I. 背 景

固形腫瘍の治療においては，外科的切除が第一選択とされている。その治療成績の向上を図る目的で種々の術後補助化学療法が検討されている。欧米とは手術手技が異なり，日本では術後補助化学療法に経口フッ化ピリミジン系抗癌剤が主に用いられていた。しかしエビデンスという観点からは問題点が指摘されていた。近年，経口フッ化ピリミジン系抗癌剤であるUFTにおいて，肺癌[1]，直腸癌[2]，乳癌[3]および胃癌[4]の領域で術後補助化学療法としての有用性が報告された。一方，1999 年に承認となったS-1は経口フッ化ピリミジン系抗癌剤として，効果増強と消化管での副作用軽減を目的に開発された薬剤で，胃癌で46.5%という高い奏効率を有し，早期に承認となった薬剤である。今回，直腸癌の術後補助化学療法の成績向上を目的として，UFTをコントロールとしS-1の有用性を検討することを計画した。

II. 対象症例および投与法

組織学的に直腸(Rsを除く)の腺癌と診断され，Table 1を満たす症例に対して以下の治療をFAXによる中央登録方式で無作為に選択して行う。

1. 対照群（A群）

手術+UFT: UFTは400 mg/m²/day（tegafur相当量）を1日2回に分けて経口投与する。これを5日間連日経口投与し，その後2日間休薬するスケジュール(5投2休法）で1年間投与する。

2. 試験群（B群）

手術+S-1: S-1は80 mg/m²/day (tegafur相当量) を1日2回に分けて経口投与する。これを4週間連日経口投与し，その後2週間休薬するスケジュールで1年間投与する（Fig. 1)。

III. 試験デザインの解説

本試験の目的は，治癒切除を受けたStage IIおよびStage III（TNM分類）の直腸癌（Rsを除く）症例を対象として，術後補助化学療法としてのS-1（tegafur・gimeracil・oteracil potassium）療法の有用性をUFT (tegafur・uracil）療法を対照としてランダム化比較試験にて検証することである。primary endpointは無再発生存期間 (relapse-free survival: RFS) であり，secondary endpointは生存期間 (overall survival: OS) である。

S-1はUFTを上回る効果が期待できる反面，副作用に関してもUFTより高頻度で発現することが予想される。また，どちらの薬剤も経口剤であり外来治療であるという点を含め，利便性に関しては同等と考えられる。したがって，S-1療法はprimary endpointであるRFSにおいて，UFT療法に勝る結果がでて初めて本試験終了後の標準的治療の一つと位置付けられる。このためS-1療法がUFT療法に比してRFSの延長効果があるか否かの優越性を検証することとした。

本試験の主たる仮説は「試験群（S-1療法）のRFSが

Table 1 Eligibility criteria

1) Histologically confirmed rectal cancer except Rs
2) Histological Stage II A (T 3, N 0, M 0), II B (T 4, N 0, M 0), III A (T 1-2, N 1, M 0), III B (T 3-4, N 1, M 0), and III C (any T, N 1, M 0) located in rectal except Rs (according to TMN classified, UICC 6th edition, 2002)
3) Patients without synchronous multiple colorectal carcinoma deeper than histological depth of invasion, sm Synchronous colorectal carcinoma with histological tumor invasion of mucosa is permitted, if patients have underwent curative resection.
4) Curative resection with D 2 or more lymph node dissection
5) Resection of histological curability A was performed
6) Age≦20 and≧80 years
7) No prior treatment (chemotherapy and radiotherapy etc) except for resection
8) Adjuvant chemotherapy will be started within 7 weeks after the operation
9) Oral administration of UFT or S-1 is possible
10) Sufficient organ functions drawn within 14 days of entry
a) WBC≧3,500/mm³
b) Hemoglobin≧9.0 g/dl
c) Platelet≧100,000/mm³
d) Total bilirubin≦1.5 mg/dl
e) AST, ALT<100 IU/l
f) Serum creatinine≦1.0 mg/dl
Written informed consent is required

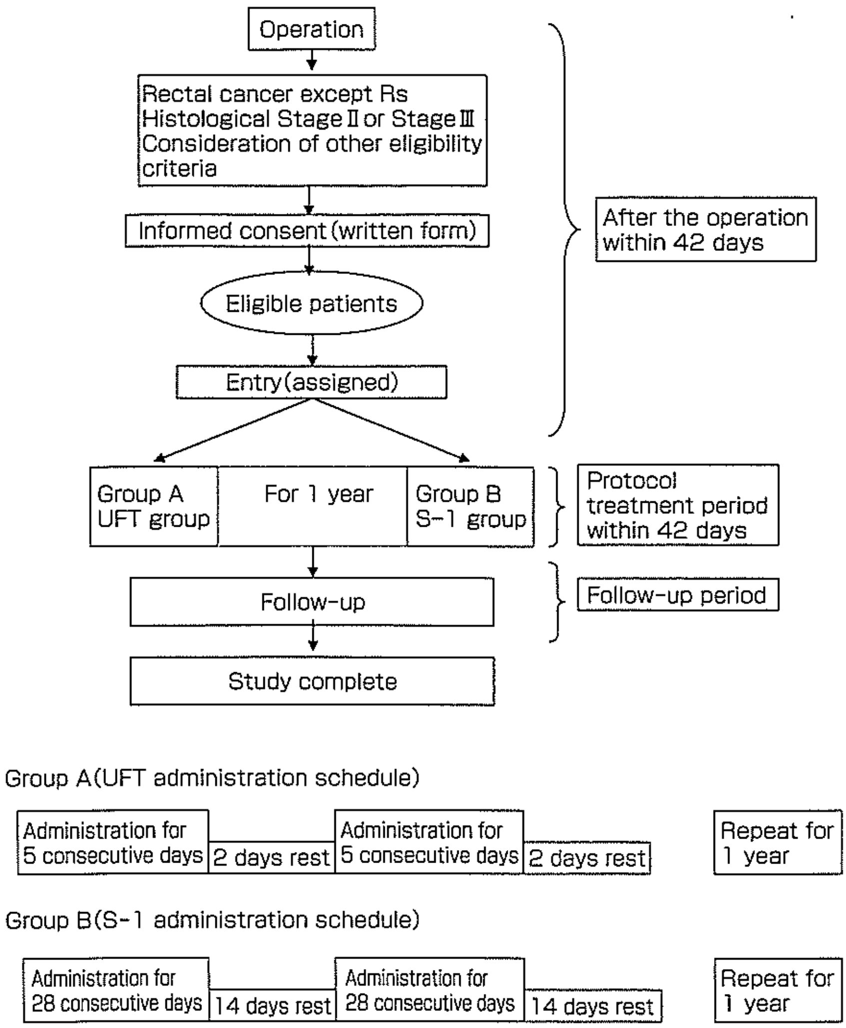

Fig. 1 Flow chart of the registration of the study
Patients were randomized either to Group A (UFT group) or Group B (S-1 group).
Group A: UFT was administered orally for 5 days (400 mg/m²/day) followed by two days rest for a year.
Group B: S-1 was orally administered for 4 weeks (80 mg/m²/day) followed by two weeks rest for a year.

対照群（UFT 療法）に対して有意に上回った場合，S-1 療法をより有用な治療法と判断する」としている。これまでの報告によると UFT 療法の直腸癌術後補助化学療法の成績では，TAC-CR において 5 年無再発生存率が 73.6%，大腸癌術後補助化学療法研究委員会: National Surgical Adjuvant Study of Colorectal Cancer（N・SAS-CC）では 3 年無再発生存率が 78%であった。

本試験での primary endpoint の解析の中心は死亡・再発をイベントに含む 5 年無再発生存率であり，N・SAS-CC では 3 年無再発生存率であるため，上記二つの結果より下回ることが予想されるために UFT 群の 5 年無再発生存率を 70%と仮定した。一方，S-1 群の予後改善効果を臨床的に意味があると思われるハザード比 0.7 と仮定した。したがって登録 3 年，追跡 5 年，$\alpha=5\%$（両側），検出力 80%として，S-1 群の優越性の検証に必要な症例数を算出すると 1 群当たり 381 例，両群 762 例となる。

IV. 考　察

1．欧米における直腸癌の術後補助化学療法

直腸癌について欧米では全直腸間膜切除（total mesorectal excision）の普及をみるのみで，直腸間膜外リンパ節転移（側方転移）に対する外科治療は試みられていない。その理由として，1950 年代の Stearns ら[5]，Bacon ら[6]の側方骨盤リンパ節郭清の試みが否定的な結果であったこと，側方転移は外科治療の対象ではなく全身病の一部ととらえ，放射線化学療法の対象と考えられていることおよび動脈硬化が高度である欧米人にとって側方郭清の侵襲と機能障害は高度であることなどがあげられ，成績向上に対する欧米の戦略は術前，術後の放射

線治療を含む補助療法に向けられてきた。

Stage IIおよびStage IIIの直腸癌に対する術後放射線照射および5-FUをベースとする化学療法の効果は，Gastrointestinal Tumor Study Group (GITSG) 7175[7]，Mayo/North Center Cancer Treatment Group (NCCTG) 79-47-51[8]およびNational Surgical Adjuvant Breast and Bowel Project (NSABP) R-01[9]によって確立された。こうした研究から，外科切除後に放射線療法と化学療法とを併用すれば，無病期間および全生存とも増大することが明らかになった。また，この試験成績の発表後，National Cancer Institute (NCI)（米国国立癌研究所）は，1990年のConsensus Development Conference（コンセンサス開発会議）で，Stage IIおよびStage IIIの直腸癌患者に対して術後併用療法による治療を推奨するという結論に達した。

これに引き続いて，GITSG[10]，Intergroup 86-47-51試験[11]，Intergroup試験0144[12]，SWOG-9304[13]，NSABP R-02[14]が実施され，NSABP R-02によってAmerican Society of Clinical Oncology (ASCO) では術後放射線療法の果たすべき役割に関する議論が始まった。National Cancer Data Baseに登録された5,987人のStage IIIの解析の結果では，5年生存率は手術単独でStage IIIA 39.0%，Stage IIIB 21.7%，Stage IIIC 12.2%，補助化学放射線療法を実施しても，Stage IIIA 60.0%，Stage IIIB 40.9%，Stage IIIC 28.9% (American Joint Committee on Cancer Sixth edition) と，日本に比べてその予後は悪い[15]。なお，2006年のNational Comprehensive Cancer Networkのガイドライン[16]によると，Stage IIおよびStage III症例には術前に5-FU＋放射線治療を行った場合は術後に5-FU±Leucovorin（もしくはFOLFOX）を使用し，術前放射線療法を行わなかった場合は，術後に5-FU±Leucovorin（もしくはFOLFOX）次いでcontinuous 5-FU/放射線療法にさらに5-FU±Leucovorin（もしくはFOLFOX）と，結腸癌における術後補助化学療法のエビデンスも取り入れたものとなっている。ただしFOLFOXはあくまで結腸癌でのエビデンスであり，直腸癌にはエビデンスはない。

2．日本における直腸癌の術後補助化学療法

日本では骨盤内自律神経を温存しつつ側方郭清を行う骨盤自律神経温存側方骨盤リンパ節郭清（自律神経温存D3郭清術）が普及している。全直腸間膜切除を含んだ手術が一般に行われている欧米に対して手術成績が良好で，局所再発率が低いことを理由に，術後放射線治療あるいは化学放射線治療は十分に検討されていない。しかしながら，日本で普及されている側方郭清を伴う自律神経温存D3郭清術と，欧米での標準手術である直腸間膜切除（mesorectal excision）での比較試験は行われておらず，そのため現在これらの術式の有効性および手術に関連する合併症の発生率などを検討するために，ランダム化比較試験が実施されている[17]。

術後補助化学療法については，1970年代より結腸癌と直腸癌を対象として手術単独に対する比較試験が行われてきた。その結果，結腸癌では術後補助化学療法の有用性を示した試験はほとんど皆無であったのに対し，直腸癌においては，Dukes BおよびCを対象とした四つの試験[18-21]により術後補助化学療法の有用性が示唆された。

しかしこれまでの国内の臨床試験では試験デザイン・運営管理が不十分であること，転移/再発例では有効性が認められていない少量投与法が採用されていたこと，多施設少数症例登録であり施設間差が大きいことなどから，術後補助化学療法群が手術単独群と比較して明確に有用であるという結果は得られていない。そこで，これらの術後補助化学療法の有用性を検討する目的で，1996年よりDukes C大腸癌を対象とした手術単独群とUFT投与群（400 mg/m^2 5日投与2日休薬1年間）との比較試験が，N・SAS-CCにて行われた。その結果，直腸癌においては中間解析の段階で3年無再発生存率が手術単独群60%，UFT投与群78% ($p=0.0014$)，3年生存率が手術単独群81%，UFT投与群91% ($p=0.0048$) と明らかに予後を改善することが判明し，早期公表すべきとの答申がなされて2004年のASCOにて報告された[2]。これらのことより，UFTはStage III直腸癌の治癒切除例に対する術後補助化学療法として，明らかなエビデンスを示している。さらに，これまで施行されたDukes B (Stage II)，Dukes C (Stage III) の直腸癌に対してUFTを使用した五つの無作為化比較試験（集学・特7[20]，集学・特15-1[22]，集学・特15-2[22]，TAC-CR[21]，N・SAS-CC[2]）のmeta-analysisでも，5年無再発生存率が手術単独群57.9%，UFT群67.6%と，UFT群が有意に無再発生存率を改善させたとの報告が2005年のASCOにて報告されている[23]。

3．本試験設定の意義

直腸癌については，欧米と日本とで放射線療法の有無と手術内容，予後に差がある。一部では，進行直腸癌の欧米での標準治療は，すでに術後にFOLFOXを行うことが標準であると考えられがちであるが，実際は上記のように放射線治療を含むレジメが術前もしくは術後に必ず導入され，術後に5-FU±LeucovorinやFOLFOX単独治療を行うことに対する標準治療としてのエビデンスはない。日本と欧米ではリンパ節の郭清方法に関する考え方に大きな違いがあり，直腸癌に関しては，欧米での

直腸癌術後補助療法のエビデンスをそのまま国内の臨床に当てはめることには問題がある。したがって日本独自のレジメを確立していくことが重要と考えている。

上記のようにN・SAS-CCで直腸癌において，UFTはStage III直腸癌の治癒切除例に対する術後補助化学療法として，明らかに予後を改善することが判明したこと，また，五つの無作為化比較試験のmeta-analysisでUFTが有意に無再発生存率を改善させていることから，日本においてUFTは直腸癌術後補助化学療法の標準治療の一つとなり得ると考えている。

またS-1はUFTと同じ作用機序を有するDIF製剤(dihydropyrimidine dehydrogenase inhibitory fluoropyrimidine)であるが，配合されているgimeracilにより5-FUの血中濃度を高く維持し，持続静注療法と同様な薬物動態を示すことが知られており，UFTを上回る効果が得られる可能性が期待される。

以上のことから，治癒切除を受けたStage IIA (T3, N0, M0), Stage IIB(T4, N0, M0), Stage IIIA(T1-2, N1, M0), Stage IIIB(T3-4, N1, M0)およびStage IIIC (any T, N2, M0) (TNM分類，UICC第6版，2002年) の直腸癌 (Rsを除く) 症例を対象に，術後補助化学療法としてのS-1療法の有用性を比較評価する目的で，本比較試験が計画された。今回の試験ではStage II, Stage III直腸癌を対象としており，Stage IIIのみを対象としているN・SAS-CCと異なる。しかしmeta-analysisではDukes BでもUFTの有効性が認められていることや，最終的にはStage IIIのサブセット解析も可能であることから登録スピードも考慮してStage II, Stage IIIを対象としている。

おわりに

UFTに関しては近年，肺癌，乳癌，胃癌，直腸癌に対して術後補助化学療法としての有用性を示す報告が行われている。一方S-1は胃癌，大腸癌などに対して高い奏効率を示して承認されたが，術後補助化学療法としての有用性に関してはまだ報告されていない。本研究により，S-1術後補助化学療法としての高いエビデンスを世界に発信できることを期待している。

文献

1) Kato H, Ichinose Y, Ohta M, *et al*: The Japan Lung Cancer Research Group on Postsurgical Adjuvant Chemotherapy—A randomized trial of adjuvant chemotherapy with uracil-tegafur for adenocarcinoma of the lung. *N Engl J Med* **350**: 1713-1721, 2004.
2) Akasu T, Moriya Y, Yoshida S, *et al*: Adjuvant oral uracil and tegafur (UFT) improves survival after complete mesorectal excision (ME) for pathologic TNM stage III rectal cancer (RC): Results of the National Surgical Adjuvant Study (NSAS)-Colorectal Cancer (CC) 01 randomized trial. *J Clin Oncol, 2004 ASCO Annual Meeting Proc* (post-meeting edition) **22**(14 S): 3524, 2004.
3) Noguchi S, Koyama H, Uchino J, *et al*: Postoperative adjuvant therapy with tamoxifen, tegafur plus uracil, or both in women with node-negative breast cancer: a pooled analysis of six randomized controlled trials. *J Clin Oncol* **23**(10): 2172, 2005.
4) Kinoshita T, Nakajima T and Ohashi Y: National Surgical Adjuvant Study Group for Gastric Cancer (N-SAS-GC) Adjuvant chemotherapy with uracil-tegafur (UFT) for serosa negative advanced gastric cancer: Results of a randomized trial by national surgical adjuvant study of gastric cancer. *J Clin Oncol, 2005 ASCO Annual Meeting Proc* **23**(16 S): 4021, 2005.
5) Stearns Jr MW and Deddish MR: Five-year results of abdominopelvic lympho node dissection for carcinoma of the rectum. *Dis Colon Rectum* **2**: 169-172, 1959.
6) Bacon HE, Dirbis F, Myers TB, *et al*: Extensive lymphadenectimy and high ligation of the inferior mesenteric artery for carcinoma of the left colon and rectum. *Dis Colon Rectum* **1**: 457-465, 1958.
7) Thomas PR and Lindblad AS: Adjuvant postoperative radiotherapy and chemotherapy in rectal carcinoma: a review of the Gastrointestinal Tumor Study Group experience. *Radiot Oncol* **13**(4): 245-252, 1988.
8) Krook JE, Moertel CG, Gunderson LL, *et al*: Effective surgical adjuvant therapy for high-risk rectal carcinoma. *N Engl J Med* **324**(11): 709-715, 1991.
9) Fisher B, Wolmark N, Rockette H, *et al*: Postoperative adjuvant chemotherapy or radiation therapy for rectal cancer: results from NSABP protocol R-01. *J Natl Cancer Inst* **80**(1): 21-29, 1988.
10) Gastrointestinal Tumor Study Group: Radiation therapy and fluorouracil with or without semustine for the treatment of patients with surgical adjuvant adenocarcinoma of the rectum. *J Clin Oncol* **10**(4): 549-557, 1992.
11) O'Connell MJ, Martenson JA, Wieand HS, *et al*: Improving adjuvant therapy for rectal cancer by combining protracted-infusion fluorouracil with radiation therapy after curative surgery. *N Engl J Med* **331**(8): 502-507, 1994.
12) Tepper JE, O'Connell MJ, Niedzwiecki D, *et al*: Adjuvant therapy in rectal cancer: analysis of stage, sex, and local control—final report of intergroup 0114. *J Clin Oncol* **20**(7): 1744-1750, 2002.
13) Smalley SR and Southwest Oncology Group: Phase III randomized study of three different regimens containing fluorouracil in patients with stage II or III rectal cancer, SWOG-9304, clinical trial, closed.
14) Wolmark N, Wieand HS, Hyams DM, *et al*: Randomized trial of postoperative adjuvant chemotherapy with or without radiotherapy for carcinoma of the rectum: National Surgical Adjuvant Breast and Bowel Project Protocol R-02. *J Natl Cancer Inst* **92**(5): 388-396, 2000.
15) Frederick LG, Andrew KS, and Norton HJ: New tumor-node-metastasis staging strategy for node-positive (Stage III) rectal cancer: An analysis. *J Clin Oncol* **22**: 1778-1784, 2004.
16) National Comprehensive Cancer Network: Clininal practice guideline in oncology version 2, 2006.
17) Japan Clinical Oncology Group: 臨床病期II, IIIの下部直腸癌に対する神経温存D3郭清術の意義に関するラン

ダム化比較試験実施計画書（ME vs ANP-D 3）.

18) 北條慶一，梶谷 鐶: 大腸癌の補助的化学療法（梶谷班一次方式）の成績一厚生省がん研究助成金による大腸癌各種治療法に関する総合的研究班（班長 梶谷鐶）の第一次共同研究の報告一. 癌と化学療法 **13**(10): 3063-3073, 1986.

19) The Colorectal Cancer Chemotherapy Study Group of Japan: Five-years result of randomized controlled trial of adjuvant chemotherapy for curatively resected colorectal carcinoma. *Jpn J Clin Oncol* **25**: 91-103, 1995.

20) がん集学的治療研究財団: 特定研究7「大腸癌術後補助化学療法としてのフッ化ピリミジン系抗癌薬剤の有用性に関する臨床比較研究」報告書, 1996.

21) Kato T, Ohashi Y, Nakazato H, *et al*: Efficacy of oral UFT as adjuvant chemotherapy to curative resection of colorectal cancer: Multicenter prospective randomized trial. *Langenbecks Arch Surg* **386**: 575-581, 2002.

22) Watanabe M, Nishida O, Kunii Y, *et al*: Randomized controlled trial of the efficacy of adjuvant immunochemotherapy and adjuvant chemotherapy for colorectal cancer, using different combinations of the intracutaneous streptococcal preparation OK-432 and the oral pyrimidines 1-hexylcarbamoyl-5-fluorouracil and uracil/tegafur. *Int J Clin Oncol* **9**(2): 98-106, 2004.

23) Hamada C, Sakamoto J, Yasutomi M, *et al*: Meta-analysis Project Group Japanese Society for Cancer of the Colon and Rectum—An individual patient data meta-analysis of adjuvant chemotherapy with UFT in patients with curatively resected rectal cancer. *J Clin Oncol, 2005 ASCO Annual Meeting Proc* **23**(16 S): 3571, 2005.

Journal of Gastroenterology and Hepatology (2006) 21, 814–818 DOI: 10.1111/j.1440-1746.2005.04028.x

GASTROENTEROLOGY

Impact of loss of heterozygosity of encoding phosphate and tensin homolog on the prognosis of gastric cancer

EIJI OKI,* YOSHIHIRO KAKEJI,* HIDEO BABA, ERIKO TOKUNAGA, TOSHIHIKO NAKAMURA, NAOYUKI UEDA, MOTONORI FUTATSUGI, MANABU YAMAMOTO, MASAHIKO IKEBE AND YOSHIHIKO MAEHARA

Departments of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan

Abstract

Background and Aim: Encoding phosphate and tensin homolog (PTEN) is a cancer suppressor gene and it has been assumed that gene mutation and loss of heterozygosity (LOH) occurs frequently in various types of carcinoma. However, the role of LOH of PTEN and its outcome variables in gastric cancer have not been well established. In the present study, we investigated the roles of PTEN, LOH and their outcomes.

Methods: Fresh frozen tumor samples from 119 gastric cancer patients with a primary diagnosis of gastric carcinoma were evaluated for LOH of PTEN using an automated sequencer. Results were compared with pathological parameters. The median follow-up period was 559 days.

Results: Loss of heterozygosity of PTEN was observed in 17.1% of patients (13/76) diagnosed with gastric cancer. No particular relationship was found with any clinicopathological factor. However, the prognosis of patients with LOH of PTEN was significantly poor. Multivariate analyses revealed that vascular invasion, invasion depth, LOH of PTEN, histology and lymph node metastasis were correlated with survival of the patient.

Conclusions: Even though mutation of PTEN in gastric cancer has rarely been reported, according to our findings, LOH of PTEN frequently occurs in gastric cancers and is correlated with disease-related deaths. The LOH of PTEN is an independent prognostic factor and PTEN is a candidate as a haplo-insufficient tumor suppressor in gastric cancers.

Key words: Akt, encoding phosphate and tensin homolog, gastric cancer, loss of heterozygosity.

INTRODUCTION

The encoding phosphate and tensin homolog (PTEN) gene, a tumor suppressor candidate, is located on chromosome 10q23 and has an extensive homology with the cytoskeletal proteins auxilin and tensin.[1,2] Mutations of PTEN have been observed frequently in various neoplasms, including glioblastoma, melanoma, prostate cancer and breast cancer.[1–7] In glioblastoma, melanoma and prostate cancer, PTEN mutations and allelic deletions are observed during the late stages, whereas in thyroid and endometrial cancers, PTEN mutations/alterations are found during the early stages and include endometrial hyperplasia and benign thyroid tumors.[3–6,8,9] Germline mutations of PTEN are found in patients with Coden syndrome, a familial syndrome associated with predisposition for multiple benign hamartomas, and malignant thyroid and breast neoplasms.[10]

The PTEN gene encodes an enzyme with phosphatase activity towards acidic protein substrates and the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3).[11] The phosphatase activity of PTEN is crucial in controlling the phosphatidylinositol

Correspondence: Eiji Oki, Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Higashi-ku, Fukuoka 812-8582, Japan. Email: okieiji@surg2.med.kyushu-u.ac.jp

*These authors contributed equally.

Accepted for publication 17 March 2005.

3-kinase (PI3-K) signal transduction pathway and in the activation of the protein kinase B (PKB/Akt) proto-oncogene. This indicates that PTEN exerts its tumor-suppressor function by negatively regulating the anti-apoptotic PI3-K/AKT signaling pathway.[12,13] The role of PTEN as a tumor-suppressor gene has been attributed to its ability to modulate cell cycle progression and cell motility.[14] It has been demonstrated that PTEN can dephosphorylate focal adhesion kinase (FAK) and inhibit the mitogen-activated protein kinase pathway.[15,16]

Although gastric cancer is one of the leading causes of cancer-related deaths in Japan,[17] its pathogenesis and progression are not yet clearly understood. Deletion or downregulation of tumor-suppressing genes play important roles in the multiple steps of tumorigenesis and the progression of gastric carcinoma. Because *p53* is the most frequently mutated tumor suppressor gene in gastric cancer, most studies on the relationship between the development of gastric cancer and abnormalities of tumor suppressor genes have mainly focused on *p53*.[18] Owing to frequent gene mutation and loss of heterozygosity (LOH), PTEN has been reported as one of the new tumor suppressor genes in various types of cancer. Previous reports have shown protein loss and promoter methylation of PTEN in gastric cancers.[19–22] However, the prognostic significance of the loss of PTEN in gastric cancer has not been well established. In the present study, we provide information on the LOH of PTEN, which is correlated with disease-related deaths of gastric cancer patients.

METHODS

Tissue samples

Pairs of primary gastric carcinoma tissue and corresponding normal mucosa were obtained consecutively from 119 patients who underwent surgery in the Department of Surgery II at Kyushu University Hospital from 1996 to 2000. Informed consent was obtained from all patients prior to their inclusion in the study. In all cases, the histopathological type of the tumor was adenocarcinoma. Cancer tissues and well-separated normal gastric mucosa obtained by gastrectomy were snap frozen immediately and kept in liquid nitrogen. Genomic DNA was prepared by proteinase K digestion and phenol/chloroform extraction, which was followed by ethanol precipitation.

Loss of heterozygosity analysis

Loss of heterozygosity was analyzed using a DNA sequencer with microsatellite markers. The oligonucleotide primers for D10S796 were synthesized and purified by HPLC. The sequences of the primers used for the polymerase chain reaction (PCR) are as follows: D10S1765-forward, 5′-CAATGGAACCAAATGTG GTC; D10S1765-reverse, 5′-AGTCCGATAATGC CAGGATG. The PCR reactions using genomic DNA were performed using a TAKARA GeneAmp PCR Reagent Kit (Takara, Tokyo, Japan) and run on the Perkin-Elmer GeneAmp PCR system 9700 (Norwalk, CT, USA). The thermal conditions of the system were as follows: one cycle at 95°C for 4 min; 35 cycles at 95°C for 0.5 min, 55°C for 0.5 min, 72°C for 0.5 min; and one cycle at 72°C for 10 min. The DNA derived from cancer tissues was amplified with 6-carboxy-x-rhodamine (ROX)-labeled 5′ primer and cold 3′ primer, whereas DNA from normal tissues was amplified with 6-carboxy-24,44,74,4,7-hexachloro-fluorescein (HEX)-labeled 5′ primer and cold 3′ primer. The PCR reactions and running conditions with the Perkin-Elmer Genetic Analyzer 310 were as described previously.[23,24]

Statistical methods

Clinicopathological data were stored in an IBM 3090 mainframe computer (IBM, Armonk, NY, USA). Biomedical computer programs (BMDP; Statistical Solutions, Saugus, MA, USA) were used for all statistical analyses. The BMDP programs P4F and P3S were used for Chi-squared and Mann–Whitney *U*-tests to compare characteristics among groups. The BMDP program P1L was used for Kaplan–Meier analysis of survival rates and the Mantel-Cox test was used to test the equality of survival curves. The median follow-up period was 559 days. The BMDP program P2L was used for simultaneous multivariate adjustments of all covariates in Cox regression analysis. The level of statistical significance was set at $P < 0.05$.

RESULTS

One hundred and nineteen cases were analyzed in the present study and data were processed using GeneScan software (ABI; Applied Biosystems, Foster City, CA, USA). However, in 43 cases, the lengths of the microsatellite sequences of the paternal and maternal alleles were almost the same and, hence, it was difficult to discriminate between these alleles. These 43 cases were excluded from the diagnoses because the analysis of LOH, which was assumed for the declination of one allele, was theoretically impossible (Fig. 1b). Thus, LOH of PTEN was observed in 17.1% of the remaining 76 cases (13/76), where any case in whom the peak value of one gene locus diminished more than 30% in the carcinoma was judged as having LOH (Fig. 1a). However, clinicopathological differences due to the presence of LOH of PTEN were not evident (Table 1).

In the present study, we also investigated the relationship between LOH of PTEN and a patient's survival. The Kaplan–Meier overall survival curve (Fig. 2) demonstrates that patients in whom PTEN showed LOH had a significantly lower survival rate than patients whose tumors had biallelic PTEN (retention of heterozygosity (ROH)). After 5 years, only 22.0% of patients were alive in the LOH group; however, 50.9% patients were alive in the ROH group.

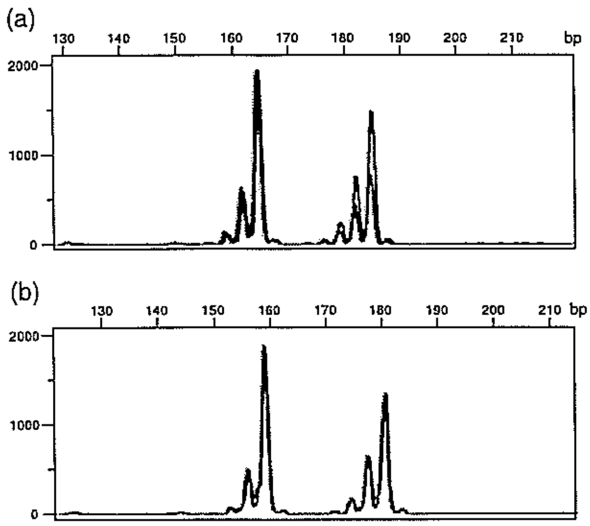

Figure 1 Genomic DNA was extracted from each tumor specimen and from each corresponding normal tissue. Each pair of DNA samples was subjected to loss of heterozygosity (LOH) analysis. Electrophoretic profiles judged to be (a) LOH and (b) ROH (retention of heterozygosity) are shown. The green line represents normal tissues, whereas the red lines represent cancer tissue.

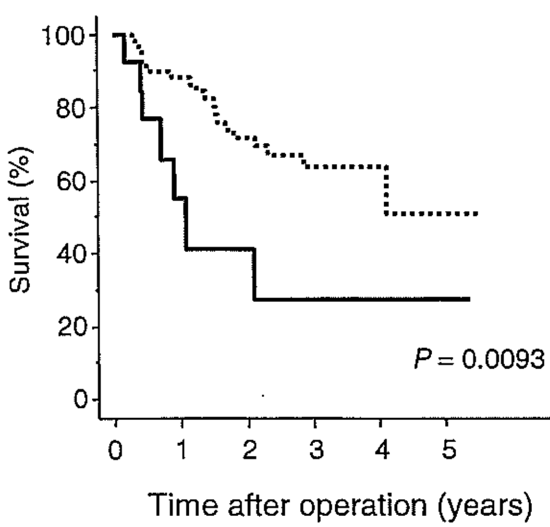

Figure 2 Kaplan–Meier overall survival curves. The solid line is for patients who had loss of heterozygosity (LOH) of encoding phosphate and tensin homolog (PTEN). The dotted line is the survival curve of patients whose tumors had biallelic PTEN.

To search for an independent prognostic factor for gastric cancer, we conducted multivariate Cox's regression analysis. The factors examined included gender, age, tumor size, macroscopic appearance, histological type, depth of invasion, lymphatic or vessel invasion, lymph node metastasis, liver metastasis, peritoneal dissemination and LOH of PTEN. As shown in Table 2, vessel invasion, depth of invasion, LOH of PTEN, histological difference and lymph node metastasis were proven to be independent covariates.

Table 1 Clinicopathological features of gastric cancers and loss of heterozygosity of encoding phosphate and tensin homolog

Variable	PTEN LOH ($n = 13$)	PTEN ROH ($n = 63$)	P
Gender			
No. males	11	49	NS
No. females	2	14	
Age (years)	66.8	62.5	NS
Histology			
Intestinal (n)	4	28	NS
Diffused (n)	8	35	
Serosal invasion			
Negative (n)	5	28	NS
Positive (n)	8	35	
Histological lymph node metastasis			
Negative (n)	4	25	NS
Positive (n)	9	38	
Vascular involvement			
Negative (n)	5	32	NS
Positive (n)	8	31	
Peritoneal dissemination			
Negative (n)	12	61	NS
Positive (n)	1	2	
Liver metastasis			
Negative (n)	11	60	NS
Positive (n)	2	3	
Stage			
I + II (n)	4	29	NS
III + IV (n)	9	34	

PTEN, encoding phosphate and tensin homolog; LOH, loss of heterozygosity; ROH, retention of heterozygosity; NS, not significant.

DISCUSSION

In gastric carcinoma, deletion or downregulation of tumor suppressor genes play important roles in the development of tumors. The role of PTEN, identified as a novel tumor suppressor gene, has been investigated in a variety of cancers. Loss of heterozygosity and mutation of PTEN have been reported in melanoma, glioblastoma, renal cancer, lung cancer and breast cancer.[1,2,6] It has been reported that PTEN mutations re rarely observed in gastric cancers, but diminished protein expression or LOH is often detected.[19–22]

In the present study, LOH of PTEN was investigated in 119 gastric cancer patients using the high-resolution microsatellite analysis method. Our method to determine LOH is based on high-resolution fluorescent microsatellite analysis developed previously.[23] In this system, electrophoretic profiles of PCR-amplified microsatellite sequences are markedly improved and

Table 2 Cox regression analysis of data of patients with gastric cancer

Explanatory variables (observed value)	*P*	Relative risk (95% CI)
Vascular invasion (negative, positive)	0.0004	9.93824 (2.3380, 39.7374)
Serosal invasion (negative, positive)	0.0004	6.2879 (2.1446, 18.4349)
LOH of PTEN (ROH, LOH)	0.0008	8.065 (0.0377, 0.4076)
Histology (intestinal, diffuse)	0.0059	4.458 (0.0788, 0.6384)
Lymph node metastasis (negative, positive)	0.0077	7.7977 (1.0121, 60.0713)

PTEN, encoding phosphate and tensin homolog; LOH, loss of heterozygosity; ROH, retention of heterozygosity; CI, confidence interval.

optimized for judgment of LOH and microsatellite instability.[23] However, 43 cases were excluded from analysis in the present study because the sequence lengths of their paternal and maternal microsatellite alleles were completely matched and could not be separated from the band pattern. Therefore, the rate of false positives is very low and, consequently, accurate positive rates are provided. In the present study, LOH of PTEN was found in 17.6% of the 76 patients analyzed. This percentage is not high when compared with results of previous reports.[19,21,22]

Consequently, there were no specific differences in the clinicopathological factors with the LOH of PTEN. However, there was a significant correlation between a patient's prognosis and LOH of PTEN. On multivariate analysis, LOH of PTEN was proved to be an independent covariate of patient survival. It is still not clear why the PTEN gene mutation is rarely observed in gastric cancer and why LOH is so frequently detected. One possible explanation is that PTEN may be a haploinsufficient tumor suppressor gene in gastric cancer, similar to mouse lymphoma, in which PTEN has been proven to be a haploinsufficient tumor suppressor.[25] It is known that PTEN is important not only as a tumor suppressor, but also as a signal transduction molecule. It is also possible that the dephosphorylattion of PI3-K becomes difficult when PTEN is aberrant. Therefore, AKT located downstream of PI3K is continuously phosphorylated and AKT conveys a resistant signal for apoptosis. As a result, a cell becomes resistant to various treatments, such as chemotherapy. In Japan, many patients with gastric cancers at more advanced stages than Stage II receive adjuvant chemotherapy postoperatively. It is speculated that the presence of LOH of PTEN reflects the chemotherapeutic effect. In those cases, a deficiency of PTEN is connected with significant hyposensitivity to postoperative treatment rather than gastric carcinogenesis. In brief, because patients with LOH of PTEN are hyposensitive to anticancer drugs, careful follow up is necessary in the clinical situation. However, in our analysis, no significant findings were obtained when the prognoses were compared between cases with postoperative chemotherapeutic treatment and cases with operation only (data not shown).

Previous reports indicated that mutations of PTEN were found in breast and prostate cancers more frequently than in gastrointestinal cancer. Poor prognoses and resistance to chemotherapy have also been reported.[26–29] For gastric cancers, there have been reports published on the correlation between the diminished protein expression of PTEN and lymph node metastasis or tumor infiltration. Yang *et al.* reported that low expression of PTEN protein was related to clinicopathological stage and the metastasis of gastric cancers.[20] It has also been reported that the LOH rate was significantly higher in advanced tumors than in early stage tumors and that LOH occurs more frequently in poorly differentiated tumors than in well- or moderately differentiated tumors. Byun *et al.* reported that 47% of gastric cancer patients had LOH of PTEN.[19] However, no report has clarified relationship between LOH of PTEN and the outcome of gastric cancer. The results of the present study indicate the significant importance of PTEN for tumor development and the treatment of gastric cancers. Further extensive studies on PTEN in gastric cancer patients are necessary.

In conclusion, LOH of PTEN occurs in 17.1% of gastric cancers and is correlated with disease-related deaths. According to the findings of the present study, LOH of PTEN is an independent prognostic factor and PTEN is a candidate haploinsufficient tumor suppressor in gastric cancers. The present study has shed light on the significant importance of PTEN for the treatment of gastric cancers in addition to *p53*.

ACKNOWLEDGMENTS

The authors are grateful to J Tuchihashi, K Miyamato, N Nakamura, Y Kubota, Y Matsumoto and T Shishino for their technical assistance. This study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1 Steck PA, Pershouse MA, Jasser SA *et al.* Identification of a candidate tumour suppressor gene, *MMAC1*, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* 1997; 15: 356–62.

2 Li J, Yen C, Liaw D *et al.* PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997; 275: 1943–7.

3 Tashiro H, Blazes MS, Wu R *et al.* Mutations in PTEN are frequent in endometrial carcinoma but rare in other

common gynecological malignancies. *Cancer Res.* 1997; 57: 3935–40.
4 Guldberg P, thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J. Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma. *Cancer Res.* 1997; 57: 3660–3.
5 Whang YE, Wu X, Suzuki H *et al.* Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc. Natl Acad. Sci. USA* 1998; 95: 5246–50.
6 Wang SI, Puc J, Li J *et al.* Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Res.* 1997; 57: 4183–6.
7 Teng DH, Hu R, Lin H *et al.* MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. *Cancer Res.* 1997; 57: 5221–5.
8 Maxwell GL, Risinger JI, Gumbs C *et al.* Mutation of the PTEN tumor suppressor gene in endometrial hyperplasias. *Cancer Res.* 1998; 58: 2500–3.
9 Dahia PL, Marsh DJ, Zheng Z *et al.* Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors. *Cancer Res.* 1997; 57: 4710–13.
10 Marsh DJ, Dahia PL, Zheng Z *et al.* Germline mutations in PTEN are present in Bannayan–Zonana syndrome. *Nat. Genet.* 1997; 16: 333–4.
11 Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 1998; 273: 13 375–8.
12 Furnari FB, Lin H, Huang HS, Cavenee WK. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc. Natl Acad. Sci. USA* 1997; 94: 12 479–84.
13 Han SY, Kato H, Kato S *et al.* Functional evaluation of PTEN missense mutations using *in vitro* phosphoinositide phosphatase assay. *Cancer Res.* 2000; 60: 3147–51.
14 Ramaswamy S, Nakamura N, Vazquez F *et al.* Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc. Natl Acad. Sci. USA* 1999; 96: 2110–15.
15 Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994; 265: 1582–4.
16 Gu J, Tamura M, Yamada KM. Tumor suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathways. *J. Cell Biol.* 1998; 143: 1375–83.
17 Maehara Y, Kakeji Y, Oda S, Baba H, Sugimachi K. Tumor growth patterns and biological characteristics of early gastric carcinoma. *Oncology* 2001; 61: 102–12.
18 Fenoglio-Preiser CM, Wang J, Stemmermann GN, Noffsinger A. TP53 and gastric carcinoma: A review. *Hum. Mutat.* 2003; 21: 258–70.
19 Byun DS, Cho K, Ryu BK *et al.* Frequent monoallelic deletion of PTEN and its reciprocal associatioin with PIK3CA amplification in gastric carcinoma. *Int. J. Cancer* 2003; 104: 318–27.
20 Yang L, Kuang LG, Zheng HC *et al.* PTEN encoding product: A marker for tumorigenesis and progression of gastric carcinoma. *World J. Gastroenterol.* 2003; 9: 35–9.
21 Sato K, Tamura G, Tsuchiya T *et al.* Analysis of genetic and epigenetic alterations of the PTEN gene in gastric cancer. *Virchows Arch.* 2002; 440: 160–5.
22 Kang YH, Lee HS, Kim WH. Promoter methylation and silencing of PTEN in gastric carcinoma. *Lab. Invest.* 2002; 82: 285–91.
23 Oda S, Oki E, Maehara Y, Sugimachi K. Precise assessment of microsatellite instability using high resolution fluorescent microsatellite analysis. *Nucleic Acids Res.* 1997; 25: 3415–20.
24 Maehara Y, Oda S, Sugimachi K. The instability within: Problems in current analyses of microsatellite instability. *Mutat. Res.* 2001; 461: 249–63.
25 Mao JH, Wu D, Perez-Losada J, De Nagase HI, Rosario R, Balmain A. Genetic interactions between Pten and p53 in radiation-induced lymphoma development. *Oncogene* 2003; 22: 8379–85.
26 Sun H, Enomoto T, Fujita M *et al.* Mutational analysis of the PTEN gene in endometrial carcinoma and hyperplasia. *Am. J. Clin. Pathol.* 2001; 115: 32–8.
27 Kanamori Y, Kigawa J, Itamochi H *et al.* PTEN expression is associated with prognosis for patients with advanced endometrial carcinoma undergoing postoperative chemotherapy. *Int. J. Cancer* 2002; 100: 686–9.
28 Depowski PL, Rosenthal SI, Ross JS. Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. *Mod. Pathol.* 2001; 14: 672–6.
29 Lee JI, Soria JC, Hassan KA *et al.* Loss of PTEN expression as a prognostic marker for tongue cancer. *Arch. Otolaryngol. Head Neck Surg.* 2001; 127: 1441–5.

World Journal of Surgical Oncology

BioMed Central

Research

Open Access

Relationship of hypoxia-inducible factor 1α and p21$^{WAF1/CIP1}$ expression to cell apoptosis and clinical outcome in patients with gastric cancer

Ken Mizokami, Yoshihiro Kakeji*, Shinya Oda and Yoshihiko Maehara

Address: Department of Surgery and Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan

Email: Ken Mizokami - mizoken@xc4.so-net.ne.jp; Yoshihiro Kakeji* - kakeji@surg2.med.kyushu-u.ac.jp; Shinya Oda - soda@nk-cc.go.jp; Yoshihiko Maehara - maehara@surg2.med.kyushu-u.ac.jp

* Corresponding author

Published: 13 December 2006

World Journal of Surgical Oncology 2006, 4:94 doi:10.1186/1477-7819-4-94

This article is available from: http://www.wjso.com/content/4/1/94

Received: 09 September 2006

Accepted: 13 December 2006

Abstract

Background: Hypoxia-inducible factor-1α (HIF-1α) plays an essential role in oxygen homeostasis. The expression of HIF-1α-inducible genes is associated with tumor progression. p21 mediates cell cycle arrest and is one of the downstream genes targeted by HIF-1.

Patients and methods: We examined the relationship between HIF-1α and p21 expression, apoptosis and tumor progression using tissue specimens obtained surgically from 126 patients with gastric cancer.

Results: Immunohistochemical analysis indicated that loss of p21 expression correlated positively with patient age and tumor size. Lymph node metastasis was significantly more frequent in tumors with loss of p21 expression ($P = 0.022$). HIF-1α-positive/p21-negative tumors had a lower apoptotic index than any other tumor samples, and patients with HIF-1α-positive/p21-negative tumors also had a significantly poorer prognosis than the other patient populations.

Conclusion: These results suggest that loss of HIF-1α-dependent p21 expression results in decreased apoptosis, increased cell survival and more aggressive tumors.

Background

The unregulated growth of cancer cells often results in hypoxic conditions in tumor cell masses. Tumor hypoxia results from an imbalance between elevated consumption of oxygen in the rapidly cycling tumor cells and insufficient oxygen supply due to the lack of a physiological vascular network. Multicellular organisms have evolved cellular mechanisms that mediate a cascade of adaptive molecular responses to hypoxia. HIF-1α is a transcription factor that activates gene expression by binding to the hypoxia responsive element (HRE), a cis-acting DNA sequence present upstream of several genes essential for the cellular response to hypoxia [1]. HIF-1α-responsive genes also function in the glycolysis pathway and in hematopoiesis and angiogenesis, through all of which cells acquire an hypoxia-adapted metabolism and increased oxygen supply [2]. Recently, HIF-1α has emerged as a key regulator in the growth of gastric cancer [3].

Apoptosis is an evolutionarily conserved cell death mechanism that also occurs in the adaptive cellular response to hypoxic stress. Apoptosis, too, is an important safeguard against tumor development. Tumors that exhibit loss of the p53 tumor-suppressor gene exhibit reduced levels of hypoxia-induced cell death and an associated increase in tumor progression [4]. The p21 gene (WAF1) was cloned in a genetic screen for downstream effectors of p53 and separately in a screen for upstream regulators of cyclin-dependent kinases (CDKs) as CDK-interacting protein (CIP1) [5]. The p21 promoter can be transactivated by HIF-1 in a human prostate cancer cell line, indicating that p21 is an HIF-1 target gene [6]. Furthermore, hypoxia-induced p21 expression was abrogated in cells lacking HIF-1α, but not in parental cells [7].

HIF-1α may therefore promote both cell survival and growth arrest through the induction of hypoxia-responsive genes. In the present study, we examined the role of HIF-1α in hypoxic control of tumor progression, by examining the relationship between HIF-1α expression, p21 expression and apoptosis in tissue specimens from patients with gastric cancer.

Materials and methods

Clinical materials

Subjects were 126 patients with gastric cancer (85 men, 41 women; age range, 27 to 88 years; mean age, 65.2 years) who underwent gastrectomy at our institution in 1994. Curative resection was performed in 77 patients and non-curative resection in 49. Resected tissue specimens were fixed in a 10% formaldehyde solution and embedded in paraffin. Sections (4 μm thickness) were mounted on glass slides. All samples were examined macroscopically and histologically, based on criteria proposed by the General Rules for the Gastric Cancer Study [8]. Histological examination was carried out on tissue preparations stained with hematoxylin and eosin (H&E). In the current study, tumors were divided into two histological types: differentiated type, comprising papillary adenocarcinoma and tubular adenocarcinoma, and undifferentiated type, comprising poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma. Two paraffin blocks were prepared for all patients" one containing both tumor tissue and adjacent normal tissue, and the other containing tumor tissue invading to the deepest level of the stomach wall.

Immunohistochemical staining

All specimens were immunostained with a monoclonal antibody against $p21^{WAF1/CIP1}$ (SX118, diluted 1:50, DAKO, Glostrup, Denmark), p53 (Do-7, diluted 1:50, DAKO, Glostrup, Denmark), and HIF-1α (NB 100–105, diluted 1:100, Novus Biologicals, Littleton, CO, USA)[9]. After deparaffinization and rehydration, the slides for p21 and p53 immunostaining were autoclaved in citrate buffer (0.01 M, pH 6.0) at 120°C for 10 minutes; 0.001 M EDTA (pH 8.0) was used for HIF-1αimmunostaining to facilitate reactivity of the fixed embedded tissue antigen with the antibody. Endogenous peroxidase was blocked by incubating the samples in methanol containing 0.3% hydrogen peroxide for 10 minutes. Samples were then rinsed in phosphate-buffered saline (PBS) and incubated with normal rabbit serum for 30 minutes. Sections were incubated with the aforementioned primary antibodies for 2 hours at room temperature, then rinsed three times in PBS. For detection, we used a Histofine SAB-PO (M) kit (Nichirei Corp., Tokyo, Japan). The sections were then incubated with biotinylated rabbit anti-mouse immunoglobin (Ig; IgG, IgA, and IgM; Nicchirei Corp) for 10 minutes, washed three times in PBS and treated with peroxidase-conjugated streptavidin for 10 minutes. After a final washing in PBS, the peroxidase label was detected by incubating the sections in diamino-benzidine tetrahydrochloride (DAB) for 3 minutes. Nuclear counter-staining was done using Mayer's hematoxylin solution. For negative controls, primary antibodies were replaced with non-immune, normal serum. Automated immunohistochemistry was also carried out to support the immunostaining described above, using a Ventana Discovery™ System (Ventana Medical Systems, Inc., Tucson, AZ, USA).

Evaluation of immunostaining

p21 protein was present in the nuclei of tumor cells (Figure 1A). In some cases, normal gastric mucosa expressed p21 protein, and nuclear staining could be detected in the superficial reaches of the tumor, but not in the deeper regions. We counted the number of p21-positive cells in the whole tumor section, and evaluated the number of p21-postive cells according to the depth of layers. The pattern of HIF-1α immunostaining in the tumor was nuclear and/or cytoplasmic (Figure 1B). Nuclear staining of HIF-1α was absent in normal tissue excluding cytoplasmic staining. In all samples, p53 protein was undetectable in normal tissue, and present in the nuclei of tumor cells (Figure 1C). A cell with nuclear immunostaining for p21, p53 or HIF-1α (weak or strong) was scored as positive. Based on these criteria, areas of focal staining with the highest percentage of p53- and p21-positive nuclear staining within deep tumor tissue were estimated. A tumor was scored as p21- or p53-positive when more than 10% of the tumor cells had nuclear staining, in keeping with previous reports [10]. HIF-1α expression was frequently evident in regions around the invading edges of the tumor, and necrotic areas such as those close to a deep ulcer. We scored tumors as positive for HIF-1α overexpression if nuclear staining was detected in more than 10% of the tumor cells, irrespective of cytoplasmic reactivity at any level [9,11].

Evaluation for apoptosis

We scored apoptotic cells as those showing the typical characteristics of apoptosis when tissue specimens were stained with H&E (Figure 1D). Apoptotic cells were iden-

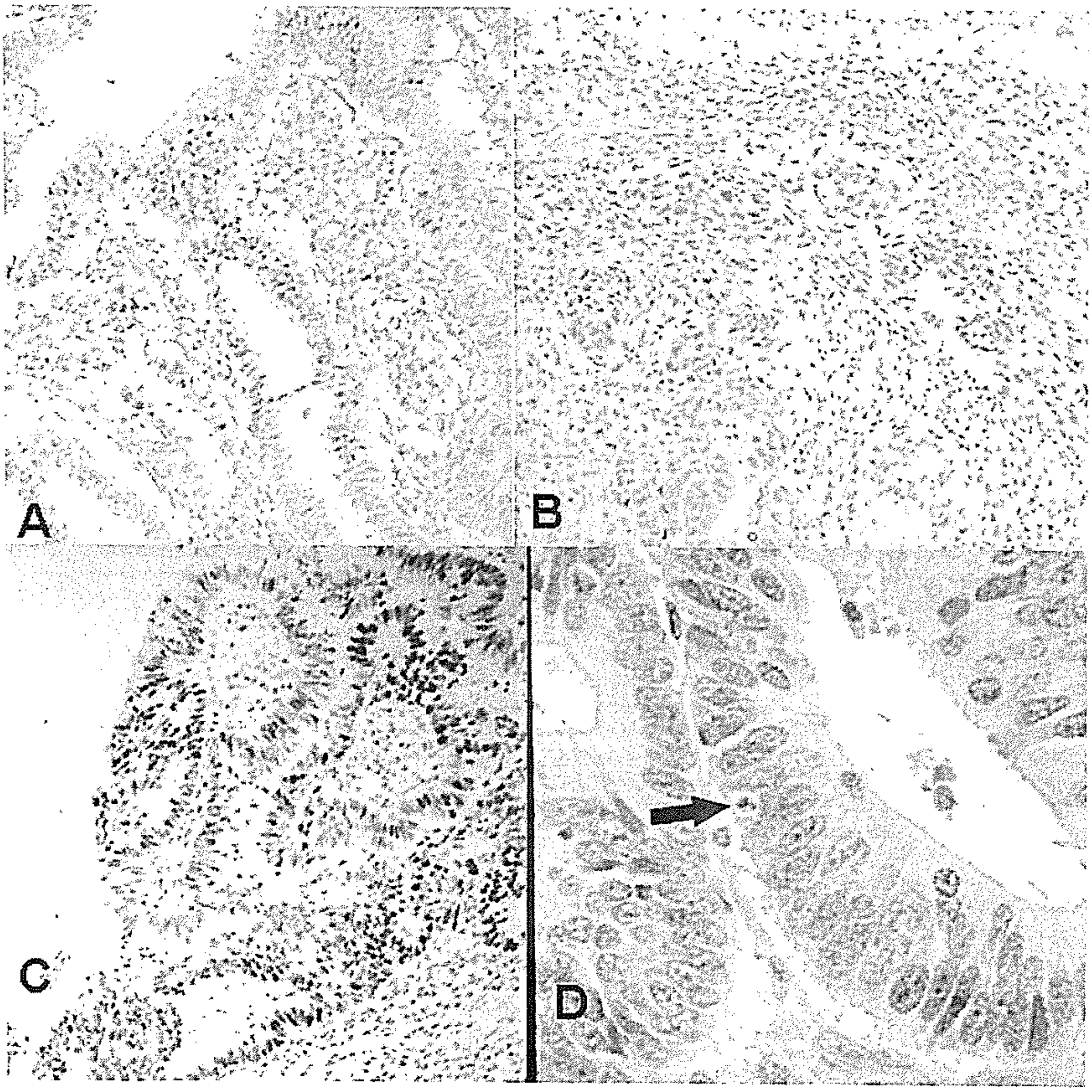

Figure 1
A) p21 expression in adjacent tumor tissue. Nuclear immunostaining is evident (original magnification, 100×). B) HIF-1α expression in invading regions of tumor. Variability in intensity of nuclear immunostaining is evident, accompanied by cytoplasmic staining (original magnification, 100×). C) Nuclear immunostaining of p53 in tumor tissue (original magnification, 100×). D) Tumor tissue stained with H&E (original magnification, 200×). Tumor tissue contains cells (arrow) with characteristic features of apoptosis.

tified based on the characteristic features of apoptosis: compaction and migration of nuclear and cell outlines, nuclear fragmentation and protuberance, and the presence of apoptotic bodies [12]. Apoptotic cells were counted in tissue specimens from all patients. Five high-power fields (×400) with the most abundant distribution of tumor cells were selected for counts and between 1000 and 1500 tumor cells were counted. The apoptotic index was then calculated as the percentage of apoptotic cells. Areas with extensive necrosis were avoided. A single pathologist at each institution reviewed the slides and counted apoptotic cells according to the criteria described above.

Statistical analysis

The BMDP Statistical Package program (BMDP, Los Angeles, CA) for IBM (Armonk, NY) 3090 mainframe computers were used for all statistical analyses [13]. Data sets were compared by chi-square and Student's t-tests using the BMDP 4F and 3S programs. The BMDP 1L program was used to analyze survival time using the Kaplan-Meier method. Statistical significance was set at the $P < 0.05$ level.

Results

p21 expression and clinico-pathologic factors

Table 1 shows the correlation of the expression or loss of expression of p21 with several clinico-pathologic factors. Overall, 71 (56.3%) of the 126 tumor specimens were negative for p21 expression. In tumors from patients under 65 years of age, loss of p21 expression was significantly more frequent than in tumors from patients over the age of 65 ($P = 0.026$). Loss of p21 expression was significantly more frequent in tumors with large size ($P = 0.03$), as compared to smaller tumors. With regard to metastases, tumors with loss of p21 expression tended to show increased frequency of lymphatic invasion ($P = 0.089$, Table 1) and a significantly higher frequency of lymph node metastasis ($P = 0.022$, Table 2) than p21-expressing tumors. Table 3 shows the relationship between p21 expression, and the expression of p53 and HIF-1α. Overexpression of p53 was detected in 57 (45.2%) tumors, and HIF-1α overexpression was detected in 49 (38.9%) tumors. Immunohistochemical analysis did not reveal a correlation between p21 expression and p53 expression, or p21 and HIF-1α overexpression ($P = 0.444$ and 0.609, respectively).

Apoptosis associated with HIF-1α and p21 expression

The mean apoptotic index of all 126 tumors was 8.95 ± 6.24 (range 0–37). Tumors were divided into four different populations based on HIF-1α and p21 expression and the mean apoptotic index for each group was calculated (Table 4). Tumors that were HIF-1α-positive/p21-negative had the lowest apoptotic index. There was a significant difference between tumors that were HIF-1α-positive/p21-negative, and those that were HIF-1α-negative/p21-negative ($P = 0.037$).

Clinical outcome associated with HIF-1α and p21 expression

The mean follow-up time for patients was 55 ± 28 (± 1 S.D.) months (range, 1–82 months). The 5-year survival rate of patients with p21-negative tumors was lower than that of p21-positive tumors, but the difference was not statistically significant (data not shown). The 126 patients were again divided into four populations based on HIF-1α expression and p21 expression, and we examined the relationship between HIF-1α expression and prognosis in p21-positive or -negative tumor samples. Table 5 shows the 1-, 3- and 5-year survival rates for patients and the correlation with HIF-1α and p21 expression. Patients with HIF-1α-positive/p21-negative tumors had a significantly poorer prognosis than the other study populations. In particular, in patients with HIF-1α-positive tumors, those who had lost expression of p21 had a significantly poorer prognosis than those with p21 expression ($P = 0.042$).

Discussion

Our findings show that loss of p21 expression correlated positively with younger patient age, and larger tumor. Moreover, many patients with p21-negative tumors had lymph node metastasis when compared to those with p21-positive tumors, at a significantly higher frequency. These results suggest that the loss of expression of p21 is involved in the processes of tumor growth and metastasis, in agreement with previously reports [14,15].

HIF-1α overexpression has been linked to a poor clinical outcome in some types of human cancers [16-18]. However, some reports have suggested that tumor expression of HIF-1α does not confer a survival advantage [18,19]. Although most of the HIF-1 target genes can promote tumor growth through their enhanced expression, HIF-1-activated genes, including p21, also have the potential to inhibit growth under hypoxic conditions [20,21]. Ectopic expression of HIF-1α in endothelial cells resulted in up-regulation of p21, reduction of CDK activities, cell cycle arrest at the G_0/G_1 check point, and subsequent apoptosis [22]. In the current study, we found that apoptotic cells were under-represented in HIF-1α-positive/p21-negative tumors. Under hypoxic conditions, HIF-1α may inhibit tumor proliferation through p21-mediated cell cycle control, resulting in the selection of cells that are resistant to apoptosis and anti-cancer treatments. Most tumor cells retain the ability to undergo apoptosis in response to hypoxic stress [23]. When the apoptotic response to hypoxia is lost, emerging tumor cells may be more resistant to treatment and may therefore contribute to subsequent tumor relapse [24]. The mechanisms by which hypoxia selects for cells resistant to apoptosis is unclear, but the involvement of the p53 mutation has been examined [25]. Reports have shown that hypoxia inhibits cell growth, and may cause apoptosis through a p53-dependent pathway [26]. HIF-1α has also been shown to promote p53-dependent apoptosis [27], but other studies have shown that growth arrest in response to hypoxia is p53-independent [26]. In the current study, we found no

Table 1: Relationship between p21 expression and clinico-pathologic factors

Factors	p21(+) (n = 55)	p21(-) (n = 71)	P value
Age (years)			
<65	20	40	**0.026**
65≦	35	31	
Gender			
Male	38	47	0.731
Female	17	24	
Depth of invasion			
t1	33	35	0.231
t2,3,4	22	36	
Histology			
Differentiated	35	39	0.325
Undifferentiated	20	32	
Tumor size			
<3 cm	23	22	**0.03**
3 cm≦	22	49	
Lymphatic invasion			
Negative	26	23	0.089
Positive	29	48	
Venous invasion			
Negative	42	57	0.595
Positive	13	14	

evidence of a relationship between p53 and p21 expression. We also evaluated the relationship between HIF-1α and p53 expression to cell apoptosis, but found no statistical significance between HIF-1α and p53 expression (data not shown). Our previous study showed that the combination of HIF-1α overexpression with nonfunctional p53 tended to indicate a dismal prognosis [28].

In patients with HIF-1α-positive tumors, the correlation between loss of p21 expression and poor clinical outcome may reflect a physiological difference in the ability of p21-positive versus p21-negative tumors to survive under hypoxic conditions. Although HIF-1α-dependent transcriptional activation has been associated with tumor growth, our results suggest that concomitant expression of p21 and HIF-1α may retard tumor growth to some degree.

The molecular mechanism underlying HIF-1α expression in cancer warrants particular attention [29]. The widespread occurrence of upregulated HIF-1α in common cancers and the involvement of hypoxia pathways in tumor angiogenesis certainly argue for its importance and wide applicability. Chemotherapy and radiation that target HIF-1α may be effective and realistic, and in fact, this approach has been reported [30]. However, the qualitative and quantitative differences in the hypoxic response of different cell types are not well known [31]. Further research is therefore required in order to evaluate the effects of HIF-1-mediated pathways on cell proliferation and apoptosis in human cancers under hypoxic microenvironments.

In the present study, we showed that HIF-1α overexpression and loss of p21expression in gastric cancers correlated with poor patient prognosis, compared to tumors

Table 2: Relationship between p21 expression and metastasis

	p21-negative tumor (%)	P value
Lymph node metastasis		
Negative	36/75(48.0)	**0.022**
Positive	35/51(68.6)	
Liver metastasis		
Negative	68/122(55.7)	0.447
Positive	3/4(75.0)	
Peritoneal dissemination		
Negative	62/112(55.4)	0.525
Positive	9/14(64.3)	

Table 3: Relationship between p21 and p53, and HIF-1α expression

		p21		
		Positive (n = 55)	Negative (n = 71)	P value
p53	Positive (n = 57)	27	30	0.444
	Negative(n = 69)	28	41	
HIF-1α	Positive (n = 49)	20	29	0.608
	Negative(n = 77)	35	42	

Table 4: Apoptosis associated with HIF-1α and p21 expression

Factor	Apoptotic index
HIF-1α (-), p21(+) n = 35	9.6 ± 7.33***
HIF-1α (-), p21(-) n = 42	9.83 ± 6.96**,***
HIF-1α (+), p21(+) n = 20	8.85 ± 4.04*,**,***
HIF-1α (+), p21(-) n = 29	6.97 ± 4.22*,**,***

* P = 0.129 ** P = 0.037 *** P = 0.078 (mean ± S.D.)

Table 5: Year survival rate associated with HIF-1α and p21 expression

Factor	1-year survival	3-year survival	5-year survival
HIF-1αg(-), p21(+) n = 35	94.1	82.0	82.0***
HIF-1α (-), p21(-) n = 42	92.7	80.0	80.0**,***
HIF-1α (+), p21(+) n = 20	95.0	85.0	70.0*,**,***
HIF-1α (+), p21(-) n = 29	68.1	57.4	45.9*,**,***

* P = 0.042 ** P = 0.003 *** P = 0.003 (%)

that retained p21 expression, or had lost HIF-1α expression. A potential mechanism for this was suggested by the finding that apoptotic cells were under-represented in HIF-1α-positive/p21-negative tumors. Aggressive tumors that fail to induce p21 in an HIF-1α – dependent manner may have increased cell survival without apoptosis, and contribute to a poor prognosis for patients.

Conflict of interest

The author(s) declare that they have no competing interests.

Authors' contributions

KM carried out the immunohistochemical study and performed the statistical analysis, and drafted the manuscript.

YK conceived the study, and participated in its design and coordination and helped to draft the manuscript.

SO involved in drafting the manuscript and revising it critically for important intellectual content.

YM gave final approval of the version to be published.

All authors read and approved the final manuscript.

Funding support

Department of Surgery and Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan.

Acknowledgements

The authors thank K. Miyamoto for technical assistance.

References

1. Semenza GL: **Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1.** *Annu Rev Cell Dev Biol* 1999, **15**:551-578.
2. Semenza GL: **HIF-1 and human disease: one highly involved factor.** *Genes Dev* 2000, **14**:1983-1991.
3. Griffiths EA, Pritchard SA, Welch IM, Price PM, West CM: **Is the hypoxia-inducible factor pathway important in gastric cancer?** *Eur J Cancer* 2005, **41**:2792-2805.
4. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ: **Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours.** *Nature* 1996, **379**:88-91.
5. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B: **WAF1, a potential mediator of p53 tumor suppression.** *Cell* 1993, **75**:817-825.

6. Salnikow K, Costa M, Figg WD, Blagosklonny MV: **Hyperinducibility of Hypoxia-responsive Genes without p53/p21-dependent Checkpoint in Aggressive Prostate Cancer.** *Cancer Res* 2000, **60**:5630-7113.
7. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E, Keshet E: **Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis.** *Nature* 1998, **394**:485-490.
8. Japanese Research Society for Gastric Cancer. **Japanese classification of gastric carcinoma.** *Tokyo: Kanehara and Co, Ltd* 1995, **12**:1995.
9. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW: **Overexpression of Hypoxia-inducible Factor 1{{alpha}} in Common Human Cancers and Their Metastases.** *Cancer Res* 1999, **59**:5830-7113.
10. Noda H, Maehara Y, Irie K, Kakeji Y, Yonemura T, Sugimachi K: **Growth pattern and expressions of cell cycle regulator proteins p53 and p21WAF1/CIP1 in early gastric carcinoma.** *Cancer* 2001, **92**:1828-1835.
11. Birner P, Schindl M, Obermair A, Breitenecker G, Oberhuber G: **Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy.** *Clin Cancer Res* 2001, **7**:1661-1668.
12. Kerr JF, Winterford CM, Harmon BV: **Apoptosis. Its significance in cancer and cancer therapy.** *Cancer* 1994, **73**:2013-2026.
13. Dixon WJ : **BMDP statistical software.** *Berkeley, CA: University of California Press* 1988.
14. Ogawa M, Maeda K, Onoda N, Chung YS, Sowa M: **Loss of p21WAF1/CIP1 expression correlates with disease progression in gastric carcinoma.** *Br J Cancer* 1997, **75**:1617-1620.
15. Noda H, Maehara Y, Irie K, Kakeji Y, Yonemura T, Sugimachi K: **Increased proliferative activity caused by loss of p21(WAF1/CIP1) expression and its clinical significance in patients with early-stage gastric carcinoma.** *Cancer* 2002, **94**:2107-2112.
16. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G: **Overexpression of Hypoxia-inducible Factor 1{{alpha}} Is a Marker for an Unfavorable Prognosis in Early-Stage Invasive Cervical Cancer.** *Cancer Res* 2000, **60**:4693-7113.
17. Birner P, Gatterbauer B, Oberhuber G, Schindl M, Rossler K, Prodinger A, Budka H, Hainfellner JA: **Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis.** *Cancer* 2001, **92**:165-171.
18. Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, Millard P, Fuggle S, Harris AL: **Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients.** *Cancer Res* 2002, **62**:2493-2497.
19. Yu JL, Rak JW, Carmeliet P, Nagy A, Kerbel RS, Coomber BL: **Heterogeneous vascular dependence of tumor cell populations.** *Am J Pathol* 2001, **158**:1325-1334.
20. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL: **HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors.** *Cancer Res* 2001, **61**:6669-6673.
21. Bruick RK: **Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia.** *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**:9082-9087.
22. Iida T, Mine S, Fujimoto H, Suzuki K, Minami Y, Tanaka Y: **Hypoxia-inducible factor-1alpha induces cell cycle arrest of endothelial cells.** *Genes Cells* 2002, **7**:143-149.
23. Shimizu S, Eguchi Y, Kamiike W, Itoh Y, Hasegawa J, Yamabe K, Otsuki Y, Matsuda H, Tsujimoto Y: **Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL.** *Cancer Res* 1996, **56**:2161-2166.
24. Schmaltz C, Hardenbergh PH, Wells A, Fisher DE: **Regulation of proliferation-survival decisions during tumor cell hypoxia.** *Mol Cell Biol* 1998, **18**:2845-2854.
25. Kinzler KW, Vogelstein B: **Life (and death) in a malignant tumour.** *Nature* 1996, **379**:19-20.
26. Graeber TG, Peterson JF, Tsai M, Monica K, Fornace AJ Jr., Giaccia AJ: **Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status.** *Mol Cell Biol* 1994, **14**:6264-6277.
27. Suzuki H, Tomida A, Tsuruo T: **Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia.** *Oncogene* 2001, **20**:5779-5788.
28. Sumiyoshi Y, Kakeji Y, Egashira A, Mizokami K, Orita H, Maehara Y: **Overexpression of hypoxia-inducible factor 1alpha and p53 is a marker for an unfavorable prognosis in gastric cancer.** *Clin Cancer Res* 2006, **12**:5112-5117.
29. Ratcliffe PJ, Pugh CW, Maxwell PH: **Targeting tumors through the HIF system.** *Nat Med* 2000, **6**:1315-1316.
30. Dachs GU, Patterson AV, Firth JD, Ratcliffe PJ, Townsend KM, Stratford IJ, Harris AL: **Targeting gene expression to hypoxic tumor cells.** *Nat Med* 1997, **3**:515-520.
31. Harris AL: **Hypoxia--a key regulatory factor in tumour growth.** *Nature Rev Cancer* 2002, **2**:38-47.

Journal of Surgical Oncology 2006;94:149–154

Clinicopathologic Significance of Hypoxia-Inducible Factor 1α Overexpression in Gastric Carcinomas

KEN MIZOKAMI,[1,4] YOSHIHIRO KAKEJI,[1*] SHINYA ODA,[1] KOJI IRIE,[2] TOMOHIRO YONEMURA,[3] FUMIO KONISHI,[4] AND YOSHIHIKO MAEHARA[1]

[1]*Department of Surgery and Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan*
[2]*Department of Pathology, Saga Prefectural Hospital Koseikan, Saga, Japan*
[3]*Department of Surgery, Saga Prefectural Hospital Koseikan, Saga, Japan*
[4]*Department of Surgery, Omiya Medical Center, Jichi Medical School, Saitama, Japan*

Background: Hypoxia-inducible factor 1α (HIF-1α) plays a key role in responses to hypoxia and expression of HIF-1α downstream genes leads to both an adapted metabolism and increased oxygen supply. We investigated the clinical significance of HIF-1α expression in gastric carcinoma.
Methods: We examined HIF-1α, vascular endothelial growth factor (VEGF), and insulin-like growth factor-2 (IGF-2) expression patterns immunohistochemically in 126 specimens of gastric carcinoma. CD34 antigen levels were also examined by immunohistochemistry to determine microvessel density (MVD) within tumors and correlations between HIF-1α expression, clinicopathological features, and survival were examined.
Results: HIF-1α expression correlated with tumor size ($P < 0.005$), depth of invasion ($P = 0.018$), VEGF expression ($P = 0.03$), and intra-tumor MVD ($P < 0.005$). IGF-2 expression was more prevalent in HIF-1α positive than in HIF-1α negative tumors and the 5-year survival rate was 58.4% for HIF-1α positive patients and 81.5% for HIF-1α negative patients ($P = 0.009$). HIF-1α expression is an independent prognostic factor in gastric carcinoma ($P = 0.032$).
Conclusions: Overexpression of HIF-1α in gastric carcinomas may upregulate its downstream gene products leading to VEGF-mediated angiogenesis, and resulting in a poor prognosis for patients.
J. Surg. Oncol. 2006;94:149–154. © 2006 Wiley-Liss, Inc.

KEY WORDS: **gastric carcinoma; HIF-1α; VEGF; microvessel density; IGF-2; prognosis**

INTRODUCTION

Dysregulated, unlimited growth of tumors perturbs homeostasis and creates a physiological disruption in the surrounding tissues as well as the tumor interior. Oxygen tension also often decreases dramatically in tumors, and this hypoxia is the result of an imbalance between an elevated consumption of oxygen in tumor cells and an insufficient oxygen supply due to a lack of vascular networks in the tumor tissue. The adaptation of tumor cells to hypoxia is associated with tumor progression and metastasis, as well as resistance to both chemotherapy and radiotherapy, and there is an inverse correlation between tumor oxygenation and clinical outcome [1,2]. At the cellular level, hypoxia induces expression of a group of physiologically important genes including erythropoietin, vascular endothelial growth factor (VEGF), glycolytic enzymes, and glucose transporters, many of which are upregulated in some types of human carcinomas. Each of these genes is under the control of HIF-1, a transcriptional regulator that binds to specific hypoxia-response elements (HRE). Hypoxia-inducible factor 1α (HIF-1α) is a component of the HIF-1 complex,

*Correspondence to: Dr. Yoshihiro Kakeji, Department of Surgery and Science, Graduate School of Medical Science, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Fax: 81-92-642-5951. E-mail: kakeji@surg2.med.kyushu-u.ac.jp
Received 20 February 2005; Accepted 10 February 2006
DOI 10.1002/jso.20568
Published online in Wiley InterScience (www.interscience.wiley.com).

and its accumulation in the nucleus is a rate limiting determinant of the functional activity of HIF-1 [3]. HIF-1α is both strongly induced and stabilized under hypoxic conditions, under which it can then translocate from the cytoplasm into the nucleus, and its target genes promote cell proliferation and viability, angiogenesis, and also metabolic adaptations to hypoxia [4]. HIF-1α acts as a tumor-promoting factor by inducting expression of VEGF, probably the most potent angiogenic factor known [5]. VEGF mRNA is not induced by hypoxia in HIF-1α-deficient embryonic stem (ES) cells, and dramatic vascular regression occurs in HIF-1α-null mouse embryos [6]. Furthermore, hypoxia induces expression of various growth factors that are known to promote cell proliferation. HIF-1α induces production of insulin-like growth factor-2 (IGF-2) and expression and secretion of this growth factor may lead to an acceleration of tumor growth [7–9].

In various human carcinomas HIF-1α expression has been examined immunohistochemically, and shown to correlate with both angiogenesis and a poor prognosis for patients. The aim of this study was therefore to investigate the clinical significance of HIF-1α expression in gastric carcinoma. Our data show that HIF-1α appears to be an important factor in determining the behavior and outcome of gastric carcinomas.

MATERIALS AND METHODS

Patients

This study included 126 randomly selected Japanese patients with primary gastric carcinomas, all of whom underwent a gastrectomy procedure in the Department of Surgery, Saga Prefectual Hospital Koseikan, Saga, Japan in 1994. There were 85 men and 41 women between the ages of 27 and 88 years old (mean, 65.2 years). Curative resections were performed on 77 patients and 49 underwent non-curative surgical procedures. A thorough pathological examination was undertaken using hematoxylin and eosin-stained tissue preparations, and pathological diagnosis and classification of the resected gastric carcinoma tissues was performed according to the General Rules for the Gastric Cancer Study in Surgery and Pathology in Japan [10]. Tumors were collected and fixed in 10% formalin and embedded in paraffin. Each paraffin block contained both tumor and adjacent non-tumor tissue and for each patient the tumor tissue invading to the deepest level of the stomach wall was selected for this study.

Immunohistochemistry

A streptavidin-biotin-based immunohistochemical method was used to determine HIF-1α expression. Briefly, 4-μm thick sections prepared from paraffin-embedded blocks were deparaffinized in xylene and rehydrated using a graded series of ethanol solutions. Slides were autoclaved in 1 mM EDTA (pH 8.0) at 120°C for 10 min and endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 10 min. The sections were incubated for 2 hr at room temperature with a monoclonal antibody against HIF-1α (IgG2b, clone MAb H1α67, NB 100-105, Novus Biologicals, Littleton, CO), at a dilution of 1:100 [11]. The sections were washed three times with phosphate-buffered saline (PBS) and the sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin (Histofine SAB-PO (M) kit, Ig; IgG, IgA, and IgM; Nichirei Corp.) for 10 min followed by three washes in PBS. The slides were treated with peroxidase-conjugated streptavidin for 10 min and following PBS washes, peroxidase labeling was developed by incubating the sections in diamino-benzidine tetrahydrochloride (DAB) for 3 min. Finally, Mayer hematoxylin solution was used for nuclear counter-staining. For negative controls, we omitted the primary antibody. HIF-1α protein expression in the cytoplasm and the nucleus of tumor cells was judged as positive-staining if there was nuclear staining in more than 10% of tumor cells and was accompanied by weak or strong cytoplasmic staining, as described in previous reports [12].

Expression of VEGF and IGF-2 was investigated, using the rabbit polyclonal antibody A-20 (Santa Cruz Biotechnology, Santa Cruz, CA) and the mouse monoclonal antibody W3D9-1 (American Research Product, Belmont, MA), respectively. Specimens were incubated for 2 hr with a 1:50 dilution of the primary antibody solution at room temperature and the degree of reactivity with individual tissue sections was deemed positive if unequivocal staining of the cytoplasm was seen in more than 5% of tumor cells, as previously reported.

A procedure for detecting microvessels using an anti-CD34 monoclonal antibody was also utilized (Dako, Glostrip, Denmark). Briefly, evaluation of microvessel density (MVD) was determined, using the modified technique of Weidner et al. [13]. Each slide was scanned at low magnification (40× or 100×), and the area of the most dense neovascularization (greatest number of capillaries or small venules) was determined. Individual microvessel counts were made on a 200× field (0.739 mm^2 per field) and positive cells or cell clusters, clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements, were counted as a single microvessel. Occasional immunopositive leukocytes were excluded on morphological grounds. The distribution of areas of most intense vascularization was heterogeneous for each tumor. In each of the samples the mean values for the numbers of microvessels was calculated from five highly vascularized areas "hot spots."

Fully automated staining was also performed to confirm the results obtained from the immunostaining procedures described above, using a Ventana Discovery™ System (Ventana Medical Systems, Inc., Tucson, AZ). All evaluations of immunostaining data were performed by two independent observers (K.M. and Y.K.) without knowledge of the patient's clinical status, and two pathologists reviewed the slides.

Statistical Analysis

The BMDP Statistical Package program (BMDP, Los Angeles, CA) for the IBM 3090 mainframe computer (Armonk, NY) was used for all analyses. The association of factors was evaluated using a chi-square and a Student *t*-test and differences among survival curves, based on HIF-1α expression, were examined using the generalized Wilcoxon test. Survival curves were constructed using the Kaplan–Meier method. A multivariate analysis of prognostic factors related to overall survival was conducted using the Cox proportional hazards model. A value of $P < 0.05$ was considered statistically significant.

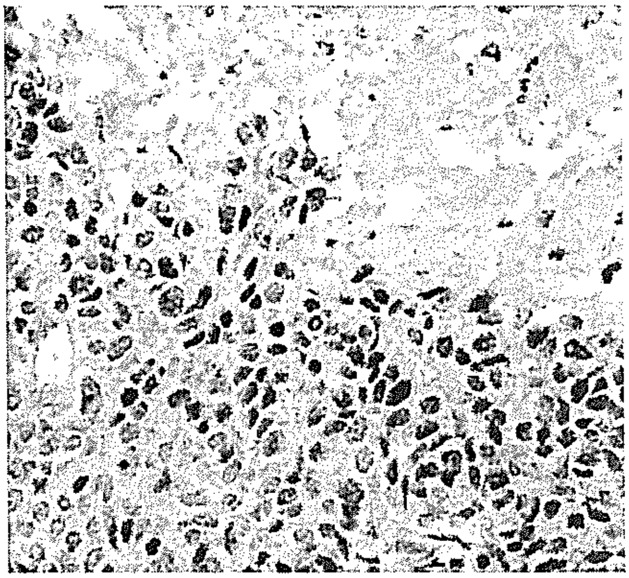

Fig. 1. The nuclear and cytoplasmic staining of HIF-1α in ulcerated regions of an advanced gastric carcinoma is shown (200×).

RESULTS

Expression of HIF-1α in gastric carcinomas was frequently detected at the invading edge of the tumor margin and at the periphery of necrotic regions within the tumor mass (Fig. 1). Of the gastric carcinoma specimens

TABLE I. Relationship of HIF-1α, VEGF, and IGF-2 to Clinicopathologic Factors in Gastric Carcinomas

	HIF-1α (−) n = 77	HIF-1α (+) n = 49	*P*-value	VEGF (−) n = 87	VEGF (+) n = 39	*P*-value	IGF-2 (−) n = 81	IGF-2 (+) n = 45	*P*-value
Age									
<65	31	29	0.038	35	25	0.013	42	18	0.202
≥65	46	20		52	14		39	27	
Gender									
Male	50	35	0.448	58	27	0.776	31	35	<0.005
Female	27	14		29	12		50	10	
Depth of invasion									
t1	48	20	0.018	51	17	0.118	45	23	0.631
t2,3,4	29	29		36	22		36	22	
Histology									
Differentiated	56	18	<0.005	58	16	0.007	49	25	0.59
Undifferentiated	21	31		29	23		32	20	
Tumor size									
<3 cm	35	10	<0.005	33	12	0.438	37	8	<0.005
≥3 cm	42	39		54	27		44	37	
Lymph node metastasis									
Negative	51	24	0.054	57	18	0.041	45	30	<0.005
Positive	26	25		30	21		36	15	
Lymphatic invasion									
Negative	40	9	<0.005	40	9	0.015	31	20	<0.005
Positive	37	40		47	30		50	25	
Venous invasion									
Negative	64	35	0.119	68	31	0.867	64	35	<0.005
Positive	13	14		19	8		17	10	