならば，そのように考えております。とくに食道癌手術では忍耐と冷静さが必要になります。1 つ 1 つ積み上げて，その集大成として手術が完成する。1 つ 1 つの基本手技が，どの 1 つのエレメントが崩れても手術は成

1. 良好な手術野を得ることが困難
　→「手術野の展開」
2. 局所解剖の認識が困難
　→「局所解剖の認識」
3. 合併症が多い
　→「血管・神経の温存」

図 143　食道癌手術が困難な理由は？

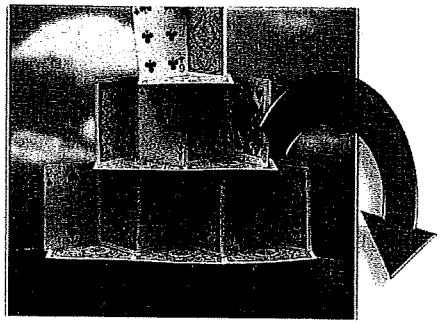

図 144　“手術は一つの作品であり，
基本手技の集大成である”

図 146　奥を広げる手術野の展開

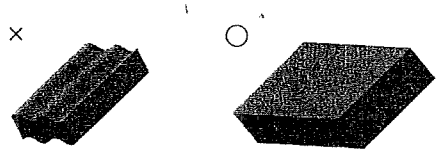

図 148　手術面の創出
“「線」ではなく「面」を創る”
＝どの方向にも等しい力で牽引する
力のひずみを生じさせない→損傷の防止

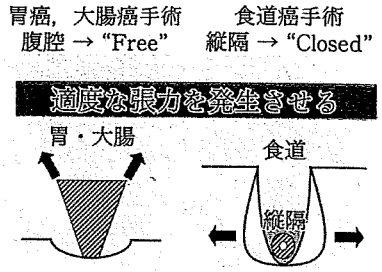

図 145　手術野の展開

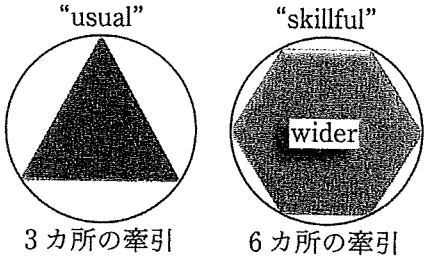

図 147　手術野の創出

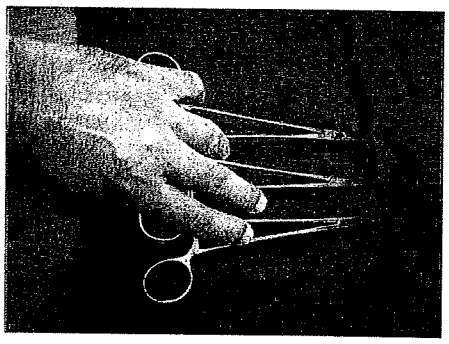

図 149　複数の鉗子をコントロール
する

立しないと考えております（図144）。

最初のテーマである「手術野の展開」ということですが，これは胃癌，大腸癌手術ではオープンな腹腔というスペースに対して，食道癌手術ではクローズドの縦隔という，非常に奥深いところの術野になります。したがって，すべての手術で共通なこととして，適度な張力を発生させて術野を作るということが大事ですが，とくに食道では，奥深くをいかに広げるかという術野の展開の工夫が必要になってまいります。胃癌，大腸癌などオープンの腹腔の手術では，手前に引き上げることによって術野を展開できますが，食道癌では，いかに奥を広げるかということが大事になるのです（図145，146）。

あとは牽引の場所，数をできるだけ多くする（図147）。それによって手術野の面積を広くできる。また，その手術野を展開する際には，このように線ができるような術野ではなく，きれいに，どの方向にも等しい力で牽引する（図148），面を作ることが，オープンであろうが鏡視下手術であろうが，同じ重要なテーマであろうと思われます。

このためには，たとえば1つの手で複数の鉗子をコントロールするということも重要な基本手技の1つであると考えております（図149）。

CCR7 and CXCR4 expression predicts lymph node status including micrometastasis in gastric cancer

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Abstract. The chemokine receptors CCR7 and CXCR4 play a major role in the mechanism of lymph node metastasis from primary tumor cells. We postulated that their expression in gastric tumor cells could predict lymph node status including lymph node micrometastasis (LNMM). We assessed CCR7 and CXCR4 expression in 93 resected gastric tumor specimens by immunohistochemistry. Dissected lymph nodes were examined by reverse transcription-polymerase chain reaction and immunohistochemistry using cytokeratin monoclonal antibody to detect LNMM in addition to hematoxylin-eosin (H&E) staining. Levels of CCR7 and CXCR4 expression were high in 26.9% (25/93) and in 32.3% (30/93), respectively of tumor cells and the levels significantly correlated with lymph node metastasis according to H&E staining (P=0.0212 and P=0.0115, respectively). We identified LNMM in 25 of 83 (30.1%) node-negative patients. Both CCR7 and CXCR4 expression significantly correlated with lymph node status including LNMM (P=0.0092 and P=0.0075, respectively). Furthermore, levels of combined CCR7 and CXCR4 expression significantly correlated with lymph node metastatic status (P=0.0021). Assessment of CCR7 and CXCR4 expression in gastric cancer is a useful tool for predicting lymph node metastatic status including LNMM.

Introduction

Chemokines are small secreted proteins that presently comprise subfamilies C, CC, CXC, and CX3C based on the arrangement of their cysteine residues in the NH_2-terminal (1). These chemokines act through their G-protein-linked receptors on target cells (2). Many chemokine receptors have been identified and their activation regulates cytoskeletal rearrangement, adhesion, and directional migration (3,4). Recent studies have demonstrated that chemokines and their receptors principally function as a signaling pathway in leukocyte trafficking and lymphocyte homing (5,6). Furthermore, these signaling pathways play an important role in tumor progression (7). Müller *et al* (8) reported that at least the chemokine receptors CCR7 and CXCR4 are highly expressed in human breast cancer cell lines and primary breast tumors and that lymph nodes, which are representative secondary metastatic sites of breast cancer, highly express their ligands CCL21 and CXCL12. These results indicate that CCR7 and CXCR4 expressed by breast tumor cells play a major role in the mechanism of lymph node metastasis from primary tumor cells.

Lymph nodes are the most common metastatic sites and nodal metastasis is recognized as an important prognostic factor in gastric cancer (9-11). Therefore, patients with lymph node metastasis have a poor prognosis, despite complete resection (R0). On the other hand, endoscopic mucosal resection (EMR) and endoscopic sub-mucosal dissection (ESD) without lymphadenectomy have been widely applied in Japan to treat mucosal gastric cancer (12-14). However, lymph node metastasis in mucosal and submucosal gastric cancer is pathologically detectable in 2-4 and 13-20%, respectively (15-19).

We previously reported that lymph node micrometastasis (LNMM) can be identified by immunohistochemistry (IHC) and by reverse transcription-polymerase chain reaction (RT-PCR) assays in patients with gastric cancer who are pathologically node-negative (pN0) according to conventional hematoxylin-eosin (H&E) staining (20,21). The clinical significance of LNMM in gastric cancer is controversial (22). However, 92% of gastric tumor cells within LNMM are Ki-67 positive (23), indicating the potential proliferative activity of LNMM in gastric cancer. Accordingly, part of the strategic surgical approach to treating gastric cancer is to detect lymph node metastasis including LNMM. However, to preoperatively assess LNMM using methods such as computed tomography and ultrasound is difficult. To date, no reports have revealed the relationship between LNMM and CCR7 and CXCR4 expression in gastric cancer. Additionally, no better biomarkers

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Key words: CCR7, CXCR4, lymph node micrometastasis, gastric cancer

for predicting lymph node status including LNMM have been identified in gastric cancer.

The present study investigates CCR7 and CXCR4 expression in gastric tumors and examines the relationship between such expression and lymph node status including LNMM.

Materials and methods

Gastric cancer cell lines. We constructed standard curves for RT-PCR assays using the gastric cancer cell line, MKN-45, which was cultured in RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (Mitsubishi Kasei, Tokyo, Japan) and 100 U/ml each of penicillin and streptomycin at 37°C in a humidified 5% CO_2 atmosphere as described (20,21).

Patients. We enrolled 93 patients (65 men and 28 women; age range, 41-84 years; average 64 years) with gastric cancer who underwent curative gastrectomy with lymphadenectomy at Kagoshima University Hospital between 2003 and 2005. Patients who had undergone preoperative radiation therapy or chemotherapy were excluded from the study. Tumors were classified and staged based on the Japanese classification of gastric carcinoma (24). Fourteen, 51 and 28 tumors were located in the upper, middle and lower thirds of the stomach, respectively. Eighty-one and 12 patients had T1 (invasion of mucosa or submucosa) and T2 (invasion of muscularis propria or subserosa)/T3 (penetration of serosa) tumors, respectively, that were histopathologically classified as differentiated (n=55; papillary, well and moderately differentiated tubular adenocarcinomas) and undifferentiated (n=38; poorly differentiated adenocarcinoma, mucinous adenocarcinoma, and signet-ring cell carcinoma). Paraffin-embedded archival tissue (PEAT) specimens obtained from these resected primary tumors were histopathologically confirmed by a surgical pathologist. All specimens were collected from the patients after informed consent had been obtained in accordance with the institutional guidelines of our hospital.

Lymph nodes. We examined 2,415 lymph nodes from 93 patients with gastric cancer (range 2-69 nodes; average 26 nodes). The negative controls for LNMM for our RT-PCR assays comprised 30 normal lymph nodes from 14 patients without cancer (gall bladder stone, n=6; gastric adenoma, n=4; gastric ulcer, n=3; Crohn's disease, n=1). The lymph nodes were cut into 2 blocks at the plane of the largest dimension. One block was suspended in 1 ml of Isogen (Nippon Gene, Toyama, Japan) and immediately stored at -80°C. The other block was fixed in 10% formaldehyde, embedded in paraffin, and then cut into 3 μm sections for H&E staining and IHC using a monoclonal antibody (mAb) to cytokeratin (CK).

RNA extraction. Thawed lymph nodes were homogenized in FastPrep (Qbiogene, Inc., Carlsbad, CA, USA) and then total RNA was extracted, isolated and purified using phenol-chloroform as described (20,21). The concentration and purity of the total RNA were determined using a GeneQuant pro UV/Vis spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK).

Primers and probes. Primer and probe sequences of carcinoembryonic antigen (CEA) and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) were designed for RT-PCR assays as described (20,21). The forward primers, donor and acceptor probe sequences, and reverse primers for CEA and GAPDH were as follows: CEA (forward), 5'-TGTCGGCATCATGAT TGG-3'; (donor and acceptor), 5'-CCTGAAATGAAGAA ACTACACCAGGGC-3'-fluorescein and 5'-LC-Red640-GCTATATCAGAGCAACCCCAACCAGC-3'-phosphorylation; (reverse), 5'-GCAAATGCTTTAAGGAAGAAGC-3'; GAPDH (forward), 5'-TGAACGGGAAGCTCACTGG-3'; (donor and acceptor), 5'-TCAACAGCGACACCCACTCCT-3'-fluorescein and 5'-LC-Red640-CACCTTTGACGCTGGG GCT-3'-phosphorylation; (reverse), 5'-TCCACCACCCTGT TGCTGTA-3'. The integrity of the RNA was confirmed by RT-PCR assays using GAPDH.

RT-PCR assay. Contamination with genomic DNA was avoided using DNase-I (Invitrogen, Life Technologies, Foster City, CA, USA) and complementary DNA (cDNA) was synthesized using the Advantage RT-for-PCR kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) as described (20,21). All RT-PCR assays were performed using the LightCycler system (Roche Diagnostics, Mannheim, Germany). The reaction mixtures contained cDNA, primers, fluorescent and LC-Red probes, $MgCl_2$, LightCycler FastStart DNA Master hybridization probes (Roche) and anti-Taq DNA polymerase antibody (TaqStart antibody, Clontech Laboratories). The amplification profile consisted of 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 15 sec, and extension at 72°C for 5 sec. All primers and probes were synthesized and purified by reverse-phase high-performance liquid chromatography and the optimal reagent concentrations and PCR cycling conditions were established at the Nihon Gene Research Laboratories (Sendai, Japan). Standard curves for each assay were generated using a threshold cycle of serially diluted MKN-45 cells as described (20,21). Quantitative data were analyzed using LightCycler software (Roche). All RT-PCR assays included positive (gastric cancer cell line), negative (normal lymph nodes from patients without cancer) and reagent controls (reagents without cDNA). Our RT-PCR assay system was optimized and established for detecting LNMM as described (20,21).

Immunohistochemical staining. We assessed LNMM in all dissected lymph nodes by IHC staining using a CK AE1/AE3 mAb (Dako Corp., Carpinteria, CA, USA) as described (20,23). The PEAT sections were deparaffinized in xylene and rehydrated in ethanol, and then endogenous peroxidase activity was blocked by 5-min incubation in methanol containing 3% hydrogen peroxide. The sections were then immersed in proteinase K (Dako Corp.) to activate the antigen and incubated with CK mAb diluted 1:200 for 30 min. The sections were washed with phosphate-buffered saline (PBS) and CK was stained using the ABC method (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) (25) and visualized using diaminobenzidine tetrahydrochloride (DAB). The negative control sections were processed identically but without the primary antibody. The positive controls were PEAT

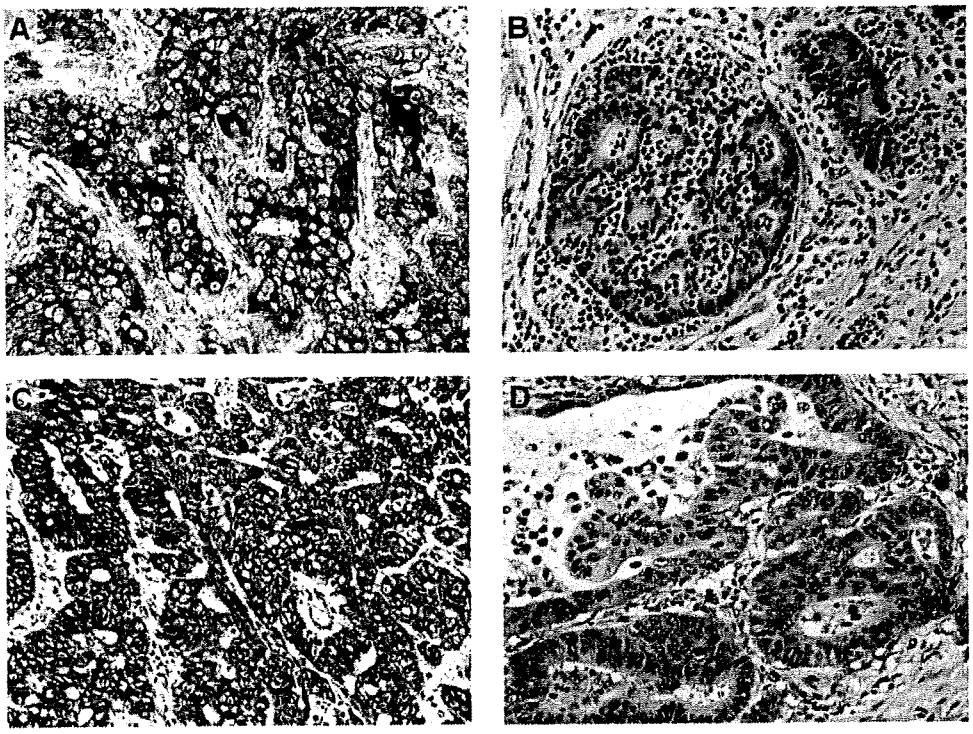

Figure 1. Representative IHC of CCR7 and CXCR4 expression in gastric cancer tissues. Tumor cells with: (A), high; and (B), low expression of CCR7; and (C), high; and (D), low expression of CXCR4. Original magnification x400.

sections of normal gastric mucosa that were consistently positive for CK.

The PEAT sections (3-μm thick) with resected primary tumors were incubated on slides at 50°C overnight, deparaffinized with xylene, and then rehydrated with a graded series of ethanol. The sections were immersed in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase, washed three times for 5 min each with PBS, and then non-specific binding was blocked with 1% bovine serum albumin in PBS at room temperature for 30 min. The sections were incubated at 4°C overnight with anti-CCR7 (BD Biosciences, San Jose, CA, USA) and CXCR4 mAbs (R&D Systems, Minneapolis, MN, USA) diluted 1:100 in PBS. After three 5-min washes in PBS, the reactions for CCR7 and CXCR4 were developed using the ABC method (Vectastain ABC kit, Vector Laboratories) (25) and visualized using DAB. Negative controls were treated with PBS without primary antibodies under the same conditions.

Two independent investigators (T. Arigami and S. Natsugoe) blinded to the clinicopathological data of the patients evaluated the IHC staining for CCR7 and CXCR4. High expression was defined as the presence of CCR7 and CXCR4 immunoreactivity in over 30% of the cancer cells (26,27). Expression of CCR7 and CXCR4 was evaluated in 10 fields each containing 100 cells using light microscopy (magnification x200).

Statistical analysis. Data were statistically compared using the χ^2 and Fisher's exact tests. All statistical calculations were performed using SAS statistical software (SAS Institute Inc., Cary, NC). A $P<0.05$ was considered statistically significant.

Results

CCR7 and CXCR4 expression in gastric tumors. Both CCR7 and CXCR4 were expressed in the cell membrane and/or cytoplasm of gastric tumors. CCR7 and CXCR4 were highly expressed in 25 (26.9%) and in 30 (32.3%) of 93 patients with gastric cancer, respectively (Fig. 1).

Correlation between CCR7 and CXCR4 expression and clinicopathological factors. CCR7 expression significantly correlated with depth of tumor, lymphatic and venous invasion, and lymph node metastasis (P=0.0024, 0.0004, <0.0001 and 0.0212, respectively), and the CXCR4 expression significantly correlated with histological type, tumor size, lymphatic and venous invasion, and lymph node metastasis (P=0.0238, 0.0162, 0.0009, 0.0005 and 0.0115, respectively; Table I).

Correlation between CCR7 and CXCR4 expression and lymph node status including LNMM. We used immunohistochemical staining with CK mAb and RT-PCR to assess LNMM in lymph nodes diagnosed as pN0 by H&E staining. We detected LNMM in 25 of 83 patients (30.1%) with such pN0 gastric cancers.

To assess the relationship between CCR7 and CXCR4 expression and lymph node status including LNMM, we classified the patients according to LNMM status as node-negative/LNMM-negative, node-negative/LNMM-positive and node-positive (Table II). Lymph node metastatic status including LNMM was significantly higher among patients with high levels, than with low levels of CCR7 and CXCR4 expression (P=0.0092 and 0.0075, respectively).

Table I. Relationship between CCR7/CXCR4 expressions and clinicopathological factors in 93 patients with gastric cancer.

Clinicopathological factors	CCR7 expression Low n=68 (%)	CCR7 expression High n=25 (%)	P-value	CXCR4 expression Low n=63 (%)	CXCR4 expression High n=30 (%)	P-value
Gender						
Male	48 (73.8)	17 (26.2)	0.8039	44 (67.7)	21 (32.3)	>0.9999
Female	20 (71.4)	8 (28.6)		19 (67.9)	9 (32.1)	
Tumor location						
Upper	9 (64.3)	5 (35.7)	0.6415	9 (64.3)	5 (35.7)	0.2746
Middle	39 (76.5)	12 (23.5)		38 (74.5)	13 (25.5)	
Lower	20 (71.4)	8 (28.6)		16 (57.1)	12 (42.9)	
Histological type						
Differentiated	36 (65.5)	19 (34.5)	0.0578	32 (58.2)	23 (41.8)	0.0238
Undifferentiated	32 (84.2)	6 (15.8)		31 (81.6)	7 (18.4)	
Depth of tumor invasion						
pT1	64 (79.0)	17 (21.0)	0.0024	58 (71.6)	23 (28.4)	0.0506
pT2-pT3	4 (33.3)	8 (66.7)		5 (41.7)	7 (58.3)	
Tumor size						
<30 mm	34 (75.6)	11 (24.4)	0.6463	36 (80.0)	9 (20.0)	0.0162
≥30 mm	34 (70.8)	14 (29.2)		27 (56.2)	21 (43.8)	
Lymphatic invasion						
Negative	60 (82.2)	13 (17.8)	0.0004	56 (76.7)	17 (23.3)	0.0009
Positive	8 (40.0)	12 (60.0)		7 (35.0)	13 (65.0)	
Venous invasion						
Negative	64 (82.1)	14 (17.9)	<0.0001	59 (75.6)	19 (24.4)	0.0005
Positive	4 (26.7)	11 (73.3)		4 (26.7)	11 (73.3)	
Lymph node metastasis[a]						
Negative	64 (77.1)	19 (22.9)	0.0212	60 (72.3)	23 (27.7)	0.0115
Positive	4 (40.0)	6 (60.0)		3 (30.0)	7 (70.0)	

pT1, invasion of mucosa or submucosa; pT2, invasion of muscularis propria or subserosa; pT3, penetration of serosa. [a]Lymph node metastasis was identified based on hematoxylin-eosin staining.

Table II. Relationship between CCR7/CXCR4 expressions and lymph node status.

	pN(-)/LNMM(-) n=58	pN(-)/LNMM(+) n=25	pN(+) n=10	P-value
CCR7 expression				
Low (n=68)	48 (82.8)	16 (64.0)	4 (40.0)	0.0092
High (n=25)	10 (17.2)	9 (36.0)	6 (60.0)	
CXCR4 expression				
Low (n=63)	45 (77.6)	15 (60.0)	3 (30.0)	0.0075
High (n=30)	13 (22.4)	10 (40.0)	7 (70.0)	

LNMM, lymph node micrometastasis.

Table III. Relationship between CCR7 and CXCR4 expression.

CCR7 expression	CXCR4 expression (%) Low (n=63)	High (n=30)	P-value
Low (n=68)	55 (59.1)	13 (14.0)	<0.0001
High (n=25)	8 (8.6)	17 (18.3)	

Relevance of CCR7 and CXCR4 expression in predicting lymph node status. Expression of CCR7 and CXCR4 were significantly correlated (P<0.0001; Table III). Based on the status of CCR7 and CXCR4, all patients were assigned to groups with low, intermediate or high expression (low expression of both CCR7 and CXCR4, low expression of either CCR7 or CXCR4 and high expression of both CCR7

Table IV. Relationship between lymph node status and expression patterns of CCR7 and CXCR4.

Expression patterns of CCR7 and CXCR4	pN(-)/LNMM(-) n=58	pN(-)/LNMM(+) n=25	pN(+) n=10	P-value
Low (n=55)	40 (69.0)	12 (48.0)	3 (30.0)	0.0021
Intermediate (n=21)	13 (22.4)	7 (28.0)	1 (10.0)	
High (n=17)	5 (8.6)	6 (24.0)	6 (60.0)	

LNMM, lymph node micrometastasis.

and CXCR4, respectively; Table IV). We found that lymph node metastatic status including LNMM was significantly higher among patients in the group with high levels, than with low levels of CCR7 and CXCR4 expression (P=0.0021).

Discussion

The chemokine receptors CCR7 and CXCR4 are expressed in tumor cells of breast cancer and malignant melanoma, as well as pancreatic, colorectal and esophageal and gastric cancers (8,28-33). The CCL21 and CXCL12 ligands for CCR7 and CXCR4 are abundant in lymph nodes (8). Wiley *et al* (34) reported that CCR7 expression enhances the metastasis of murine melanoma cells to draining lymph nodes in mice. Furthermore, Müller *et al* (8) reported that injection of an anti-CXCR4 antibody significantly reduced the metastasis of breast cancer cells to regional lymph nodes in immunodeficient mice. Thus, the CCR7 and CXCR4 signaling pathway might play crucial roles in the mechanism of lymph node metastasis from primary tumor cells. Therefore, we examined the correlation between CCR7 and CXCR4 expression and lymph node status.

We initially investigated CCR7 and CXCR4 expression in PEAT sections of primary gastric tumors and the relationships with clinicopathological factors. We found that CCR7 and CXCR4 expression in gastric tumor cells visualized and identified by IHC correlated with depth of tumor invasion, lymphatic invasion, venous invasion and lymph node metastasis determined by H&E staining. These findings indicated a close relationship between tumor progression and expression of both CCR7 and CXCR4.

We previously demonstrated the importance of LNMM when selecting therapeutic strategies for patients with gastric cancer (20,21,35). Therefore, lymph node metastatic status, including LNMM should be predicted. We postulated that CCR7 and CXCR4 expression correlated with lymph node metastatic status including LNMM in gastric cancer. Our findings showed that CCR7 and CXCR4 expression significantly correlated with lymph node status including LNMM. We then examined whether CCR7 and CXCR4 expression could predict lymph node status. We demonstrated that lymph node status, including LNMM, was more closely correlated with expression of both CCR7 and CXCR4 than with either alone. Assessment of CCR7 and CXCR4 expression in preoperative biopsy specimens might thus yield valuable information for predicting preoperative lymph node status including LNMM.

An antagonist of CXCR4 can suppress tumor migration, invasion, and lung metastasis in an animal model (36-40). Several CXCR4 antagonists, such as AMD3100, 4F-benzoyl-TE14011 and TN14003, are currently available (36-40). We showed here that expression of CCR7 and CXCR4 in gastric tumors significantly correlated with lymph node metastatic status, lymphatic and venous invasion. Therefore, CCR7 and CXCR4 antagonists might represent novel therapeutic agents that could regulate distant metastases, including those to lymph nodes in patients with advanced gastric cancer.

In conclusion, we demonstrated that CCR7 and CXCR4 are both expressed in gastric tumor cells and that their expression correlates with tumor progression and lymph node metastatic status including LNMM. Therefore, CCR7 and CXCR4 are potential markers for predicting lymph node metastatic status in patients with gastric cancer. The evaluation of CCR7 and CXCR4 expression might also serve as a useful means of predicting the presence or absence of LNMM in patients with early gastric cancer who will undergo less invasive surgery such as EMR and ESD. Furthermore, future studies on biological behavior of the gastric tumor cells expressing CCR7 and CXCR4 may allow the development of new immunotherapy inhibiting these signaling pathways for patients with gastric cancer.

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The Utility of Rapid Diagnosis of Lymph Node Metastasis in Gastric Cancer Using a Multiplex Real-Time Reverse Transcription Polymerase Chain Reaction Assay

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Key Words
Gastric cancer · Lymph node metastasis · Micrometastasis · Isolated tumor cells · Sentinel node concept · Reverse transcription polymerase chain reaction · Multiple markers

Abstract
Background: Lymph node metastasis is the most important prognostic factor in gastric cancer. However, diagnosis by hematoxylin and eosin staining or immunohistochemistry is not always sufficient for the detection of cancer cells because only representative number of slices are examined. Cancer cells may, therefore, be missed by traditional histological methods. Recently, reverse transcription polymerase chain reaction (RT-PCR) methods have been introduced for improved detection of cancer cells. The purpose of this study was to evaluate the utility of a prototype RT-PCR assay run on the Cepheid SmartCycler® system compared to conventional RT-PCR using the LightCycler® system. ***Patients and Methods:*** Forty-seven overt metastatic lymph nodes from 8 patients with advanced gastric cancer and 22 benign lymph nodes from patients without malignant tumor who received surgery were obtained with informed consent. We examined the lymph nodes by RT-PCR, using markers for CEA and CK19 and the LightCycler and SmartCycler systems. ***Results:*** In the singlex assay, the sensitivity of CEA and CK19 was 91.5 and 70.2% in the LightCycler system, and 97.9 and 95.7% in the SmartCycler system, respectively. In the multiplex assay, the sensitivity was 91.5% in the LightCycler system and 100% in the SmartCycler system, respectively. ***Conclusion:*** In this study, rapid diagnosis using RT-PCR by the SmartCycler system had higher accuracy for detecting lymph node metastasis than the conventional LightCycler system. The SmartCycler system is more effective for the diagnosis of lymph node metastasis in gastric cancer when run with the prototype assay.

Introduction

Sentinel nodes (SN) are the first lymph nodes to receive lymphatic flow from the primary tumor and metastasis initially occurs at this site [1]. Thus, metastases are likely located in SNs as the first step of lymph node metastasis. For this reason, SN navigation surgery has been introduced for patients with breast cancer and malignant melanoma [2–5]. Recently, this concept has also been ap-

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plied to gastrointestinal tract cancer [6–10]. We recently reported that the SN concept was acceptable for patients with early gastric cancer using immunohistochemical staining and real-time reverse transcription polymerase chain reaction (RT-PCR) using the LightCycler® System [11, 12].

Lymph node metastasis is one of the most important prognostic factors in a number of cancers [13–18]. However, the sensitivity of pre-operative diagnosis using multidetector-row helical computed tomography and abdominal ultrasonography of lymph node metastasis is insufficient [19, 20]. The diagnosis of lymph node metastasis during operations generally depends on hematoxylin and eosin (HE) staining at most institutions. At some institutions, it is possible to perform immunohistochemistry during the operation to detect micrometastasis in lymph nodes [21–23]. However, these methods can be highly laborious. Moreover, these methods are performed only on representative slices of the lymph nodes. If metastases exist in the remaining, uninspected mass of the lymph nodes, metastatic foci would not be detected. Isozaki et al. [24] examined lymph node metastasis in gastric cancer by histological examination using a serial sectioning method and concluded that serial sectioning enables more accurate evaluation of the extent of lymph node metastasis.

To avoid discrepant results, accurate and efficient diagnosis of lymph node metastasis during surgery is important. Additionally, the clinical significance of micrometastasis, including isolated tumor cells (ITC) is controversial. However, it has been shown that micrometastatic cells and ITC have proliferative activity [25, 26]. It is important to diagnose these tiny metastatic foci to clarify their clinical significance.

RT-PCR is a more sensitive method for the detection of micrometastasis than immunohistochemistry [27]. The sensitivity and specificity of RT-PCR depends on the markers selected. Using multiple markers in real-time RT-PCR may be a valid method for the detection of micrometastasis and ITC [28].

Recently, a prototype research assay using rapid real-time RT-PCR, (Veridex LLC, Raritan, N.J., USA) which runs on the Cepheid SmartCycler® system (Cepheid, Sunnyvale, Calif., USA) has been developed and the utility of this system has been reported [29–31]. This system is advantageous both due to its speed (approximately 40 min in total to obtain a result) and its ability to perform multiplex assays. The current study investigated the utility of the SmartCycler system compared to the LightCycler® system using carcinoembryonic antigen (CEA) and cytokeratin-19 (CK19) as markers in each system. Expression of these markers has previously been shown to have diagnostic importance in gastric cancer [12, 27, 28, 32, 33].

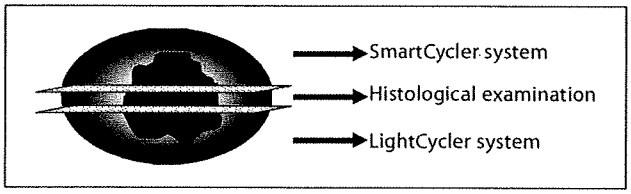

Fig. 1. All lymph nodes were cut into 3 uniform pieces. One third was used for HE staining and immunohistochemistry, one third for RT-PCR using the LightCycler system, and the remaining third for RT-PCR using the SmartCycler system.

Materials and Methods

Diseased and Benign Lymph Nodes

Forty-seven histologically diseased lymph nodes, as measured by HE staining and immunohistochemistry using an anti-CK antibody, were obtained with informed consent from 8 patients with advanced gastric cancer. Twenty-two benign lymph nodes were obtained with informed consent from patients with benign disease who underwent surgery.

Verification of Lymph Node Metastasis by HE Staining and Immunohistochemistry

All lymph nodes were cut into 3 uniform pieces. One third was used for HE staining and immunohistochemistry, one third for RT-PCR using the LightCycler system and the remaining third for RT-PCR using the SmartCycler system (fig. 1).

All lymph nodes were stained with HE and immunohistochemistry was performed using a monoclonal anti-CK antibody cocktail (AE1/AE3; Dako Corporation, Carpinteria, Calif., USA). The tissue sections were deparaffinized in xylene, rehydrated with a graded series of ethanol, and then endogenous peroxidase activity was blocked by a 5-min incubation in 3% hydrogen peroxide in methanol. The sections were subsequently immersed in proteinase K (Dako Corporation) to activate the antigen and incubated with CK monoclonal antibody diluted 1:200 for 30 min. After two 5-min washes with phosphate-buffered saline, the avidin-biotin complex and immunoperoxidase were applied (ABC method, Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, Calif., USA). Cells positive for CK were visualized using diaminobenzidine tetrahydrochloride and the sections were lightly counterstained with hematoxylin. The negative controls consisted of sections processed in the same manner but without the primary antibody. CK-positive normal gastric mucosa and primary tumor specimens were used as positive controls in all testing. Three independent observers (S.Y., Y.U. and H.A.) evaluated all immunohistochemically stained slides. Overt metastatic lymph nodes were verified as macrometastatic lymph nodes histologically.

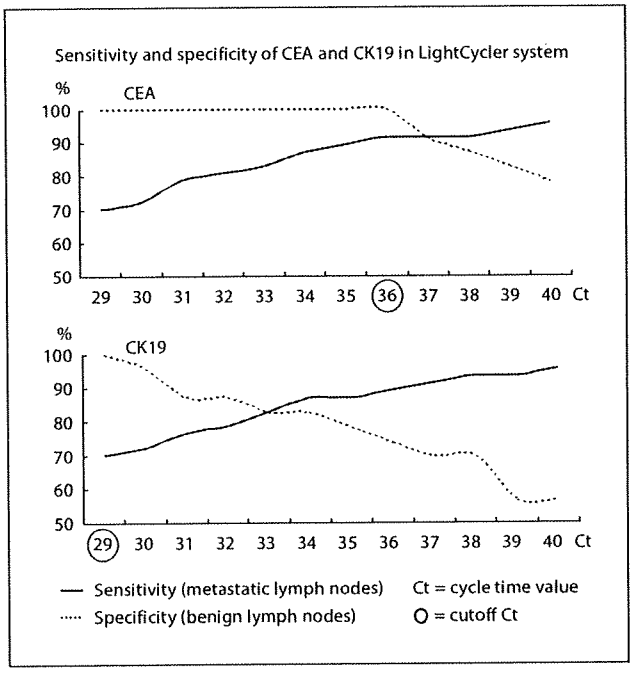

Fig. 2. Regarding the LightCycler system, we set up the provisional cutoff Ct value in the highest sensitivity with 100% specificity to avoid false positive results. The highest sensitivity with 100% specificity was 91.5% for CEA, using the cutoff value of 36 and the highest sensitivity with 100% specificity was 70.2% for CK19, using the cutoff value of 29.

Real-Time RT-PCR Assay by the LightCycler System

All samples were prepared for the LightCycler system according to the previously published manuscript [12]. Total RNA was extracted from homogenized lymph nodes using the guanidinium thiocyanate phenol-chloroform method from Isogen (Nippon Gene, Toyama, Japan). The concentration, purity and mass of total RNA were determined by measuring spectrophotometric absorption at 260 and 280 nm using GeneQuant pro UV/Vis Spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK). Total RNA was treated with DNase-I (Invitrogen, Life Technologies, Foster City, Calif., USA) to eliminate contamination with genomic DNA. Complementary DNA (cDNA) was synthesized using the Advantage RT-for-PCR kit (Clontech Laboratory Inc., Palo Alto, Calif., USA) according to the manufacturer's protocol and cDNA was then processed for PCR. This assay was performed based on the hybridization probe method. The CEA primers and probe were designed based on the method described by Gerhard et al. [34] and the CK19 primers and probe were designed at Nihon Gene Research Laboratories Inc. This assay is interpreted using threshold cycle (Ct) values.

Real-Time RT-PCR Assay by the SmartCycler System

The nodal tissue was homogenized and RNA was purified using the RNA Sample Preparation Kit (Veridex LLC). The RT-PCR assay, which included reverse transcription of the cDNA from target mRNA and amplification of the cDNA, was performed in one step using a prototype kit run on the SmartCycler system. This kit was designed to detect expression of CEA, CK19 and a housekeeping gene (internal control) and is based on the hydrolysis probe method and interpreted using Ct values. Each assay contained a positive and negative external control.

Table 1. Comparison between LightCycler and SmartCycler systems in singlex and duplex assay

		LightCycler	SmartCycler
Sensitivity	Single marker		
	CEA	91.5%	97.9%
	CK-19	70.2%	95.7%
	Double markers	91.5%	100%
Specificity		100%	100%

Statistical Analysis

For this study, the McNemar test was used to analyze data comparing the methods. $p < 0.05$ was considered significant.

Results

Expression of CEA and CK19 mRNA in Metastatic and Benign Lymph Nodes Using the LightCycler System

All lymph nodes were confirmed to express the housekeeping gene using the SmartCycler system. In benign lymph nodes, the specificity of CEA was 100% when using a Ct cutoff of 36, but when the Ct value was over 36, the specificity began to decrease. Specificity was 90.9% at a Ct of 37 (fig. 2). The highest sensitivity with 100% specificity was 91.5% for CEA, using a Ct cutoff value of 36 (table 1). We set up the provisional cutoff Ct value at 36 for CEA with the LightCycler system (fig. 2). In the same way, the specificity of CK19 was 100% up to a Ct cutoff of 29, but when the cycle time value was over 29, specificity began to decrease (fig. 2). The highest sensitivity obtained with 100% specificity was 70.2% with CK19 and a Ct cutoff set at 29 (table 1). We set up the provisional cutoff Ct value at 29 for CK19 with the LightCycler system (fig. 2).

Expression of CEA and CK19 mRNA in Metastatic and Benign Lymph Nodes Using the Prototype Assay System

In benign lymph nodes, the specificity of CEA was 100% at all times (fig. 3). The highest sensitivity with the lowest Ct cutoff of 35 was 97.9% (table 1). We set up the

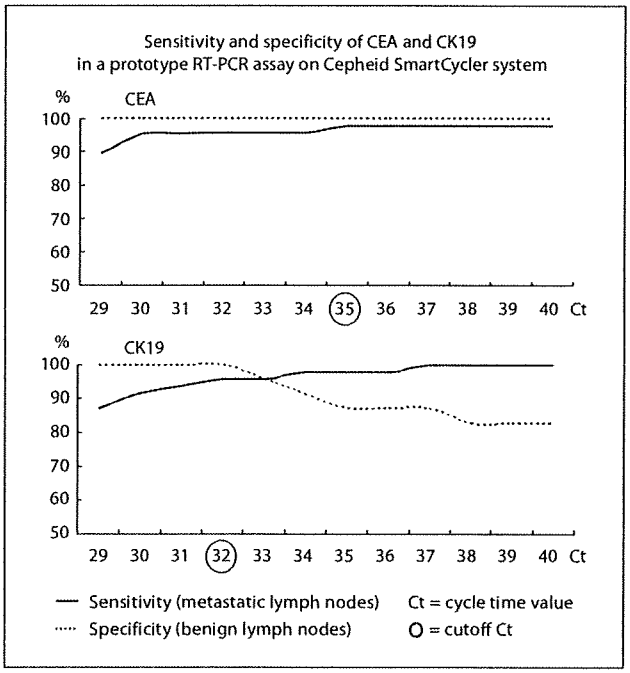

Fig. 3. Regarding the SmartCycler system, we set it up in the same way as the LightCycler system. The highest sensitivity with 100% specificity was 97.9% for CEA, using the cutoff value of 35 and the highest sensitivity with 100% specificity was 95.7% for CK19, using the cutoff value of 32.

Table 2. Evaluation of the results of the LightCycler and SmartCycler systems

	LC-CK19		p value
	negative	positive	
SC-CK19			
Negative	22	2	0.0027
Positive	14	31	
	LC-CEA		p value
	negative	positive	
SC-CEA			
Negative	23	0	0.0833
Positive	3	43	
	LC-CK19 or CEA		p value
	negative	positive	
SC-CK19 or CEA			
Negative	22	0	0.0455
Positive	4	43	

LC = LightCycler system; SC = SmartCycler system.

provisional cutoff Ct value at 35 for CEA by the prototype assay system to avoid false positive results (fig. 3). With CK19, the specificity was 100% up to a Ct of 32, but when the Ct value was over 32, the specificity began to decrease (fig. 3). The highest sensitivity with 100% specificity was 95.7% with CK19 and a Ct cutoff of 32 (table 1). We set up the provisional CK19 cutoff Ct value at 32 for the prototype assay system (fig. 3).

Comparison of the Performance between the LightCycler and Prototype Assay Systems in Singlex and Duplex Assays

As a singlex assay, using both CEA and CK19, sensitivity of the prototype assay system was equal to or higher than that of the LightCycler system. With both systems, the sensitivity was improved for the multiplex assay (compared to the singlex assay). These results are shown in table 1. Both assays used the provisional cutoff Ct values for each marker in each system with a specificity of 100% in order to avoid false positive results (table 1). The concordance of the results in both systems was compared using the McNemar test (table 2). For CK19, the sensitivity of the prototype assay system was significantly higher than that of the LightCycler system ($p = 0.0027$). For CEA, there was no significant difference between the systems. In the multiplex assay, the sensitivity of the prototype assay system was significantly higher than that of LightCycler system ($p = 0.0455$).

Discussion

Accurate diagnosis for lymph node metastasis is essential for gastric cancer, but the sensitivity of preoperative diagnosis for lymph node metastasis is not always sufficient [19, 20]. An improvement in accuracy of the intraoperative diagnosis for lymph node metastasis may ultimately lead to a better prognosis for patients. We reported that the SN concept is applicable to patients with early gastric cancer even in cases where micrometastasis is detected by immunohistochemical staining and RT-PCR [11, 12]. While lymph nodes are a three-dimensional specimen, routine intraoperative histological examination is a two-dimensional diagnostic method. Hence, a comprehensive diagnosis for lymph node metastasis is needed.

RT-PCR is a sensitive method for the detection of micrometastasis in lymph nodes, but it has been very time consuming. In conventional RT-PCR assay, approximately 3 h were required to obtain our results in this study.

Genetic diagnostic methods such as RT-PCR have recently improved, and these methods have advantages for rapid diagnosis. The transcription-reverse transcription concerted reaction using CEA as a marker enables one to obtain the result within 1 h, and the performance of transcription-reverse transcription concerted assay is at least equivalent to the LightCycler system [35]. The one-step nucleic acid amplification (OSNA) assay is characterized by mRNA quantification and requires about 21 min to complete. OSNA using CK19 as a marker generated similar results to histological staining in 98.2% of lymph nodes in breast cancer patients [36]. The fully automated, multiplex quantitative RT-PCR assay and SmartCycler system requires less than 30 min to complete. The sensitivity and specificity of this system was 94 and 100%, respectively, compared to histological examination of lymph nodes from patients with breast cancer [37].

With these diagnostic methods, selection of markers is a very important issue. Gastric cancer is comprised of various histological types. Honda et al. [38] reported that 21.7% of differentiated tumors, 34.7% of undifferentiated tumors with a tubular component and 69.7% of pure undifferentiated tumors all express high levels of CEA. Therefore, the diagnostic value of CEA for the detection of metastatic foci in lymph nodes becomes problematic. Okada et al. [28] suggested that multiple-marker RT-PCR assays are useful for detecting micrometastasis in regional lymph nodes with gastric cancer. Some markers have been reported for the diagnosis of lymph node metastasis with gastric cancer, such as CEA [12, 27, 28, 32, 39–41], CK18 [42], CK19 [33], CK20 [27, 28, 41], MUC1 [39], MUC2 [40], hTRT [39], TFF1 [43], mammaglobin B [44] and MAGE3 [28]. In the current study, assays were performed by using only CK19 and CEA.

In breast cancer and other types of solid tumors, CK19 is one of the most popular markers for the detection of circulating tumor cells, bone marrow and lymph nodes [45–52]. Though the utility of RT-PCR using CK19 was reported previously [33], the specificity of CK19 was lower compared with CEA in the LightCycler system. Rudd et al. [53] reported the existence of its pseudogene, and lower specificity compared with CEA may be caused by the pseudogene. However, we considered these 2 markers in combination (CK19 and CEA) to be useful for detecting epithelial cancer cells, and the issue of specificity is improved by using multiple markers.

In this study, the advantages of the prototype RT-PCR assay on the SmartCycler system compared with the conventional system are: the simplicity of the procedure, rapidity to get results and the improvement of the sensitivity as well as the specificity by using multiple markers in one assay.

In the near future, the improvement of genetic diagnostic methods offering both simplicity and reproducibility of the result, and newly discovered markers with high sensitivity as well as specificity may enable a more accurate diagnosis of lymph node metastasis, including micrometastasis.

In conclusion, the prototype assay system has potential benefits for diagnosis of lymph node metastasis compared with the conventional RT-PCR system because of its improved turnaround time, sensitivity and specificity.

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