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膵臓星細胞活性化におけるオートファジーの役割

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膵臓星細胞活性化におけるオートファジーの役割に関する研究

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研究要旨

膵癌が極めて予後不良である理由には早期からの浸潤、転移や化学療法に対する高い治療抵抗性がある。膵癌組織周囲に存在する膵星細胞は膵癌により活性化され、活性化星細胞は逆に癌細胞の浸潤、転移を促し、さらに **desmoplasia** と呼ばれる過剰な間質増生を促す（癌間質相互作用）。Desmoplasia では血管密度が乏しく腫瘍細胞への薬剤到達率が低下し化学療法抵抗性をもたらしている。しかしながらこの詳細なメカニズムは判明していない。我々はこのメカニズムとしてオートファジーに着目している。オートファジーが癌細胞自身の転移、浸潤、さらには治療抵抗性まで癌のあらゆる局面に重要な役割を果たしていることが明らかになっており、この抑制が癌細胞の悪性度を抑制する可能性が考えられる。近年肝炎における肝星細胞活性化にオートファジーの関与が示唆されているが、膵臓癌における星細胞活性化メカニズムは国内外で明らかになっていない。本研究では膵星細胞活性化にオートファジーが関与している事を証明し、癌細胞の浸潤、転移抑制、化学療法抵抗性の新たなメカニズムを解明できると考えている。これによりオートファジーの抑制が癌細胞、膵星細胞の活性化双方の抑制をもたらし、革新的な膵癌治療戦略を提供できると考えている。

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A. 研究目的

膵臓癌は極めて予後不良な癌腫であり、新規治療法の開発は社会的急務である。本疾患が予後不良な理由には、早期から浸潤、転移を生じる生物学的悪性度の高さや過剰な間質増生（desmoplasia）のもたらす乏血性環境を原因とする化学療法抵抗性が挙げられる。近年、癌細胞周辺の間質細胞が癌細胞の悪性度に影響している＜癌間質相互作用＞という概念に注目が集まっている。とりわけ、最近発見された膵間質に存在する膵星細胞**(Pancreatic Stellate Cells; PSCs)**は、種々の分泌因子を介し膵癌細胞の浸潤、転移を促進するとして報告された。膵星細胞が膵癌の悪性形質を誘導

する責任細胞として注目されているがそのメカニズムの報告は皆無である。我々は、近年新たな癌制御メカニズムとして注目されているオートファジーがこのメカニズムに関与していると考えている。最近、オートファジーが肝炎における肝星細胞の活性化に関与している事が初めて報告されたが、膵星細胞活性化とオートファジーが関与している報告は国内外において皆無である。今回、膵星細胞が膵癌悪性度に影響を与える新たなメカニズムを解明、新たな膵癌治療戦略を構築する。

B. 研究方法

1. ヒト膵星細胞株の樹立（仲田、大内田担当）

   すでに、膵癌患者より得られる手術切除標本を用いてヒト膵星細胞株を20株以上作成した。作成された細胞株に対して、膵星細胞の特徴とされるMyofibroblast様の形態の確認やα-SMA染色を行い、星細胞である事を確認した。

2. 活性化膵星細胞におけるオートファジー誘導の確認　（平成２４年度　仲田、水元　担当）

   膵星細胞でのオートファジー活性を共焦点顕微鏡によるGFP-LC３発現、ウエスタンブロット、更には電子顕微鏡によるオートファゴゾームの形成を観察して確認した。具体的には実験１において分離した星細胞でそれぞれ、オートファジー活性化の違いを確認した。また、これまでの報告から各種癌細胞、線維芽細胞でストレスによりオートファジーを誘導する事が報告されており、培養した星細胞がストレス投与下にオートファジーを誘導されるかを確認した

3. 膵癌細胞との共培養によるオートファジーを介した膵星細胞活性化の確認

   （大内田、水元　担当）　星細胞が膵癌細胞との共培養により活性化が促進されるかを確認、その際オートファジーが誘導されているかを実験１と同様の方法で観察した。

4. 膵星細胞に対する Atg5 ノックダウン効果の確認　（仲田担当）

   膵星細胞に対してオートファジー関連因子 Atg5 をノックダウンし、星細胞の活性化の変化を確認した。その効果を確認したところ約 80%のノックダウン効果を得た。

   Control 繊維芽細胞とノックダウン細胞におけるオートファジー誘導を比較したところ Atg5 ノックダウン細胞においてオートファジーの誘導が抑制されている事を確認した。更に Atg5 ノックダウン細胞においては脂肪滴発現が抑制され、ノックダウン繊維芽細胞において星細胞の活性化が抑制されている事が示唆された。

（倫理面への配慮）

本研究は、癌に含まれ後天的に出現する特定の細胞集団を対象としており、マイクロアレイや RT-PCR を用いた発現解析も同様に後天的な特定の分子の発現異常を解析するものであり、ゲノム解析は行わず、平成 13 年の三省の「ヒトゲノム・遺伝子解析研究に関する倫理指針」の対象になる研究ではない。しかし、臨床検体を使

用した解析を含む研究であるので、平成15 年7月の厚生労働省「臨床研究に関する倫理指針」に従い、九州大学倫理委員会で承認済みである。また、本研究に使用される切除組織は、治療のために切除された組織を一部用いるものであり、研究のために過剰に切除されることはなく、医療行為を伴う介入研究には当てはまらず、疫学研究に関する倫理指針が適用され、同指針の第3章7条の第2項の観察研究に相当し、①のイの場合に相当し、文書による説明と文書による同意は必ずしも必要としないとされているが、説明の内容と同意に関する記録が必要となるため、念のため、文書による説明を行い文書による同意を得ることとする。試料は、九州大学の個人情報保護規定を遵守し、匿名コード化し、個人情報保護を徹底し、安全、人権、プライバシーに充分に留意する。

本研究でのマウスの飼育・管理・実験は、動物愛護、生命倫理の観点に十分に配慮し、「研究機関等における動物実験等の実施に関する基本指針」および九州大学の学内規定に基づいて適切に行う。

実験用各種ウイルス・plasmid の取り扱いは、九州大学の学内規定に基づき厳正に行う。すでに P2 レベルの動物実験施設、培養実験施設を専用に確保しており、承認された計画調書に従い、安全性の確保に最大限の注意を払って研究を遂行する。

C. 研究結果

1. ヒト膵星細胞株の樹立および性状の確認

膵癌患者より得られる手術切除標本を用いてヒト膵星細胞株を作成した。作成された細胞株は、膵星細胞の特徴とされるMyofibroblast様の形態を呈し、α-SMAが陽性であることを確認した。その際、同症例標本より癌組織近傍の膵組織および癌組織より離れた膵組織から星細胞を培養し、オートファジー誘導性の違いを確認したところ、癌組織近傍より作製した星細胞において癌組織より離れた組織から培養した星細胞に比べてオートファジーが誘導される症例が一部に見られた。さらに複数の星細胞株それぞれに対してオートファジー誘導性の違い、オートファジー必須遺伝子Atg5発現の分布を確認したところ、個々の症例に由来する星細胞によりオートファジーの誘導性が異なる可能性がある事を確認した。更には、オートファジー抑制剤3-MAの投与により一部の星細胞で脂肪滴の発現が抑制され、Quiescent星細胞様性質を示す事を確認した。

2. 膵癌細胞、星細胞におけるオートファジー誘導の確立。膵癌細胞および星細胞にGFP-LC3を導入し、星細胞での容易なオートファジー発現観察を確認する手法を確立した。また、ストレス条件下に癌細胞でオートファジーが誘導される事も同時に確認した。

3. 膵癌細胞、星細胞共培養における3MA投与による膵癌細胞浸潤能力への影響(大内田、水元担当)

膵星細胞、癌細胞に 3MA を投与したところ星細胞、癌細胞でオートファジーの誘導が抑制される細胞を認める事を確認した。さらに、膵星細胞と膵癌細胞との共培養モデルを確立、星細胞との共培養において膵

臓癌浸潤が促進される事を確認した。癌細胞、膵星細胞共培養下にオートファジー抑制剤 3MA の投与で膵癌細胞の浸潤が抑制される事を確認した。

4. 膵星細胞に対する Atg5 ノックダウン効果の確認 （仲田担当）

膵星細胞に対してオートファジー関連因子 Atg5 をノックダウンし、星細胞の活性化の変化を確認した。その効果を確認したところ約 80%のノックダウン効果を得た。Control 繊維芽細胞とノックダウン細胞におけるオートファジー誘導を比較したところ Atg5 ノックダウン細胞においてオートファジーの誘導が抑制されている事を確認した。更に Atg5 ノックダウン細胞においては脂肪滴発現が抑制され、ノックダウン繊維芽細胞において星細胞の活性化が抑制されている事が示唆された。

D. 結論、考察

星細胞はDesmoplasiaを形成し、抗癌剤治療抵抗性の原因と考えられている。また、星細胞は癌細胞の転移、浸潤を促している。その為、膵星細胞の活性化を抑制することにより、膵癌細胞の浸潤を抑制出来るのではないかと考えている。同時にオートファジーは癌細胞自身の浸潤能、治療抵抗性関連する事が示唆されていると考えられる。今回の研究遂行により膵癌の浸潤性が膵星細胞、膵癌細胞双方に対するオートファジーを抑制することにより、相乗的に浸潤性を抑制する事が予想される。また、Desmoplaisa形成も抑制し、化学療法抵抗性を解決出来るのではないかと考えている。

本年度の研究成果から膵癌細胞周囲の星細胞にはオートファジーが誘導されているが、その中でも誘導の強い星細胞と誘導が弱い星細胞が認められた。また、癌細胞から離れた部位の星細胞ではオートファジーの誘導が癌細胞周囲細胞に比較して低いものがあった。癌細胞周囲の星細胞の一部が癌細胞によりオートファジーを誘導されている可能性が示唆された。今後、症例の蓄積による検討が必要である。

また、今後はオートファジー抑制により星細胞自身の活性化が抑制される事確認する。膵癌細胞および星細胞共培養下に膵癌浸潤能が増強されるがオートファジー抑制剤により浸潤能が抑制されている。今回、オートファジー抑制剤3MAを投与する事により膵臓癌の浸潤能が抑制された。本結果からでは3MAが膵癌細胞および星細胞双方のオートファジーを抑制している可能性が考えられる。今回星細胞に対してAtg5をノックダウンしたところ星細胞活性化の抑制が確認され、オートファジー及び星細胞活性化の関連性が強く示唆された。今後もその再現性多方面から検討していく予定である。

E. 健康危険情報

無し

F. 研究発表

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会定期学術集会 2013/4/11-13

18. 寅田信博、赤川進、崔林、大内田研宙、小薗真吾、水元一博、相島慎一、小田義直、田中雅夫高品質かつ簡便な凍結組織アレイ作製法-手術切除組織を効率よく保存する保存組織タブレット法を活用して- 第 113 回日本外科学会定期学術集会 2013/4/11-13

G. 知的財産権の出願・登録状況

該当なし

別添４

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Nagai E, Ohuchida K, Nakata K, et al	Feasibility and safety of intracorporeal esophagojejunostomy after laparoscopic total gastrectomy: Inverted T-shaped anastomosis using linear staplers.	Surgery	153(5)	732-8	2013
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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

MicroRNA-10a is Overexpressed in Human Pancreatic Cancer and Involved in Its Invasiveness Partially via Suppression of the *HOXA1* Gene

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ABSTRACT

Background. There is increasing evidence that microRNAs are differentially expressed in many types of cancers. Despite progress in analyses of microRNAs in several types of cancers, the functional contributions of microRNAs to pancreatic cancer remain unclear.

Methods. In the present study, the expression levels of specific microRNAs identified by microarray analyses were examined in a panel of 15 pancreatic cancer cell lines. We then investigated the functional roles of these microRNAs in the proliferation and invasion of pancreatic cancer cells.

Results. Based on the microarray data, we found frequent and marked overexpression of *miR-10a*, *miR-92*, and *miR-17-5p* in pancreatic cancer cell lines. Microdissection analyses revealed that *miR-10a* was overexpressed in pancreatic cancer cells isolated from a subset of primary tumors (12 of 20, 60%) compared with precursor lesions and normal ducts ($P < .01$). In vitro experiments revealed that *miR-10a* inhibitors decreased the invasiveness of pancreatic cancer cells ($P < .01$), but had no effect on their proliferation. Inhibition of *HOXA1*, a target of *miR-10a*, promoted the invasiveness of pancreatic cancer cells ($P < .01$).

Conclusions. The present data suggest that *miR-10a* is overexpressed in a subset of pancreatic cancers and is involved in the invasive potential of pancreatic cancer cells partially via suppression of *HOXA1*.

Pancreatic cancer is the fourth most common cause of tumor-related death in the industrialized world.[1,2] Only 10–20% of pancreatic cancer patients are candidates for surgery at the time of presentation, and fewer than 20% of patients who undergo curative resection are alive after 5 years.[3,4] Despite recent progress, there are no modalities for early detection of pancreatic cancer. With the exception of a few recent reports describing successful use of adjuvant chemotherapy, there have been no reports of effective treatments for advanced pancreatic cancer, including local and metastatic diseases.[5] To improve the prognosis of patients with pancreatic cancer, novel effective screening strategies and/or treatments are needed.

MicroRNAs (miRNAs) are small noncoding RNA gene products of approximately 22 nucleotides that are found in a variety of organisms. They play key roles in regulating the translation and degradation of mRNAs through base-pairing to partially complementary sites, predominantly in the 3′-untranslated regions of mRNAs.[6–8] Although the biologic functions of most miRNAs are not yet fully

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understood, it has been suggested that they are involved in various biologic processes, including cell proliferation, cell death, stress resistance, and fat metabolism, through regulation of gene expression.[9]

There is increasing evidence that miRNAs are mutated or differentially expressed in many types of cancers. Expression of the miRNA *let-7* is often reduced in lung cancers with poor prognoses, and deletion of miRNAs *Mir-15* and *Mir-16* occurs in 68% of patients with chronic lymphocytic leukemia.[10,11] In addition, expression of *miR-143* and *miR-145* is downregulated in colon carcinomas, and expression of the precursor *miR-155* is high in Burkitt's lymphoma.[12,13] The *miR-17-92* cluster has been reported to have oncogenic functions in human B-cell lymphomas and lung cancers.[14,15] These reports are consistent with the hypothesis that miRNAs play substantial roles in the pathogenesis of human cancers.

Recently, the development of microarrays containing all known human miRNAs has made it possible to perform miRNA expression profiling.[16] miRNA expression profiles have been shown to be potential tools for cancer diagnosis.[17] Several investigators have reported that microarray-based miRNA profiling identified miRNAs that were differentially expressed in pancreatic cancer.[18,19] However, it is difficult to conclude that the identified differences in gene expression accurately reflect the differences between cancer cells and normal ductal epithelial cells because these analyses were performed using RNA samples from primary pancreatic tissues without microdissection.

In the present study, we observed overexpression of *miR-10a* in pancreatic cancer cells, and the following data suggest that *miR-10a* is involved in the invasiveness of pancreatic cancer cells partially via suppression of *HOXA1*, which was reported to be involved in lung cancer, gastric cancer, and breast cancer.[20–22]

MATERIALS AND METHODS

Pancreatic Cell Lines and Tissues

A total of 15 pancreatic cancer cell lines including NOR-P1, which was established in our laboratory, and an immortalized human pancreatic ductal epithelial cell line (HPDE) were used.[23,24] All cells were maintained as previously described.[24,25] Tissue samples were obtained as described previously.[26] The details are described in the Supplemental materials.

Laser Microdissection

Laser microdissection was performed as described previously.[27,28] The details are described in the Supplemental materials.

miRNA Microarray Expression Analysis

Microarray analyses were carried out using a Filgen-Array miRNA 384 (Filgen, Nagoya, Japan) containing *mir*Vana miRNA Probe Set ver. No. 1 (Ambion) as shown in the Supplemental materials.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) for Analysis of miRNA Expression

Cells were analyzed by qRT-PCR with a *mir*Vana qRT-PCR miRNA Detection Kit (Ambion) or a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The details are described in the Supplemental materials.

Transfections

Cells were transfected by electroporation with a Nucleofector System (Amaxa Biosystems, Koln, Germany) as described previously.[26] The details are described in the Supplemental materials.

Cell Proliferation Assay

Cell proliferation was analyzed at various time points by measuring propidium iodide (PI) incorporation as described previously.[29] The details are described in the Supplemental materials.

Invasion Assay

The invasiveness of cancer cells was evaluated by counting the number of cells invading a Matrigel-coated transwell as reported previously.[26] The details are described in the Supplemental materials.

Quantitative Analysis of HOXA1 Levels by One-Step qRT-PCR

One-step qRT-PCR was performed using a QuantiTect SYBR Green RT-PCR Kit (Qiagen, Tokyo, Japan) with a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories) as described previously.[28] The details are described in the Supplemental materials.

Inhibition of HOXA1 Expression by RNA Interference

Inhibition of *HOXA1* expression was achieved by RNA interference with small interfering RNAs (siRNAs) as described previously.[26] The details are described in the Supplemental materials.

Western Blot Analysis

Protein expression was analyzed by western blotting as described previously.[21] The details are described in the Supplemental materials.

Statistical Analyses

For microarray data analysis, Microarray Data Analysis Tool Ver. 1.2 was used. For in vitro experiments, values were expressed as the mean ± standard deviation (SD). The details of other analyses are described in the Supplemental materials.

RESULTS

miRNA Genes Differentially Expressed Between CAPAN-1 and HPDE Cells

We used a miRNA microarray to obtain the miRNA expression profiles of a pancreatic cancer cell line, CAPAN-1, and an immortalized human pancreatic ductal epithelial cell line, HPDE. When we compared the miRNA expression profiles between these 2 cell lines, 10 miRNAs showed significant differences in their expression levels ($P < .01$, Table 1). Specifically, 8 miRNAs were upregulated and 2 miRNAs were downregulated in CAPAN-1 cells compared with their levels in HPDE cells. Of these miRNA genes, 5, namely *miR-17-5p*, *miR-10a*, *miR-92*, *miR-29b*, and *miR-450*, exhibited particularly large differences in their expression levels ($P < .001$, Table 1). It has been reported that *miR-17-5p*, *miR-29b*, and *miR-92* are overexpressed in pancreatic cancer, consistent with the present microarray data.[30]

To confirm our microarray data, we subjected 6 of the differentially expressed miRNAs to qRT-PCR analysis.

TABLE 1 10 miRNAs differentially expressed in CAPAN-1 cells vs HPDE cells

Probe name	Normalized intensity		Ratio	Type	*P* value
	CAPAN-1	HPDE			
hsa_miR_16	642.2	224.3	2.87	Up	.0021
ambi_miR_7103	329.2	150.8	2.19	Up	.0023
hsa_miR_17_5p	387.6	179.9	2.16	Up	.0002
hsa_miR_10a	338.1	164.4	2.06	Up	.0007
hsa_miR_92	355.3	180.3	1.97	Up	.0006
hsa_miR_423	308.9	174.7	1.77	Up	.0087
hsa_miR_19b	295.7	186.7	1.59	Up	.0071
hsa_miR_29b	276.9	179.7	1.55	Up	.0002
hsa_miR_450	306.1	556.7	0.55	Down	.00002
hsa_miR_205	174.3	271.5	0.65	Down	.0029

Specifically, we analyzed the expression levels of *miR-17-5p*, *miR-92*, *miR-34*, *miR-200c*, *miR-203*, and *miR-10a* in the miRNA fractions obtained from CAPAN-1 and HPDE cells. The qRT-PCR analyses revealed a greater difference in *miR-10a* expression compared with that observed in the microarray analysis. In the analyses of the expression levels of *miR-17-5p*, *miR-34*, *miR-203*, and *miR-92*, qRT-PCR showed similar results to the microarray analyses (Fig. 1a), while the fold change of *miR-203* expression differed between the microarray and qRT-PCR data.

Comparisons of Cancer-specific miRNA Expression Levels in 15 Pancreatic Cancer Cell Lines and HPDE Cells

Next, we compared the expression levels of *miR-17-5p*, *miR-92*, and *miR-10a* in 15 pancreatic cancer cell lines using a small RNA-rich fraction. We found that all 15 pancreatic cancer cell lines expressed *miR-10a*. In particular, 7 of the 15 pancreatic cancer cell lines expressed remarkably high levels of *miR-10a* and 3 cell lines (KP-3, Suit-2, and Hs766T) expressed moderate levels of *miR-10a*, whereas *miR-10a* was not expressed in HPDE cells (Fig. 1b). Similar results were obtained for *miR-92* and *miR-17-5p* (Supplemental Fig. 1a, b).

Overexpression of miR-10a in Microdissected Pancreatic Cancer Cells and Its Correlation with HOXA1 Expression

Recently, Weiss et al. reported that miR-10a was an important mediator of metastasis formation in pancreatic tumor cells.[31] Therefore, we focused on the expression and function of *miR-10a* in pancreatic cancer. To confirm overexpression of *miR-10a* in primary pancreatic cancer cells derived from resected pancreatic tumors in vivo, we performed microdissection to isolate pancreatic IDC cells and normal ductal cells from bulk pancreatic tissues and measured their levels of *miR-10a* expression. Recently, PanIN lesions have been recognized as precursor lesions for conventional pancreatic cancer.[32] Therefore, to investigate the changes in *miR-10a* expression during pancreatic carcinogenesis, we also microdissected PanIN cells (PanIN-1B from 9 lesions; PanIN-2 from 2 lesions) and measured their levels of *miR-10a* expression. We found that IDC cells expressed significantly higher levels of *miR-10a* than PanIN cells and normal ductal cells (PanIN cells, $P < .01$; normal ductal cells, $P = .001$; Fig. 1c). Furthermore, 14 of 20 IDC samples (70%) expressed higher levels of *miR-10a* than any of the normal samples, consistent with the results for the cultured cells. However, there were no differences in *miR-10a* expression between PanIN and

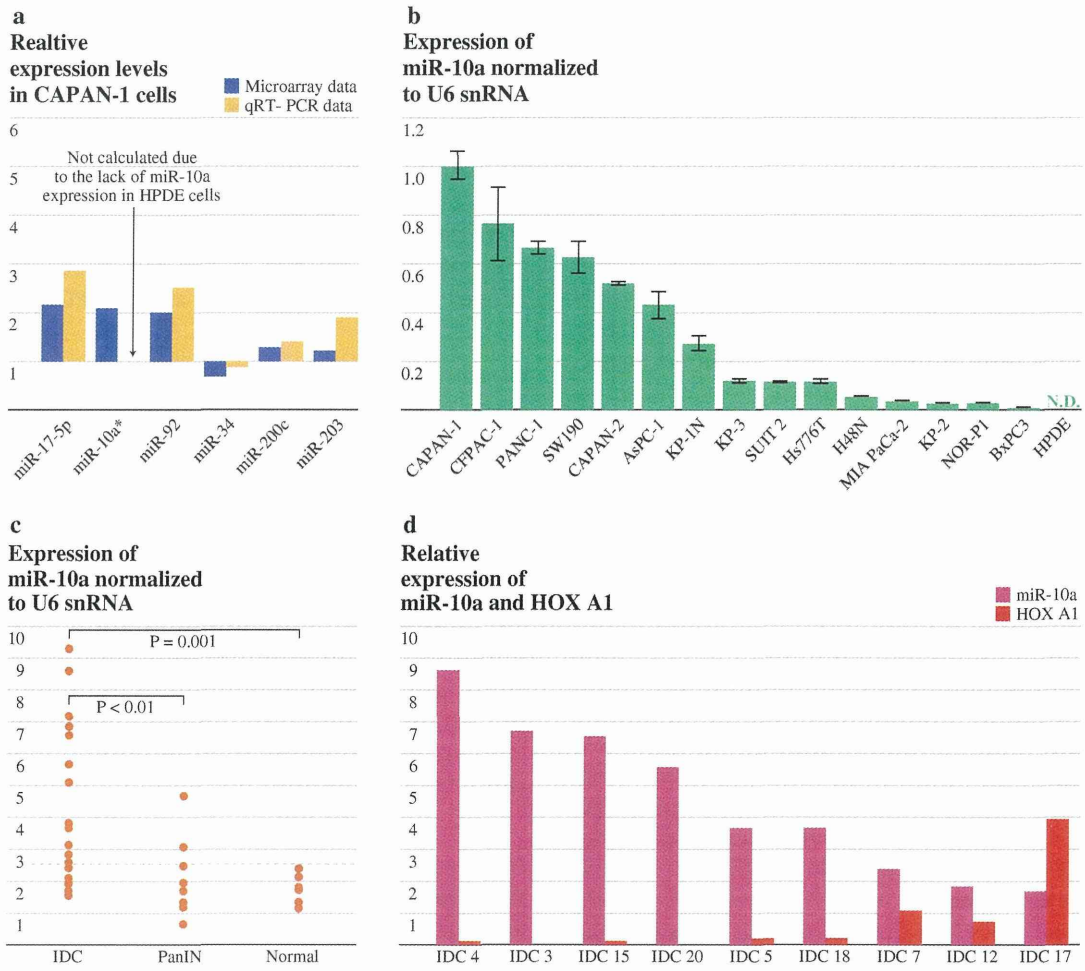

FIG. 1 **a** Comparisons of microarray and qRT-PCR data. Total RNAs with miRNA fractions were extracted from CAPAN-1 and HPDE cells and subjected to qRT-PCR analysis to measure the levels of indicated miRNAs. Most of the qRT-PCR data are consistent with the microarray data. **b** miR-10a expression in 15 pancreatic cancer cell lines and HPDE cells. *ND* not detected. **c** Overexpression of miR-10a in microdissected pancreatic cancer cells derived from resected pancreatic tumors. IDC cells were isolated from 20 pancreatic tumor sections, as well as PanIN cells from 11 sections and normal epithelial cells from 10 sections with the histologic appearance of normal pancreas. **d** There is a significant inverse correlation between miR-10a and HOXA1 expressions in IDC cells microdissected from primary pancreatic tumors ($P = .018$, $r = .833$; Spearman rank correlation test)

normal ductal cells (Fig. 1c). Taken together, overexpression of *miR-10a* was found in invasive cancer cells but not in early precursor lesions or normal ductal cells.

Recently, *miR-10a* was reported to downregulate *HOXA1* mRNA in the human chronic myeloid leukemia blast crisis cell line K562.[33] Therefore, to investigate the correlation between *miR-10a* and *HOXA1* expressions in vivo, we measured the *miR-10a* and *HOXA1* expression levels in IDC cells microdissected from primary pancreatic cancer tumors and found an inverse correlation between *miR-10a* and *HOXA1* expressions ($P = .018$, $r = .833$; Fig. 1d). We also measured *HOXA1* expression in PanIN cells ($n = 4$) and normal ductal cells ($n = 3$), but did not detect any measurable levels of *HOXA1* expression.

Effects of miR-10a on Cancer Proliferation and Invasion

In a preliminary study, we found that the expression of *miR-10a* changed depending on the degree of confluence of cultured cells. Hwang et al. also reported that cell-cell contact globally activates microRNA biogenesis.[34] These data suggest that expression of *miR-10a* is also regulated in a confluence-dependent manner.

Next, we investigated the functional role of *miR-10a* in pancreatic cancer cells by performing inhibition analyses in vitro. We inhibited *miR-10a* activity using an antisense oligonucleotide (anti-*miR-10a* inhibitor) specific for *miR-10a*. To clarify the specific activity of *miR-10a*, we also

analyzed the effects of a nonsense miRNA inhibitor (negative control inhibitor) and an anti-*miR-92* inhibitor. First, we examined the effects of the anti-*miR-10a* inhibitor on *miR-10a* activity in pancreatic cancer cells by measuring the levels of *HOXA1* mRNA. CAPAN-1 and PANC-1 cells expressing high levels of *miR-10a*, SUIT-2 cells expressing moderate levels of *miR-10a*, and MIA PaCa-2 cells expressing very low levels of *miR-10a* were transfected with the nonsense inhibitor, anti-*miR-10a* inhibitor, or anti-*miR-92* inhibitor, cultured for 3 days, and harvested at the same culture condition (90% confluence) for analysis of *HOXA1* mRNA expression. *HOXA1* mRNA expression was remarkably increased in the 3 pancreatic cancer cell lines highly or moderately expressing *miR-10a* after transfection of the anti-*miR-10a* inhibitor (Fig. 2), consistent with the results of a previous study.[33] The nonsense inhibitor did not affect the expression of *HOXA1*. To investigate the effect of the anti-*miR-10a* inhibitor on HOXA1 protein expression, we performed western blotting and found an increase in HOXA1 protein expression after transfection of the anti-*miR-10a* inhibitor (Fig. 2). These data suggest that transfection of the anti-*miR-10a* inhibitor efficiently suppressed *miR-10a* activity in the present study. In contrast, there were no significant changes in *HOXA1* expression in low-*miR-10a*-expressing MIA PaCa-2 cells after transfection of each inhibitor. Furthermore, we investigated the effect of the anti-*miR-10a* inhibitor on HOXA2 expression and found no changes in HOXA2 expression (Fig. 2).

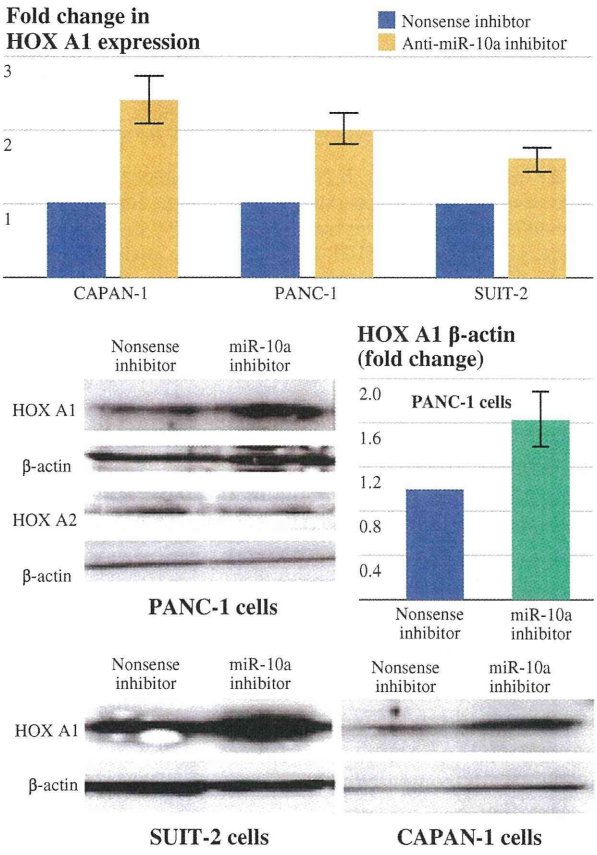

FIG. 2 Effects of an anti-*miR-10a* inhibitor on *HOXA1* expression. The indicated cells were harvested at 3 days after a second transfection with an anti-*miR-10a* inhibitor or a nonsense inhibitor. Total RNA and protein were extracted from the cells and subjected to qRT-PCR and western blotting to measure the *HOXA1* mRNA and protein expressions, respectively. *HOXA1* mRNA expression was normalized to the corresponding *18S* rRNA expression

Next, we investigated the effects of *miR-10a* on the proliferation of pancreatic cancer cells. High-*miR-10a*-expressing CAPAN-1 and PANC-1 cells, moderate-*miR-10a*-expressing SUIT-2 cells, and low-*miR-10a*-expressing MIA PaCa-2 cells were transfected with the nonsense inhibitor, anti-*miR-10a* inhibitor, or anti-*miR-92* inhibitor, seeded in 24-well plates at densities of 2–5 $\times$ 10^4 cells/well and cultured for 1–4 days. Proliferation activity was assessed by measuring PI incorporation at the indicated times after the transfection. The anti-*miR-10a* inhibitor had no significant effects on the growth of SUIT-2, PANC-1, CAPAN-1, or MIA PaCa-2 cells (Supplemental Fig. 2), although it appeared to slightly promote the proliferation of PANC-1 cells. We also performed colony formation and soft agar assays to evaluate the effects of the *miR-10a* inhibitor on cell growth at very low densities or anchorage-independent growth and found no effects.

We investigated the effects of *miR-10a* on the invasiveness of pancreatic cancer cells using an in vitro invasion assay. PANC-1, CAPAN-1, SUIT-2, KP-2, and MIA PaCa-2 cells were transfected with the nonsense inhibitor, anti-*miR-10a* inhibitor or anti-*miR-92* inhibitor, seeded in Matrigel-coated inner wells at a density of 1 $\times$ 10^5 cells/well and cultured for 24–72 h. The number of invading PANC-1 cells transfected with the anti-*miR-10a* inhibitor was remarkably lower than the numbers of invading PANC-1 cells transfected with the nonsense inhibitor or anti-*miR-92* inhibitor (Fig. 3a), and the difference was significant ($P < .01$, Fig. 3b). Similarly, the number of invading SUIT-2 cells transfected with the anti-*miR-10a* inhibitor was lower than the numbers of invading SUIT-2 cells transfected with the nonsense inhibitor or anti-*miR-92* inhibitor (Fig. 3a), and this difference was also significant ($P < .01$, Fig. 3b). Similar results were obtained for the invasiveness of CAPAN-1 cells transfected with the anti-*miR-10a* inhibitor ($P < .01$).

Subsequently, we performed the same experiments using another type of *miR-10a* inhibitor, namely a miRCURY LNA knockdown probe (LNA-10a). We found similar inhibitory effects of LNA-10a on the invasion of PANC-1 cells. These data suggest that *miR-10a* may have a specific role in the invasion of pancreatic cancer cells. In

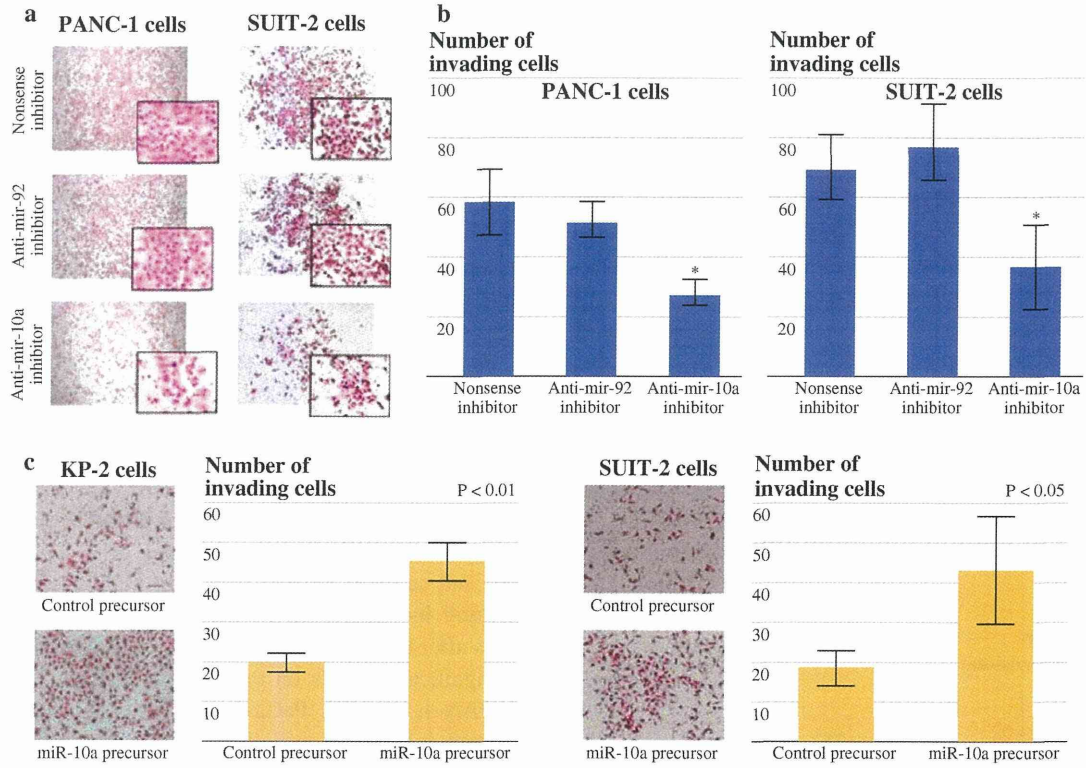

FIG. 3 Effects of an anti-*miR-10a* inhibitor or a *miR-10a* precursor on the invasive potential of pancreatic cancer cells. After the second transfection with indicated inhibitors, the cells were seeded into the inner wells coated with Matrigel and incubated for 24–72 h. The numbers of cells invading the Matrigel-coated membranes were counted. **a** Representative photomicrographs of PANC-1 and SUIT-2 cells (original magnification, ×40; right lower insets, ×200) treated with the indicated inhibitors. **b** The anti-*miR-10a* inhibitor suppresses the invasion of PANC-1 (upper) and SUIT-2 (lower) cells ($^{*}P < .01$). **c** The *miR-10a* precursor was transfected into KP-2 cells expressing low levels of *miR-10a* and SUIT-2 cells expressing moderate levels of *miR-10a*. The cells were seeded for invasion assays at 2 days after transfection and the numbers of invading cells were counted at 36 hours after seeding

contrast, there were no differences in invasiveness between low-*miR-10a*-expressing MIA PaCa-2 cells and KP-2 cells transfected with any of the inhibitors.

We further investigated the effects of a *miR-10a* precursor on the proliferation and invasion of low-*miR-10a*-expressing KP-2 cells. The *miR-10a* precursor significantly increased the number of invading KP-2 cells compared with the control precursor ($P < .01$, Fig. 3c, upper), although there were no differences in the proliferation of KP-2 cells transfected with the *miR-10a* precursor or control precursor (Supplemental Fig. 3). We also investigate the effects of a miR-10a precursor on the invasion of moderate-miR-10a expressing SUIT-2 cells and found similar results (Fig. 3c, lower).

Involvement of HOXA1 in miR-10a-Induced Invasiveness of Pancreatic Cancer

In the present study, we confirmed that *HOXA1* was a target of *miR-10a* (Fig. 2). To investigate the involvement of *HOXA1* in the mechanism of *miR-10a*-induced invasiveness of pancreatic cancer cells, we evaluated the effects of inhibiting *HOXA1* expression on the invasiveness of pancreatic cancer cells. We inhibited *HOXA1* expression using siRNAs targeting *HOXA1* (siRNA-1 and siRNA-2), which reduced the *HOXA1* mRNA levels in PANC-1 cells to less than 20% of the control level from 24–48 hours after transfection. We also found that these siRNAs significantly decreased the protein levels of HOXA1 (Supplemental Fig. 4). We performed cell proliferation and invasion assays using these siRNA-transfected cells. The siRNAs targeting *HOXA1* significantly increased the numbers of invading cells compared with a control siRNA ($P < .01$, Fig. 4a), but had no effect on proliferation. We further investigated the effects of these siRNAs on the invasiveness of SUIT-2 cells and found similar results (Fig. 4a).

We also investigated whether the effects of *miR-10a* were mainly or partially exerted through inhibition of *HOXA1* expression. As shown in Fig. 4b, *miR-10a* inhibitor-suppressed invasion of PANC-1 and SUIT-2 cells was partially recovered at 36 hours after transfection of the

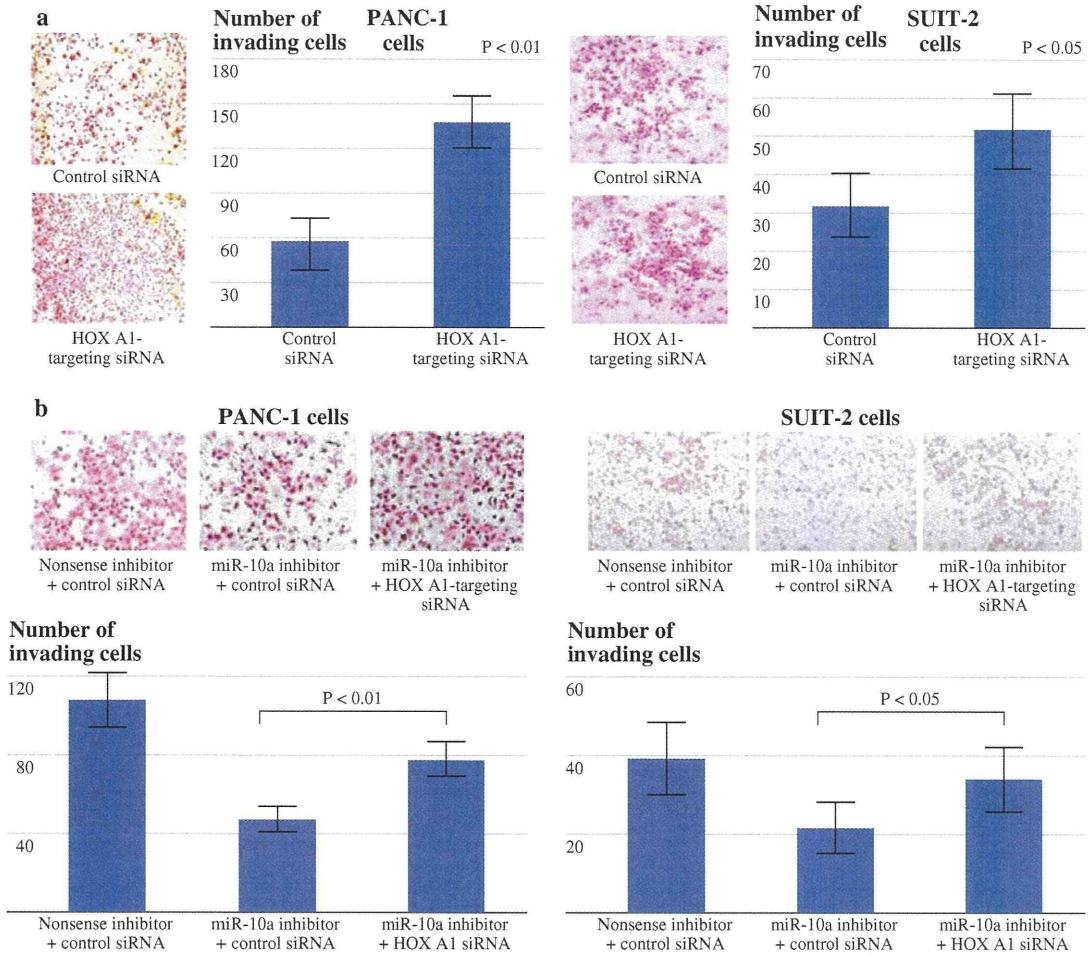

FIG. 4 Effects of a siRNA targeting *HOXA1*, a target gene of *miR-10a*, on the invasive potential of pancreatic cancer cells. **a** The cells were subjected to invasion assays at 24 h after transfection with 100 pmol of siRNA and cultured for an additional 24 h. Representative micrographs of PANC-1 (*upper*) and SUIT-2 (*lower*) cells (original magnification, ×40) transfected with a control siRNA or a siRNA targeting *HOXA1*. **b** PANC-1 (*upper*) and SUIT-2 (*lower*) cells were transfected with the *HOXA1*-targeting siRNA or control siRNA after transfection with a *miR-10a* inhibitor or a nonsense inhibitor and seeded for invasion assays

HOXA1-targeting siRNAs (PANC-1, $P < .01$; SUIT-2, $P < .05$).

DISCUSSION

In the present study, we have provided the evidence that a cancer-specific miRNA, *miR-10a*, regulates the invasiveness of pancreatic cancer cells and that *miR-10a* is overexpressed in primary pancreatic cancer cells microdissected from a subset of resected pancreatic tumors. Recently, several investigators have also reported that miR-10a and miR-10b, which is a close relative of miR-10a, positively regulates metastasis and/or cell invasion in pancreatic cancer or breast cancer.[31,35] These findings suggest that both miR-10a and miR-10b may play important roles using similar mechanisms during cancer progression.

Bloomston et al. revealed that *miR-10a*, *miR-92*, and *miR-221* are overexpressed in pancreatic cancer.[18] Similarly, Szafranska et al. demonstrated that *miR-16*, *miR-196a*, *miR-130b*, and *miR-221* are overexpressed in pancreatic cancer.[19] These data are partially consistent with the present results. In the present study, we used immortalized human pancreatic ductal epithelial cell line HPDE for comparison. Although HPDE cells are derived from normal cells, they have been immortalized by transfection with papilloma virus-derived *E6* and *E7* genes. Therefore, HPDE cells may exhibit changes in the expression levels of some cancer-related genes. Hence, the present miRNA profiles only provide a partial list of the miRNAs differentially expressed in pancreatic cancer, but provide useful reference information for analyses of primary tissues.

Garzon et al. reported that *miR-10a* inhibits *HOXA1* expression, consistent with the present results.[33] We also

found that inhibition of *HOXA1* promoted the invasiveness of pancreatic cancer cells and that 60% of microdissected pancreatic cancer samples expressed *miR-10a* without *HOXA1* expression, while pancreatic normal duct and PanIN-1A cells did not express significant levels of *HOXA1*. These data suggest the possibility that *HOXA1* is upregulated at the late stage of carcinogenesis and then downregulated at the more invasive stage, although further studies are required to identify the roles of HOXA1 during each stage of pancreatic carcinogenesis and cancer progression. It has been reported that *HOXA1* is decreased in lung cancer and transcriptionally silenced by CpG island hypermethylation in lung adenocarcinoma and gastric cancer.[20–22] On the other hand, it has been reported that HOXA1 increases the proliferation and survival of mammary carcinoma cells.[36,37] Taken together, HOXA1 may play different roles in carcinogenesis and cancer progression depending on the tumor types. In 2009, Weis et al. reported that miR-10a promoted the invasion of pancreatic cancer cells through suppression of HOXB1 and HOXB3 using other pancreatic cancer cell lines with zebrafish models.[31] Therefore, HOXB1 and HOXB3 may be involved in miR-10a-regulated invasion in our models, although further examination is needed.

In conclusion, we used a microarray strategy to identify a specific miRNA, *miR-10a*, that is overexpressed in a subset of pancreatic cancers and involved in the invasive potential of pancreatic cancer cells. Strategies to reduce *miR-10a* expression may be useful for limiting tumor invasion for high-*miR-10a*-expressing pancreatic cancers. In addition, we demonstrated that *HOXA1*, a target gene of *miR-10a*, is involved in tumor cell invasion. Identification of such target genes may provide valuable insights into the mechanisms of cancer invasion as well as novel diagnostic or therapeutic approaches for pancreatic cancer.

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ORIGINAL ARTICLE

Feasibility of laparoscopic gastrectomy for advanced gastric cancer with positive peritoneal cytology

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Abstract
Purpose The role of gastrectomy for patients with positive peritoneal cytology, but a negative macroscopic peritoneal implant (P−/cy+), remains unclear. The aim of this study was to evaluate laparoscopic gastrectomy for P−/cy+ patients.
Methods This study reviewed a prospectively maintained gastric cancer database of gastric-cancer patients those underwent surgical resection. P−/cy+ gastric cancer that had invaded the subserosa, or deeper layers, of the stomach wall without distant organ metastases was considered operable in this institution. P−/cy+ patients underwent either open or laparoscopic gastrectomy with D2 lymphadenectomy. The short-term results were examined to assess differences in outcome between the two groups.
Results Eighteen P−/cy+ patients without distant organ metastases underwent surgery between 2000 and 2010. Laparoscopic gastrectomy was performed in nine patients and open gastrectomy in nine patients. The estimated blood loss was significantly smaller, the resumption of food intake earlier, and the length of postoperative hospital stay shorter in the patients that underwent laparoscopic gastrectomy than in the patients that underwent open gastrectomy. There were no significant differences in the 2-year survival rates between the groups.
Conclusion Laparoscopic gastrectomy for P−/cy+ patients is a minimally invasive and safe oncologic procedure with good short-term results.

Keywords Gastric cancer · Peritoneal lavage cytology · Laparoscopic gastrectomy · Prognosis · Chemotherapy

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Introduction

Although gastroscopic examinations are now widely performed, a large number of gastric cancers are still only diagnosed at an advanced stage. A primary tumor that has invaded into the subserosal or serosal layers of the gastric wall is likely to spread into the peritoneal cavity and, consequently, become implanted within the peritoneum (peritoneal dissemination). Free cancer cells in the peritoneal cavity that originate from either the primary lesion or metastatic lymph nodes are thought to be the main cause of peritoneal dissemination (P+). Therefore, positive peritoneal cytology (cy+) is a predictor of a poor prognosis [1–5]. However, Boku et al. [6] reported a 3-year survival rate after gastrectomy in patients with positive peritoneal cytology, but negative macroscopic peritoneal implantation (P−/cy+), to be 25 %, and Miyashiro et al. [7] reported a survival rate of P−/cy+ patients after radical gastrectomy to be significantly longer than that of P+ patients. Although the role of gastrectomy as a treatment for P−/cy+ patients remains unclear, it may provide a prognostic benefit by allowing the early induction of chemotherapy [8–12]. Extraperigastric (D2) lymphadenectomy is also performed when P−/cy+ patients undergo gastrectomy [7]. However, radical gastrectomy, including D2 lymphadenectomy, is extremely invasive and may thus be detrimental to P−/cy+ patients.

Laparoscopy has gained widespread clinical acceptance for the treatment of gastric cancer [13–18]. This approach offers important advantages in comparison to open surgery, including reduced intra-operative blood loss, reduced postoperative pain and accelerated recovery, an earlier return to normal bowel function with an earlier resumption of oral intake, early discharge from hospital, and lower financial costs [14, 19]. The advantages of laparoscopic gastrectomy for early gastric cancers have been evaluated and have now been broadly accepted, but laparoscopic gastrectomy for advanced cancers remains limited to only a few institutions because of the technical difficulty associated with D2 lymphadenectomy and questions associated with the oncologic adequacy of such laparoscopic procedures [20]. Moreover, the advantages of laparoscopic gastrectomy in comparison to open gastrectomy for P−/cy+ patients have not yet been reported. Therefore, laparoscopic gastrectomy for P−/cy+ gastric cancer can only be accepted as a safer alternative to open surgery, if it results in an equivalent postoperative course.

The purpose of the present study was to compare the short-term results of gastrectomy using either a laparoscopic or open approach in P−/cy+ gastric cancer patients, to elucidate the safety and feasibility of laparoscopic procedures for this patient group.

Methods

The study reviewed a prospectively maintained gastric cancer database of gastric cancer patients those underwent surgical resection. Two-hundred and twenty-four patients underwent surgery for gastric cancer that had invaded the subserosa or deeper layers of the stomach wall, between January 2000 and December 2010, at the Department of Surgery and Oncology, Graduate School of Medicine, Kyushu University. The pouch of Douglas was washed with 100 ml of physiologic saline solution immediately after laparotomy or the insertion of the laparoscope. The fluid was collected and immediately centrifuged at 2000 rpm for 3 min. The sediment was smeared onto four glass slides. The slides were stained using the Giemsa and Papanicolaou methods and a diagnosis was made by cytologists blinded to the clinical information. The results were classified as positive when at least one cancer cell was detected. A suspicion of malignancy was classified as negative. The identification of cancer cells was based on the nuclear size [including the nuclear/cytoplasm (N/C) ratio], anisokaryosis, membrane pattern, nucleoli pattern, and chromatin density [21]. Positive peritoneal cytology without macroscopic peritoneal dissemination (P−) and distant organ metastases was considered operable. The patients with massive ascites, suspected to be peritoneal dissemination, were excluded from surgery. Gastrectomy removed the distal two-thirds of the stomach or the entire stomach, and D2 lymphadenectomy was performed. Splenectomy was indicated when the tumor was located at the greater curvature of the upper stomach, or involved lymph nodes around the left gastroepiploic or short gastric arteries. A partial resection of the transverse-colon was indicated if the tumor had invaded the transverse mesocolon. The specimens were inserted into a retrieval bag, withdrawn through the extended port-site, and then the intraperitoneal space was washed with 20 L of physiologic saline before closing the abdomen [10]. Laparoscopic gastrectomy with D2 lymphadenectomy has been performed for P−/cy+ patients without tumor invasion to adjacent organs since January 2008. The macroscopic and cytological results showed that there were 19 P−/cy+ patients without distant organ metastases (13 males and 6 females; mean age 64.6 ± 17.2 years; range 22–89 years). The cytological results were confirmed to be consistent with the final pathology results after surgery. Radical gastrectomy was performed in 18 patients, and pancreatoduodenectomy (due to invasion of the pancreas) was performed in one patient (this patient was excluded from the study). The choice of surgical procedure (open versus laparoscopic) was based solely on the patient's individual decision after being informed of the methods and risks involved. All patients provided their informed written consent. The performance status was evaluated with the scale of the Eastern Cooperative Oncology Group (ECOG) [22]. Patients underwent preoperative chemotherapy when peritoneal metastasis was suspected by preoperative examinations. P−/cy+ patients with a good performance status (ECOG score 0 or 1) received postoperative chemotherapy with either 80 mg/m^2 S-1 divided into two daily doses for 28 days repeated every 42 days after 2008 or infusional 500 mg/m^2 fluorouracil plus 10 mg/m^2 cisplatin for 120 h repeated every week before 2008 within eight postoperative weeks.

The study protocol was approved by the local ethics committee, and contained a critical pathway program to avoid any possible bias. The clinical stage and pathological features of the primary tumors were defined according to the criteria of the American Joint Commission on Cancer [23].

Statistical analysis

The clinicopathological characteristics, duration of surgery, number of resected lymph nodes, estimated blood loss, postoperative complications and length of postoperative hospital stay, time to recovery of bowel function (time to first flatus) and resumption of food intake, induction rate of postoperative chemotherapy, and 2-year overall survival time were examined to assess differences in the