た治療戦略には及ばないというのが現時点でのコンセンサスと考えられる。したがって，根治切除不能大腸癌に遭遇した場合，臨床上重要と考えられることは，第一治療として切除と非切除のどちらを選択するかではなく，原発巣による症状の程度，耐術能，化学療法の感受性などを注意深く観察し，総合的に治療法を適時選択していく知識と経験であると考えられる（図1）。

おわりに

原発巣切除は，姑息的であっても生存期間を延長するとの報告が多かった。しかし，今後，分子標的治療薬をはじめとする化学療法の進歩によって，切除の位置付けが変化していく可能性もあるので，常に最新の情報を収集し，適切な治療方針を選択していくことが重要と考えられる。

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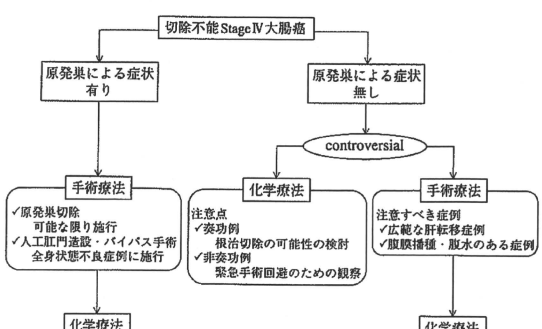

図1　切除不能StageⅣ大腸癌に対する治療方針

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Summary

The value of primary tumor resection in patients with unresectable StageⅣ colorectal cancer

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Primary tumor resection followed by systemic chemotherapy in patients with StageⅣ colorectal cancer is the standard treatment strategy. However, this strategy is gradually changing due to recent advances in systemic chemotherapy for StageⅣ colorectal cancer. For patients presenting symptoms caused by the primary tumor, resection is considered necessary as the first-line treatment. On the other hand, in asymptomatic colorectal cancer, there are several reports that most cases do not require any surgical intervention for primary-tumor-related complications if patients undergo the latest chemotherapies, which are combinations of infusional 5-FU/leucovorin with oxaliplatin or irinotecan. Furthermore, the addition of molecular target agents to the above combinations has provided clinically meaningful improvement in response rates and occasionally enables curative resection. The discussion about the value of primary tumor resection for unresectable StageⅣ colorectal cancer hereafter will grow heated.

●原 著●

切除不能進行・再発大腸癌に対する二次治療としてのBevacizumab併用化学療法

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Bevacizumab in Combination with mFOLFOX6 or FOLFIRI for Previously Treated Metastatic Colorectal Cancer: Motoi Koyama[*1], Akihiko Murata[*1], Yutaka Kimura[*1], Yoshiyuki Sakamoto[*1], Hajime Morohashi[*1], Norihisa Kimura[*1], Fujihiko Gasa[*1], Junya Sato[*2], Kazushi Terui[*2], Akemi Awatsu[*3] and Kenichi Hakamada[*1] ([*1]*Dept. of Gastroenterological Surgery, Hirosaki University Graduate School of Medicine*, [*2]*Dept. of Pharmaceutical Sciences*, [*3]*Dept. of Nursing, Hirosaki University Hospital*)

Summary

We retrospectively investigated the safety and efficacy on outpatient chemotherapy including bevacizumab (BV) as second-line therapy for inoperable metastatic colorectal cancer. Analytical subjects were thirty patients treated with chemotherapy including BV as second-line therapy after first disease progression. All patients were treated with BV 5 mg/kg. Concurrent therapy was given mFOLFOX6 (2 patients) and FOLFIRI (28 patients). The BV treatment frequency and all course treatment frequency including the prior regimens averaged 20 and 37 times, respectively. The overall response rate was 24. 1% (PR, 7 patients; SD, 17 patients; PD, 5 patients), and the median duration of progression-free survival was 8. 0 months. The median duration of survival after addition of BV was 20. 3 months. The adverse events were 84% (>grade 3, 9%), BV-associated adverse events were GI perforation (1 patient), GI hemorrhage (1 patient), grade 3 hypertension (1 patient) and grade 2 epitaxis (2 patient). Although it is necessary to be careful about GI hemorrhage and GI perforation, we could safely continue the treatment with BV on outpatient chemotherapy. We confirmed that the chemotherapy including BV as second-line therapy had high antitumor effect and patient benefit. **Key words**: Chemotherapy of colorectal cencer, Second-line therapy, Bevacizumab (*Received Oct. 21, 2009/Accepted Dec. 9, 2009*)

要旨 切除不能進行・再発大腸癌に対する二次治療におけるbevacizumab（以下BV）を併用した外来化学療法の安全性と抗腫瘍効果をretrospectiveに検討した。対象は初回治療増悪後の二次治療として施行したBV併用療法の30例であった。BVは全例5 mg/kgで投与し，併用療法はmFOLFOX6が2例，FOLFIRIが28例。BV施行回数は平均20回で，前治療を含めた全コース施行回数は平均37回。BV併用二次治療における抗腫瘍効果はPR 7例，SD 17例，PD 5例（奏効率24%）で，無増悪生存期間の中央値は8.0か月，BV開始後の生存期間中央値は20.3か月であった。有害事象は93%であったが，grade 3以上は20%で，BVに特有な有害事象は消化管出血・穿孔・高血圧が各1例でみられた。BV併用療法は消化管出血や穿孔を念頭において治療に当たる必要があるが，外来化学療法で安全に継続可能であり，二次治療においても抗腫瘍効果は高く有用性が確認できた。

はじめに

切除不能進行・再発大腸癌に対する化学療法として従来のFOLFOX/FOLFIRI療法にbevacizumab（以下BV）が併用され，現在では標準治療として位置付けられている[1-7]。当教室では2007年8月からBVを導入しており，今回は二次治療として施行されたBVを併用した外来化学療法の安全性と抗腫瘍効果をretrospectiveに

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表 1 Bevacizumab 併用療法（二次治療）

性別	男性 15 例，女性 15 例
平均年齢	61.4 歳（47～78 歳）
原発巣	結腸 13 例，直腸 17 例
同時性/異時性	同時性 12 例，異時性 18 例
標的臓器	1 臓器 13 例，2 臓器 9 例，3 臓器 8 例
BV 投与量	全例 5 mg/kg
併用療法	mFOLFOX6 2 例，FOLFIRI 28 例
全コース施行回数	37.2 回（7～79 回）
BV 施行回数	19.5 回（3～48 回）
BV 併用化学治療継続中	8 例
BV 併用化学治療中止	22 例
中止理由	病勢増悪 19 例
	有害事象 2 例
	患者希望 1 例

BV: bevacizumab

検討した。

Ⅰ．対象および方法

1．対象と方法

2006 年 1 月～2008 年 12 月に経験した外来化学療法は 80 例で，一次治療は mFOLFOX6，二次治療は FOLFIRI を基本レジメンとしている。BV 併用化学療法は 80 例中 38 例に施行され，一次治療の BV 併用療法が 8 例であり，初回治療増悪後の二次治療として施行された BV 併用療法の 30 例を今回の対象とした。BV は全例 5 mg/kg で投与し，併用療法として mFOLFOX6 が 2 例，FOLFIRI が 28 例で施行された（表 1）。一次治療の mFOLFOX6 増悪後にセカンドレジメンとして BV 併用 FOLFIRI に移行した症例が 17 例であり，二次治療として FOLFIRI を継続中に，当院で BV 使用が可能となった 2007 年 8 月以降にサードレジメンとして BV 併用 FOLFIRI に移行した症例が 11 例であった。mFOLFOX6 と併用した 2 例は，ファーストレジメンの mFOLFOX6 による神経毒性のためにセカンドレジメン FOLFIRI に変更して，初回増悪後にサードレジメンとして BV 併用 FOLFOX に移行した症例であった。

患者背景は男性 15 例，女性 15 例で，平均年齢は 61 歳。原発巣は結腸 13 例，直腸 17 例で，標的臓器は 1 臓器が 13 例，2 臓器が 9 例，3 臓器以上が 8 例であった。

2．治療法

治療対象は以下の適応とした。① ECOG PS が 0～1，② 主要臓器機能が保持されている，③ 心血管系や脳梗塞・出血の既往がない。

3．評価項目

抗腫瘍効果の評価には Response Evaluation Criteria in Solid Tumors（RECIST）を用いた。有害事象の grade は National Cancer Institute Common Toxicity Criteria（NCI-CTC）version 3.0 を採用した。

表 2 抗腫瘍効果

（全症例: 29 例）	CR 0 例，PR 7 例，SD 17 例，PD 5 例
	奏効率（CR＋PR） 24%
	病勢制御率（CR＋PR＋SD）83%
（セカンドレジメン: 16 例）	CR 0 例，PR 4 例，SD 8 例，PD 4 例
	（奏効率 25%，病勢制御率 75%）
（サードレジメン: 13 例）	CR 0 例，PR 3 例，SD 9 例，PD 1 例
	（奏効率 23%，病勢制御率 92%）

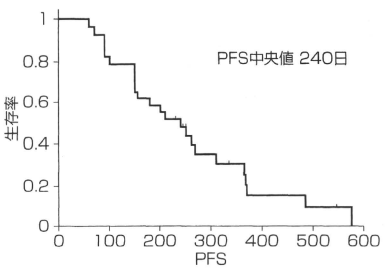

図 1 Bevacizumab 併用療法の二次治療における無増悪期間（PFS）

Ⅱ．結　果

1．抗腫瘍効果

BV 開始前の FOLFOX/FOLFIRI 療法の前治療コース施行回数は平均 18（4～52）回であった。BV 施行回数は平均 20（3～48）回であり，前治療を含めた全コース施行回数は平均 37（7～79）回。BV 併用化学療法を治療継続中は 8 例であり，中止例 22 例の中止理由の内訳は病勢増悪が 19 例，有害事象が 2 例，患者希望が 1 例であった。病勢増悪の 19 例中 10 例は三次治療として cetuximab 併用化学療法に移行した（表 1）。

BV を 4 回以上施行した 29 例の抗腫瘍効果は PR 7 例，SD 17 例，PD 5 例。奏効率（CR＋PR）は 24%であり，病勢制御率（CR＋PR＋SD）は 83%であった。セカンドレジメンとして施行した 16 例では PR 4 例，SD 8 例，PD 4 例（奏効率 25%，病勢制御率 75%），サードレジメンの 13 例では PR 3 例，SD 9 例，PD 1 例（奏効率 23%，病勢制御率 92%）で，両群の抗腫瘍効果に差異はみられなかった（表 2）。

BV 併用後の二次治療における無増悪期間（PFS）中央値は 240 日（8.0 か月）で（図 1），前治療を除いた二次治療からの全生存期間中央値（MST）は 610 日（20.3

表3 有害事象

有害事象	grade 1	grade 2	grade 3	grade 4	全イベント
ヘモグロビン		4（13%）			4（13%）
白血球	5（17%）	6（20%）	3（10%）		14（47%）
血小板	11（37%）				11（37%）
疲労倦怠感	17（57%）	4（13%）			21（70%）
悪心・嘔吐	9（30%）	3（10%）			12（40%）
食欲不振	10（33%）				10（33%）
顔面潮紅		1（ 3%）			1（ 3%）
口内炎	3（10%）	3（10%）			6（20%）
脱毛	2（ 7%）				2（ 7%）
下痢	5（17%）	1（ 3%）			6（20%）
末梢神経障害	5（17%）				5（17%）
消化管出血	1（ 3%）		1（ 3%）		2（ 7%）
鼻出血	4（13%）	2（ 7%）			6（20%）
高血圧	1（ 3%）	1（ 3%）	1（ 3%）		3（10%）
消化管穿孔			1（ 3%）		1（ 3%）
全有害事象	7（23%）	15（50%）	6（20%）		28（93%）

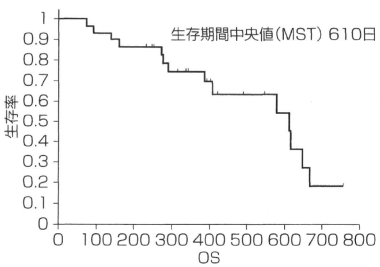

図2 Bevacizumab併用後の二次治療における全生存期間（OS）

か月）であった（図2）。

2．有害事象

BV併用の二次治療における有害事象は28例（93%）でみられたが，grade 3以上は6例（20%）であり，その内訳は白血球・好中球減少の血液毒性が3例で，非血液毒性として消化管出血・消化管穿孔・高血圧が各1例であった。全gradeにおけるBVに特有な有害事象としては，鼻出血6例（20%），高血圧3例（10%），消化管出血2例（7%），消化管穿孔1例（3%）がみられたが，動静脈血栓症は認めなかった（表3）。

Ⅲ．考　察

最近では生物学的に重要な経路を標的とする新規治療薬の開発により，切除不能進行・再発大腸癌に対する治療の選択肢は大きく広がり，患者の生存期間および無増悪生存期間は著しく延長している[1,2]。BVは血管内皮増殖因子（VEGF）に結合してこの作用を阻害する遺伝子組換えヒト化モノクローナル抗体であり[3]，一次または二次化学療法にBVを併用することでさらなる生存期間が得られることが報告され[4-7]，現在では標準治療として位置付けられている。しかし，BVの至適な投与方法や投与順序については明確になっていない[8]。

本邦においても，切除不能進行・再発大腸癌に対する化学療法として，従来のFOLFOX/FOLFIRI療法に加えてBVを併用した化学療法が一次治療として標準化されつつある。しかし一方で，多くの施設では外科医が主体となって大腸癌化学療法を担っているのが現状である。したがって実際の診療においては，手術直後であることや外来化学療法における安全性を理由として，一次治療からBV併用療法を使用できない場合も少なくない。これまでにBV併用の二次治療における文献的な報告はE3200試験などのみであり[4]，本邦の報告例は1983～2009年の期間について医学中央雑誌で「ベバシズマブ」「大腸癌」「二次治療」をキーワードとして検索し得る限りでは認められない。そこで今回は，初回治療増悪後の二次治療として施行されたBV併用の外来化学療法における安全性と抗腫瘍効果をretrospectiveに検討した。

大腸癌術後の化学療法患者が増加の一途をたどる現状において，当教室では外来化学療法で継続可能なレジメンを選択し，一次治療はmFOLFOX6，二次治療はFOLFIRIを基本レジメンとして，最近では一次治療からBVを併用している。2006年1月に外来化学療法室が開設して以来，2008年12月までに経験した大腸癌化学療法は80例で，遠隔転移の肝肺転移が切除可能となった6例を除いた74例のなかで，FOLFOXのみは8例で，FOLFOX/FOLFIRIは28例であった。その多くの症例は，当院でBV使用が可能となった2007年8月以

前に治療中止や転院となった症例であり，BV 導入後は二次治療の FOLFIRI 施行中でもサードレジメンとして BV 併用 FOLFIRI 療法に移行した。今回の対象は，BV 併用療法を施行した 38 例のうち，二次治療として施行された BV 併用の外来化学療法の 30 例である。

今回の二次治療における抗腫瘍効果は，奏効率は 24%，BV 開始後の PFS は 8.0 か月で，MST は 20.3 か月であった。一次治療における BV 併用化学療法では，奏効率 26～58%，PFS は 9.0～11.2 か月で，MST は 16.6～27.3 か月と報告されている[5-7,9-12]。今回の抗腫瘍効果は奏効率では劣っているが病勢制御率は 83%と良好であり，MST は前治療期間を除いた二次治療でありながら 20 か月という結果が得られた。さらに，BV が導入される以前の FOLFOX4/6 や FOLFIRI による二次治療の治療成績は，奏効率 4～15%，PFS 2.5～4.7 か月と報告されており[4,13-15]，今回の BV 併用二次治療の奏効率 24%，PFS 8.0 か月と比較すると，二次治療からであっても BV を併用する有用性が確認できた。

BV 併用化学療法の有害事象は，これまでの欧米の大規模試験や本邦の市販後調査における報告では[4,11,12,16]，高血圧 13.5～29.9%（grade 3 以上 0.4～6.2%），出血 11.8～31.0%（grade 3 以上 1.3～3.4%），蛋白尿 4.6～10.4%（grade 3 以上 0.1～1.1%），消化管穿孔 0.9～2.0%（grade 3 以上 0.9～1.8%），静脈血栓症 1.3%（grade 3 以上 1.3%），動脈血栓症 0.4～3.4%（grade 3 以上 0.3～3.4%）とされている。今回の BV 併用の外来化学療法に際しては，有害事象対策として点滴施行前に末梢血・生化学検査を行い，月一度の尿検査と D-ダイマーの測定を定期的に行った。今回の有害事象は全 grade を総合すると 84%であったが，grade 3 以上は 9%と少なかった。これは，前治療の FOLFOX/FOLFIRI を施行している段階で，血液毒性に対する 5-FU 減量などの対策が講じられていたことに起因すると考えられる。一方で，BV に特有な有害事象とされている高血圧・鼻出血の多くは grade 1～2 であったが，grade 3 の消化管出血と消化管穿孔の各 1 例を経験し，入院治療を必要とした。

消化管出血の 1 例は BV 併用の二次治療を 3 コース施行後に少量の一時的な下血が出現したが，貧血の進行もないため治療を継続していた。6 コース施行した 3 日後に，約 400 mL の下血が出現して同日入院となった。大腸内視鏡検査で骨盤内リンパ節再発の病巣が腸管に浸潤して，腸管内に再発腫瘍が露出していた。貧血に対して輸血による治療を必要としたが，自然に止血が得られた。1 週間後に退院となったが，この後は化学療法を希望せず，best supportive care を継続中である。また，消化管穿孔の 1 例は超低位前方切除後の肝転移・局所再発に対して，BV 併用の二次治療を 6 コース施行後に肛門周囲膿瘍が出現した。肛門縁から 4 cm の前回吻合部付近に局所再発が存在して，腫瘍自体が穿孔して肛門周囲の坐骨直腸窩に膿瘍を形成していた。入院として切開ドレナージを行い，後日に腸管ストーマ造設術を施行した。BV による腸管穿孔・創傷治癒遅延の影響が否定できないために，膿瘍が治癒した後に，BV を cetuximab に変更して，cetuximab 併用化学療法に変更した。しかし，cetuximab 4 回施行後に同様の肛門周囲膿瘍が出現して再度切開ドレナージを行った。したがって，化学療法の治療効果による腫瘍縮小・壊死に伴って腸管穿孔が引き起こされたものと推測された。

今回，grade 3 の消化管出血と消化管穿孔を経験したが，必要に応じて入院加療を併用して治療を継続することが可能であった。BV 併用化学療法は二次治療であっても外来化学療法は継続可能なレジメンであるが，これら特有な有害事象を念頭において治療に当たる必要がある。

切除不能進行・再発大腸癌に対する二次治療としての BV 併用化学療法について，安全性と抗腫瘍効果の両面から検討した。今回の結果から，BV 併用療法は安全性からも外来化学療法が可能であり，二次治療においても抗腫瘍効果は高く有用性が確認できた。

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RESEARCH ARTICLE Open Access

Anti-apoptotic effect of claudin-1 in tamoxifen-treated human breast cancer MCF-7 cells

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Abstract

Background: Claudin-1 is a membrane protein of tight junctions, and is associated with the development of various cancers. However, the significance of claudin-1 expression in cancer cells is not well understood. Here, we showed for the first time the anti-apoptotic effect of claudin-1 in human breast cancer MCF-7 cells.

Methods: Human breast cancer MCF-7 and T47 D cells were treated with or without tamoxifen, siRNA against claudin-1, or tamoxifen and claudin-1 siRNA. The samples were analyzed by RT-PCR, Western blotting or immunofluorescent staining.

Results: The expression of claudin-1 was upregulated in tamoxifen-treated MCF-7 cells, whereas the expression of claudin-1 was not altered in tamoxifen-treated T47 D cells. Knockdown of claudin-1 by siRNA increased the amount of poly (ADP-ribose) polymerase (PARP) regardless of tamoxifen treatment in MCF-7 cells, but not T47 D cells. In the cell membranes of the MCF-7 cells, tamoxifen treatment increased the amount of claudin-1, but decreased the amount of β-catenin. Claudin-1 siRNA increased the amount of E-cadherin in the cytoplasm of the MCF-7 cells as well as the amount of β-catenin in their cell membranes.

Conclusion: These results indicate that claudin-1 has anti-apoptotic effects, and is involved in the regulation of the expression and subcellular localization of β-catenin and E-cadherin in MCF-7, but not T47 D cells.

Background

Breast cancer is the second most common cause of female mortality in United States. The breast cancer incidence and mortality rates were about 190,000 and 40,000, respectively, in 2009 [1]. The majority of breast cancers are sporadic, and most risk factors for the disease are related to estrogen exposure. This suggests that insufficient apoptosis in cancer cells is involved in their survival as insuffcient apoptosis leads to the development of chemotherapy resistance and carcinogenesis [2].

Tamoxifen is one of most widely used anti-estrogen drugs for the treatment of human breast cancer [3]. Tamoxifen treatment leads to a rapid decrease in number of S-phase cells, an accumulation of cells in the G1-fraction [4], and the induction of apoptosis *in vivo* and *vitro* [5-7]. Tamoxifen induces apoptosis through several distinct pathways including a mitochondria-dependent pathway, the induction of c-Myc, the activation of members of the mitogen-activated protein kinases (MAPK) family, and the upregulation of p53 [7-11]. However, the detailed molecular mechanisms by which tamoxifen induces apoptosis are not well understood.

Tight junctions and adherens junctions proteins, including claudins, E-cadherin, β-catenin, and ZOs proteins, are responsible for the maintenance of epithelial cell-cell adhesion and defining cell polarity, and are also involved in cell signaling events [12]. Changes in claudin expression are also involved in invasion, metastasis, and colony formation in various cancer cells [13-15]. In a previous study, the mRNA expression of claudin-1 was decreased in the tumor group compared with the control (normal) group in breast cancer tissues [16]. Decreased expression of claudin-1 was also correlated with breast cancer recurrence [17]. However, the relationship between claudin-1 and chemotherapy is poorly understood.

In the present study, we investigated the relationship between claudin-1 and tamoxifen treatment in human

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breast cancer MCF-7 and T47 D cells. The expression of claudin-1 was upregulated by tamoxifen treatment in MCF-7 cells. Combination treatment with both claudin-1 siRNA and tamoxifen significantly increased the amount of cleaved PARP. Knockdown of claudin-1 affected the expression and subcellular localization of β-catenin and E-cadherin in MCF-7 cells. Our results suggest that claudin-1 has an anti-apoptotic effect, involving the regulation of β-catenin and E-cadherin, in MCF-7 cells.

Methods

Cell culture and treatment

MCF-7 and T47 D cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in Dulbecco's Modified Eagle's Medium-high glucose (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When the MCF-7 cells were treated with 40 μM of tamoxifen (Sigma) for 20 h, apoptotic reactions were detected as described below. However, the incubation with 40 μM of tamoxifen for more than 24 h resulted in the severe toxicity to cells, and more than 90% of cells were detached from the plates (data not shown). Therefore, we treated the cells with 40 μM of tamoxifen for 20 h in the follow experiments. In addition, we treated MCF-7 cells with 1, 10 or 20 μM of tamoxifen for 48 h in some experiments to observe the longer effects.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was isolated using an RNeasy RNA isolation kit (QIAGEN, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of total RNA using ReverTra Ace (TOYOBO, Osaka, Japan). RT-PCR was performed using an aliquot of first-strand cDNA as a template under standard conditions with Taq DNA polymerase (QIAGEN). The primers were designed to perform optimal RT-PCR by DNASIS software, and the primers used were as follows: claudin-1-F: 5'-CAGCTGTTGGGCTTCATTCTC-3', claudin-1-R: 5'-ATCACTCCCAGGAGGATGCC-3'; claudin 4-F: 5'-ATGGCCTCCATGGGGCTACA-3', claudin 4-R: 5'-AGCGAGTCGTACACCTTGCA-3'; E-cadherin-F: 5'-ACATTGTCACCTCGCAGAC-3', E-cadherin-R: 5'-GCGGATTGTAGAAGTCTTGG-3'; GAPDH-F: 5'-CCACCCATGGCAAATTCCATGGCA-3', GAPDH-R: 5'-AGACCACCTGGTGCTCAGTGTAGC-3'. The amplified products of claudin-1, claudin-4, E-cadherin, and GAPDH were 277 bp, 208 bp, 336 bp, and 696 bp, in length, respectively. The cDNA for claudin-1, claudin-4, E-cadherin, and GAPDH were amplified for up to 25 cycles. The PCR products were separated on 1.5% (w/v) agarose gels.

The real-time PCR was carried out using SYBER Green Master Mix (Applied Biosystems, Tokyo, Japan). The primers used as follows: claudin-1-F: 5'-AGATGAGGATGGCTGTCATTGG -3', claudin-1-R: 5'-CATGCTGTGGCAGCTAAAATAGC-3'; E-cadherin-F: 5'-ACATTGTCACCTCGCAGAC-3', E-cadherin-R: 5'-GCGGATTGTAGAAGTCTTGG-3'; 18 S rRNA-F: 5'-GTAACCCGTTGAACCCCATT-3', 18 S rRNA-R: 5'-CCATCCAATCGGTAGTAGCG-3'. The amplified products of claudin-1, E-cadherin, and 18 S rRNA were 72 bp, 336 bp, and 150 bp, in length, respectively.

Short interference RNA (siRNA)

Short interference RNA (siRNA) against claudin-1 were synthesized by QIAGEN. The sequences for the sense and anti-sense claudin-1 siRNA were 5'-r (GCAUGGUAUGGCAAUAGAA) d (TT) -3' and 5'-r (UUCUAUUGCCAUACCAUGC) d (TG) -3', respectively. We also used another siRNA against claudin-1 (claudin-1 siRNA2). The sequences for the sense and anti-sense claudin-1 siRNA2 were 5'-r (CGAAAUUGUUACAAUAGAA) d (TT)-3' and 5'-r (UUCUAUUGUAACAAUUUCG) d (TT)-3'. The negative control (scrambled) siRNA sequences were 5'-r (UUCUCCGAACGUGUCACGU) d (TT)-3' and 5'-r (ACGUGACACGUUCGGAGAA) d (TT)-3'. For the siRNA transfection experiments, MCF-7 and T47 D cells were seeded at 5×10^4 cells per 35-mm well. Twenty-four h later, the siRNA were transfected into the cells using the Lipofectamine RNA iMAX reagent (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were incubated for 48 h and subjected to various analyses.

Western Blotting

The cells transfected with siRNA were lysed using M-PER lysis buffer (PIERCE, Rockford, IL, USA). Protein concentrations were determined using the bicinchoninic acid (BCA) assay. The obtained lysates (10 μg protein) were subjected to SDS-PAGE, and the acquired proteins were transferred to PVDF membranes (Immobilon P, Millipore, Tokyo, Japan). The membranes were then incubated with antibodies specific for claudin-1 (1:10,000), claudin-4 (1:20,000), and claudin-7 (1:2,000), which were purchased from Invitrogen; E-cadherin (1:1,000), which was purchased from Takara, Shiga, Japan; β-catenin (1:30,000), Bcl-2 (1:2,000), and p21 (1:1,000), which were purchased from EPITOMICS, CA, USA; cyclin D1 (1:1,000), which was purchased from Merck, Darmstadt, Germany; PARP (1:1,000) and cleaved caspase-8 (1:10,000), which were purchased from Cell Signaling Technology, Inc Danvers, MA, USA; Bax (1:1,000), which was purchased from Santa Cruz,

CA, USA; p53 (1:2,000), which was purchased from Abcam, Cambridge, UK; and actin (1:30,000) (Sigma), followed by treatment with horseradish peroxidase-conjugated secondary antibody (IBL, Gunma, Japan). Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO) was used to dilute the primary antibody. The ECL, ECL-plus, or ECL-advance Western Blotting Detection System (Amersham, Uppsala, Sweden) was used for detection. The intensity of the bands was quantified by using the National Institute of Health Image computer program. The signal intensities were compensated by actin as internal controls.

Immunofluorescent staining

MCF-7 and T47 D cells were seeded on a 4-chamber slide glass and incubated overnight. The cells were then washed with phosphate-buffered saline (PBS) and fixed with ice-chilled methanol for 30 min, before being permeabilized with 0.2% Triton-X-100 in PBS for 30 min. The permeabilized cells were then washed in PBS twice and treated with 5% normal horse serum in PBS for 30 min (to minimize the non-specific adsorption of antibodies), before being incubated with anti-claudin-1 (1:200), anti-β-catenin (1:300), or anti-E-cadherin (1:300) antibodies at 4°C overnight. The cells were then incubated for 1 h with goat anti-rabbit IgG antibody conjugated to Alexa 488 dye (Molecular Probes, Inc, Tokyo, Japan), while nuclear staining was performed using 4', 6-diamidino-2-phenylindole (DAPI) or Hoechst 33258. Hoechst 33258 staining was used to examine nuclear condensation. The cells were visualized using confocal laser scanning microscopy (Zeiss, LSM 710, Wetzlar, Germany), and the number of cells that were intensely stained with Hoechst 33258 was counted.

Results

Tamoxifen treatment induces apoptosis and upregulates the expression of claudin-1 in MCF-7 cells

We investigated the endogenous expression of claudin-1 in two breast cancer cell lines by Western blotting. The endogenous protein expression of claudin-1 was weak in MCF-7 cells, whereas it was abundantly expressed in T47 D cells (Figure 1A and 1B). In addition, E-cadherin was abundantly expressed in both MCF-7 and T47 D cells. Next, we examined the relationship between tamoxifen and claudin-1. We examined how the protein expression of claudin-1 was affected by tamoxifen treatment. MCF-7 and T47 D cells were treated with various concentrations of tamoxifen for 20 h. Cell lysates were prepared from cells and subjected to Western blot analysis. The protein expression of claudin-1 was slightly increased by treatment with 20 μM tamoxifen in MCF-7 cells, and the cells treated with 30 or 40 μM of tamoxifen treatment showed clear increases in their claudin-1 protein levels. Twenty, 30, or 40 μM of tamoxifen treatment also increased the amounts of cleaved PARP and caspase-8 in the MCF-7 cells, but decreased their expression of β-catenin. The protein expression of E-cadherin was decreased in MCF-7 cells after treatment with 30 or 40 μM of tamoxifen. We also examined whether the longer exposure to tamoxifen affects the expression of claudin-1 in MCF-7 cells. The expression of claudin-1 and the amount of cleaved PARP were significantly increased in the cells treated with 20 μM of tamoxifen for 48 h (Figure 1C and 1D). In T47 D cells, the amounts of cleaved PARP and caspase-8 were increased, whereas the expression of E-cadherin was decreased after 40 μM of tamoxifen treatment. However, the protein expression levels of claudin-1, claudin-4, claudin-7, and β-catenin in T47 D cells were almost unaffected by tamoxifen treatment. We also examined whether the expression of claudin-1 mRNA was affected by tamoxifen treatment. MCF-7 and T47 D cells were treated with various concentrations of tamoxifen for 20 h, and RNA samples were prepared from the cells and subjected to RT-PCR and real-time PCR analyses for claudin-1, claudin-4, E-cadherin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18 S rRNA (Figure 2A and 2B). The mRNA levels of claudin-1 were significantly increased in MCF-7 cells after treatment with 30 or 40 μM of tamoxifen, whereas tamoxifen treatment did not affect the expression of claudin-1 in T47 D cells. The mRNA expression of E-cadherin was decreased in both MCF-7 and T47 D cells after treatment with 40 μM of tamoxifen. On the other hand, tamoxifen treatment did not affect the expression of claudin-4 in MCF-7 or T47 D cells.

Nuclear condensation is one of the features of apoptosis. Using immunofluorescent staining, we examined whether tamoxifen treatment induces nuclear condensation. MCF-7 and T47 D cells were treated with tamoxifen, fixed, and then stained with Hoechst 33258. Nuclear condensation was increased about 9 or 5-fold in MCF-7 or T47 D cells, respectively, treated with 40 μM of tamoxifen compared with that in the untreated-control cells (Figure 3A and 3B).

Claudin-1 has anti-apoptotic effects under tamoxifen treatment in MCF-7 cells

To understand the mechanism of the upregulation of claudin-1 expression by tamoxifen treatment, we examined whether the transfection of claudin-1 siRNA affected the expression of factors related to apoptosis. MCF-7 cells were transfected with control siRNA or siRNA against claudin-1. After 48 h of transfection, the cells were treated with 40 μM of tamoxifen for 20 h. The cell lysates were then subjected to Western blot analyses for claudin-1, cleaved PARP, cleaved caspase-8,

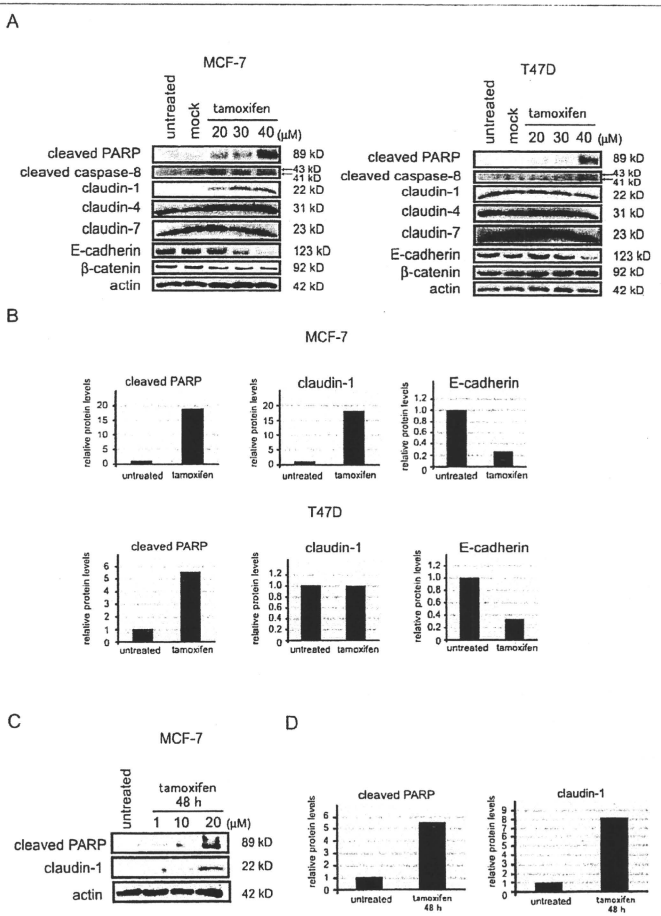

Figure 1 The protein levels of claudin-1 were increased in MCF-7 cells treated with tamoxifen. (A) MCF-7 and T47 D cells were treated with the control treatment (buffer alone: Mock) for 20 h or 20, 30, or 40 μM of tamoxifen for 20 h, cell lysates were prepared and subjected to Western blot analyses for cleaved PARP, claudin-1, claudin-4, claudin-7, E-cadherin, cleaved caspase-8, and actin. One representative of at least three independent experiments with similar results is shown. (B) The intensity of the bands for cleaved PARP, claudin-1 and E-cadherin in untreated and tamoxifen (40 μM) - treated cells were quantified. (C) MCF-7 cells were treated with or without 1, 10 or 20 μM of tamoxifen for 48 h, cell lysates were prepared and subjected to Western blot analyses for cleaved PARP, claudin-1 and actin. One representative of at least three independent experiments with similar results is shown. (D) The intensity of the bands for cleaved PARP and claudin-1 in untreated and tamoxifen (40 μM) - treated cells were quantified.

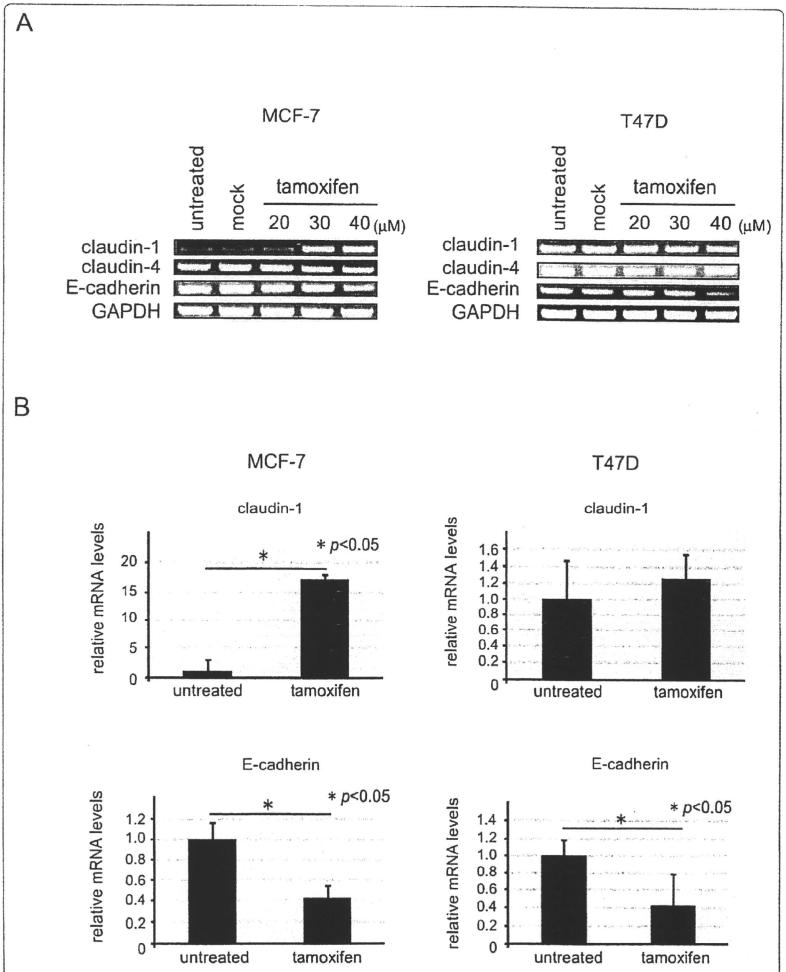

Figure 2 The mRNA levels of claudin-1 were increased in MCF-7 cells treated with tamoxifen. (A) After the MCF-7 and T47 D cells had been treated with various concentrations of tamoxifen for 20 h and subjected to RT-PCR analyses. One representative of at least three independent experiments with similar results is shown. (B) MCF-7 and T47 D cells were treated with or without 40 μM of tamoxifen for 20 h, and subjected to real-time PCR analyses for claudin-1 and E-cadherin. Each value represents the mean + SE (*bars*) of three independent experiments *$p < 0.05$, according to the *t*-test.

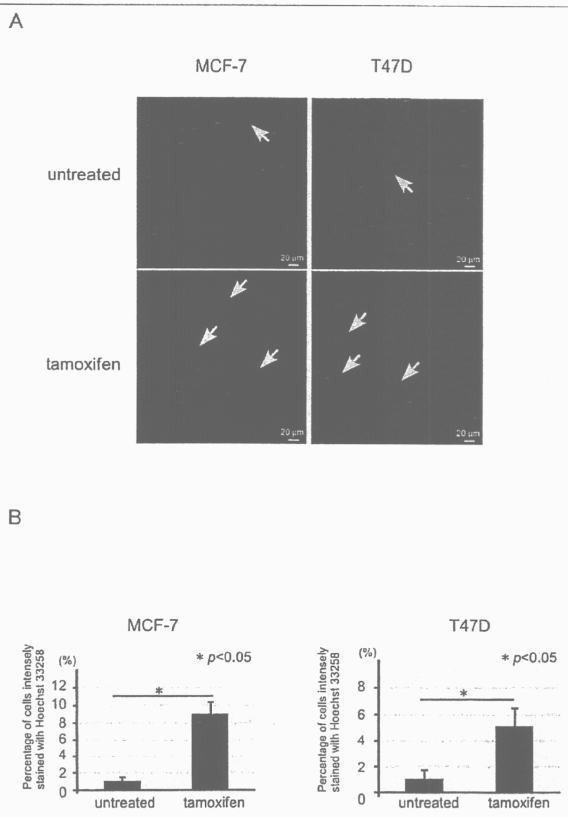

Figure 3 Nuclear condensation was detected by tamoxifen treatment both in MCF-7 and T47 D cells. (A) MCF-7 and T47 D cells were seeded in a 4-chamber slide glass and incubated overnight. The cells were treated with or without 40 μM of tamoxifen for 20 h, fixed, and stained with Hoechst 33258. The arrows show nuclear condensation. (B) Percentage of cells intensely fragmented and condensed in nuclei with Hoechst 33258 were counted as positive cells. About 100 total cells were counted in individual ten random microscopic fields at ×40 magnification. Each value represents the mean + SE (*bars*) of two independent experiments $*p < 0.05$, according to the *t*-test.

E-cadherin, β-catenin, Bax, Bcl-2, cyclinD1, p53, p21, and actin (Figure 4A and 4B). Claudin-1 knockdown by siRNA significantly reduced the expression of claudin-1 with or without tamoxifen treatment, and the transfection of claudin-1 siRNA increased the amounts of cleaved PARP and caspase-8 with or without tamoxifen treatment. The expression of E-cadherin was upregulated by claudin-1 knockdown without tamoxifen treatment, while the expression of E-cadherin in the presence of claudin-1 siRNA and tamoxifen treatment was slightly increased. On the other hand, the expression of cyclinD1 was downregulated by claudin-1

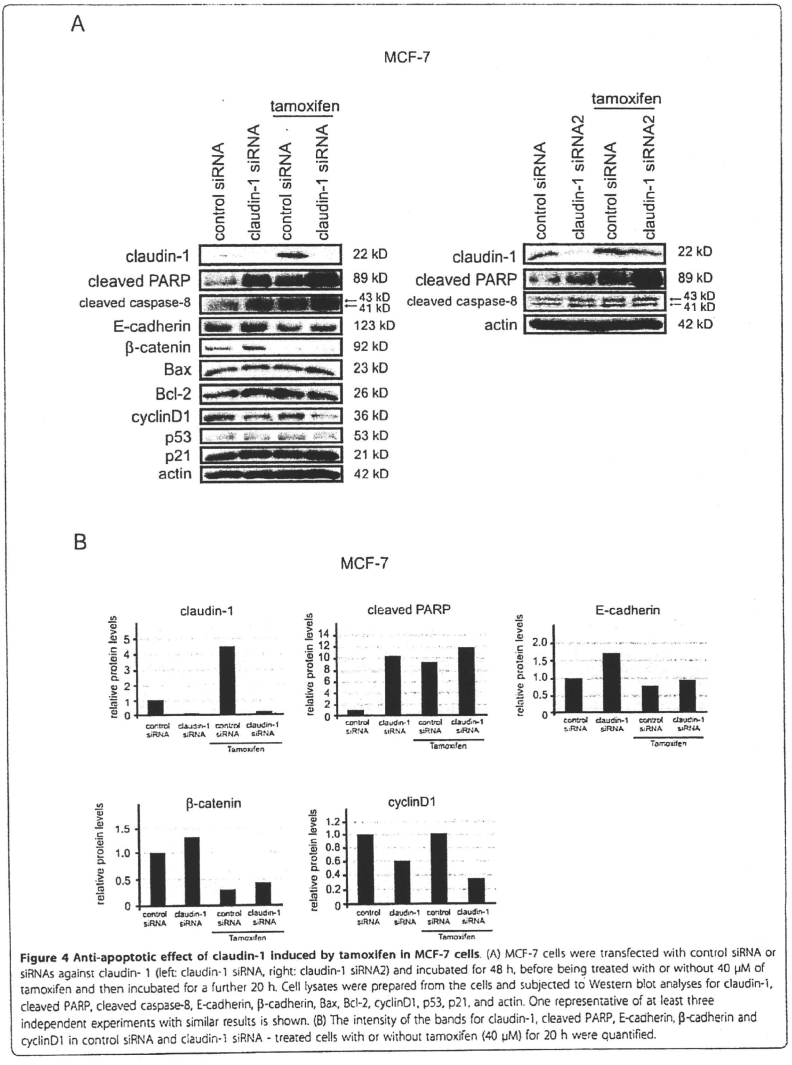

Figure 4 Anti-apoptotic effect of claudin-1 induced by tamoxifen in MCF-7 cells. (A) MCF-7 cells were transfected with control siRNA or siRNAs against claudin- 1 (left: claudin-1 siRNA, right: claudin-1 siRNA2) and incubated for 48 h, before being treated with or without 40 μM of tamoxifen and then incubated for a further 20 h. Cell lysates were prepared from the cells and subjected to Western blot analyses for claudin-1, cleaved PARP, cleaved caspase-8, E-cadherin, β-cadherin, Bax, Bcl-2, cyclinD1, p53, p21, and actin. One representative of at least three independent experiments with similar results is shown. (B) The intensity of the bands for claudin-1, cleaved PARP, E-cadherin, β-cadherin and cyclinD1 in control siRNA and claudin-1 siRNA - treated cells with or without tamoxifen (40 μM) for 20 h were quantified.

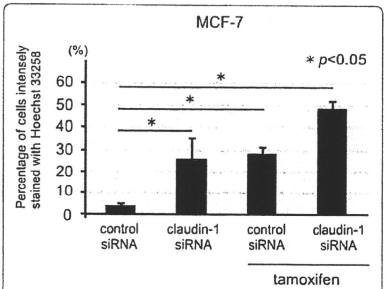

Figure 5 Nuclear condensation was increased in claudin-1 siRNA treatment of MCF-7. MCF-7 cells were treated as above, and then stained with Hoechst 33258. Percentage of cells intensely fragmented and condensed in nuclei with Hoechst 33258 were counted as previously described, and each value represents the mean + SE (*bars*) of two independent experiments $*p < 0.05$, according to the *t*-test.

knockdown with or without tamoxifen treatment, whereas tamoxifen treatment did not affect the expression of cyclinD1. The expression of β-catenin was upregulated by claudin-1 knockdown in the absence of tamoxifen treatment, but combination treatment involving claudin-1 siRNA and tamoxifen had little effect the expression of β-catenin. The expression levels of Bax, Bcl-2, p53, p21, and actin were not affected by claudin-1 knockdown, tamoxifen treatment or combination treatment involving claudin-1 siRNA and tamoxifen.

As nuclear condensation was induced in the cells treated with tamoxifen (Figure 3), we examined whether claudin-1 siRNA treatment had similar effects. Nuclear condensation was increased about 7-fold in the MCF-7 cells treated with claudin-1 siRNA as well as the control siRNA and tamoxifen-treated cells compared with that in the control (only control siRNA treatment) cells (Figure 5). Combination treatment involving claudin-1 siRNA and tamoxifen increased nuclear condensation about 16-fold compared with that in the control cells.

Next, we examined whether claudin-1 knockdown by siRNA affected the amount of cleaved PARP in T47 D cells. The amount of cleaved PARP was upregulated by tamoxifen treatment, but it was little affected by claudin-1 siRNA treatment or combination treatment involving claudin-1 siRNA and tamoxifen (Figure 6A and 6B).

Changes in the subcellular localization of E-cadherin and β-catenin in MCF-7 cells treated with claudin-1 siRNA or tamoxifen

We investigated whether the subcellular localization of E-cadherin and β-catenin was affected by claudin-1 siRNA or tamoxifen treatment using immunofluorescent staining. As shown in Figure 7A and 8A, tamoxifen treatment increased the amount of claudin-1 in the cell membranes of MCF-7 cells, while tamoxifen treatment did not affect the amount of claudin-1 in T47 D cells. Tamoxifen treatment decreased the amounts of E-cadherin in the cell membranes of MCF-7 and T47 D cells, while it increased the amount of E-cadherin in the cytoplasm of MCF-7, but not T47 D, cells. On the other hand, tamoxifen treatment decreased the amount of β-catenin in the cell membranes of MCF-7 cells, but increased it in the cytoplasm. The amount of E-cadherin in the cell membrane was decreased in MCF-7 cells treated with claudin-1 siRNA, while the amount of E-cadherin in the cytoplasm was increased (Figure 7B). Knockdown of claudin-1 also increased the amount of β-catenin in the cell membranes of MCF-7 cells. In T47 D cells, claudin-1 siRNA treatment did not affect the amount of E-cadherin or β-catenin in the cell membrane or cytoplasm (Figure 8B). These data demonstrated that claudin-1 has anti-apoptotic effects during tamoxifen treatment, which involve changes in the subcellular localization of claudin-1, E-cadherin, and β-catenin in MCF-7 cells.

Discussion

In this study, we focused on the functions of claudin-1 in human breast cancer cells. Claudins are generally located in the cell membrane and mainly contribute to cell-cell adhesion [18,19]. It was confirmed that claudin-1 is localized to the cell membrane in T47 D cells. However, little claudin-1 was localized to the cell membrane in MCF-7 cells. Tamoxifen treatment increased claudin-1 protein expression as well as its membrane localization in MCF-7 cells, whereas tamoxifen treatment did not affect the expression or subcellular localization of claudin-1 in T47 D cells. Thus, the function of claudin-1 may differ among different cell types. It has been reported that MCF-7 cells have wild type p53 but lack caspase-3. On the other hand, T47 D cells express caspase-3 but p53 is mutated [20,21]. They showed that the sensitivity of these cells against anti-cancer drugs such as staurosporine and Triphala are different. The differential expression of claudin-1 may be also related to differences in phenotype of these two cell lines.

Recent studies have shown the relationship between claudin expression and cellular resistance in tumors [22,23]. The elevated claudin-1 expression induced by 5-fluorouracil (5-FU) or TNF-α treatment is associated with the regulation of apoptosis in nasopharyngeal carcinoma and pancreatic cancer cells, although these cells low levels of protein expression and claudin-1 localization in the membrane were also observed [23,24]. In addition, knockdown of claudin-6 induces cellular

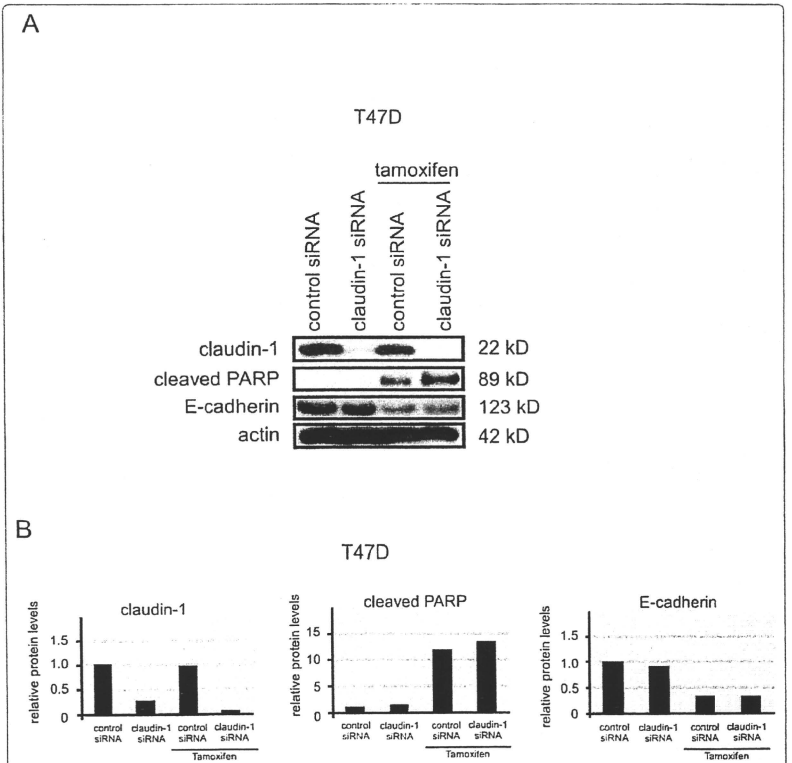

Figure 6 Claudin-1 knockdown has little effects on apoptosis in T47 D cells. (A) T47 D cells were treated with control siRNA or siRNA against claudin-1 and treated as above. Cell lysates were subjected to Western blot analyses for claudin-1, cleaved PARP, E-cadherin, and actin. One representative of at least three independent experiments with similar results is shown. (B) The intensity of the bands for claudin-1, cleaved PARP and E-cadherin in control siRNA and claudin-1 siRNA - treated cells with or without tamoxifen (40 μM) for 20 h were quantified.

resistance to apoptosis in MCF-7 cells [22]. These observations and our findings suggest that the upregulation of claudin-1 by apoptosis-inducers contributes to cellular resistance to apoptosis when claudin-1 protein is expressed at low levels and mislocalized to the cell membrane. However, it is unclear how claudin-1 is regulated by apoptosis inducers. We found that tamoxifen treatment increased the expression of claudin-1 mRNA and proteins related to apoptosis in MCF-7 cells. We speculate that tamoxifen treatment regulates the transcription of claudin-1. Further studies are needed to interpret whether tamoxifen treatment regulates claudin-1 expression.

Next, we investigated the molecular mechanisms of the apoptosis induced by claudin-1 knockdown in MCF-7 cells. Unfortunately, apoptosis-related proteins, such as Bax, Bcl-2, p53, and p21, were not affected by claudin-1 knockdown with or without tamoxifen treatment.

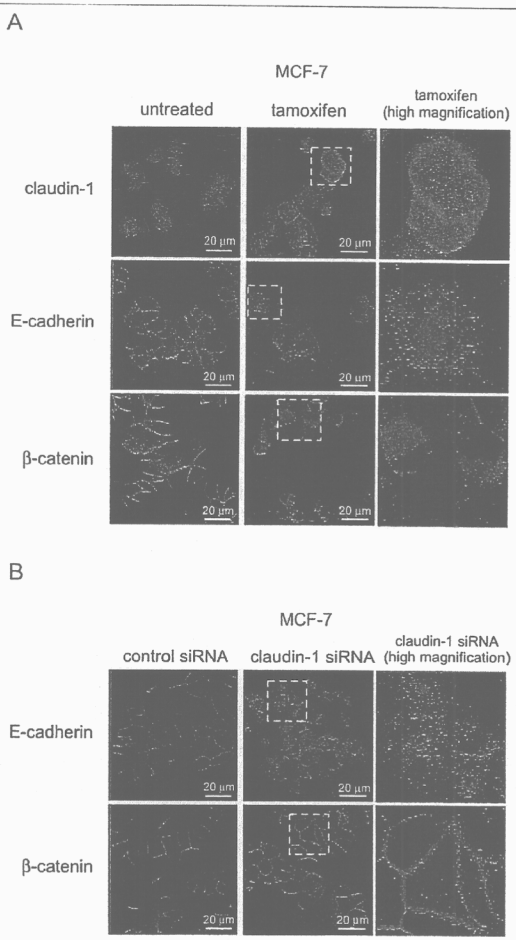

Figure 7 Subcellular localization of claudin-1, E-cadherin, and β-catenin in MCF-7 cells after tamoxifen or claudin-1 siRNA treatment. (A) MCF-7 cells were treated with or without 40 μM of tamoxifen for 20 h, fixed, incubated with anti-claudin-1, E-cadherin or β-catenin antibody, and visualized using Alexa488-conjugated secondary antibody (Green). The cells were also counterstained with DAPI (Blue) in order to localize the nucleus. A merged image that is representative of at least two independent experiments with similar results is shown. White dot box for high magnification were also shown. (B) MCF-7 cells were transfected with siRNA against claudin-1. After 48 h incubation, the cells were fixed, incubated with anti-E-cadherin or β-catenin antibody, and visualized using Alexa488. One representative merged image is shown.

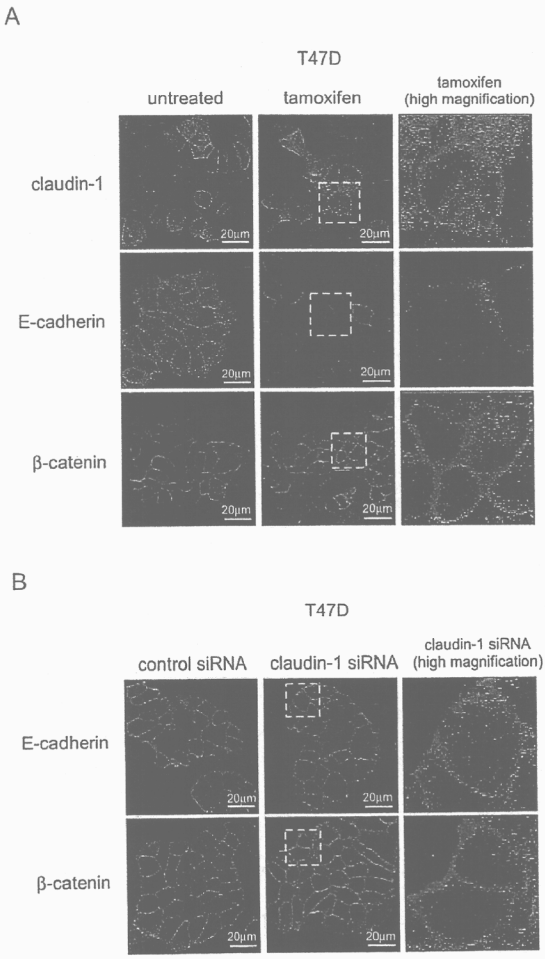

Figure 8 Subcellular localization of claudin-1, E-cadherin, and β-catenin in T47 D cells after tamoxifen or claudin-1 siRNA treatment. (A) T47 D cells were treated with or without 40 μM of tamoxifen for 20 h, incubated with anti-claudin-1, E-cadherin, or β-catenin antibody, and visualized using Alexa488. One representative merged image is shown. (B) T47 D cells were transfected with siRNA against claudin-1. After 48 h incubation, the cells were fixed, incubated with anti-E-cadherin or β-catenin antibody, and visualized using Alexa488. One representative merged image is shown.

However, the expression of cyclin D1 was downregulated by claudin-1 knockdown, regardless of tamoxifen treatment in MCF-7 cells. We speculate that the regulation of apoptosis by claudin-1 knockdown may be related to pathways other than the p21, p53, and mitochondrial-pathways. Lee et al. showed that claudin-1 has anti-apoptotic effects under 5-FU treatment, but they could not demonstrate the molecular mechanisms of claudin-1 induced apoptosis [23].

Interestingly, it has been reported that changes in the subcellular localization of β-catenin or E-cadherin may be related to the regulation of apoptosis [25-28]. 2-methoxyestradiol induces β-catenin expression in prostate cancer cells, but blocks β-catenin degradation, as well as its cytoplasmic or nuclear accumulation, resulting in cell cycle arrest and apoptosis [29]. Therefore, we performed immunofluorescent staining to analyze the changes in the subcellular localization of β-catenin and E-cadherin induced by claudin-1 knockdown or tamoxifen treatment. As expected, claudin-1 knockdown affected the subcellular localization of β-catenin and E-cadherin in MCF-7, but not T47 D cells. Tamoxifen treatment also affected the subcellular localization of β-catenin and E-cadherin. So, we speculate that knockdown of claudin-1 upregulates the protein expression of β-catenin and changes its subcellular localization in MCF-7 cells and then induces cell cycle arrest, resulting in apoptosis. However, tamoxifen treatment downregulates the expression of β-catenin in MCF-7 cells. According to these results, we suggest that tamoxifen treatment upregulates the expression of claudin-1 and that the upregulation of claudin-1 subsequently downregulates the expression of β-catenin. β-catenin may be one of the downstream factors of claudin-1 in MCF-7 cells. However, the detailed mechanism by which claudin-1 regulates the expression of β-catenin needs to be clarified.

We also examined whether other claudins are affected by tamoxifen treatment. The expression of claudin-4 and claudin-7 was not affected by tamoxifen treatment in MCF-7 and T47 D cells as shown in Figure 1A and 2A. Thus, only claudin-1 in claudin's family would be specifically affected by tamoxifen treatment, although we could not elucidate the specific effect of claudin-1 by tamoxifen treatment.

In the present study, we demonstrated the function of claudin-1 in human breast cancer MCF-7 cells. Claudin-1 has anti-apoptotic effects in tamoxifen-treated MCF-7 cells.

Conclusion

We demonstrated the function of claudin-1 in human breast cancer MCF-7 cells. Our results showed for the first time that claudin-1 has anti-apoptotic effects in tamoxifen-treated MCF-7 cells, involving the regulation of apoptosis-related factors and subcellular localization of adherens junctions.

Our data would be useful for future studies in order to establish the mechanisms of apoptosis regulation in human breast cancer. Further research needs to be clarified the relationship between tight junctions and apoptosis.

Abbreviations

RT-PCR: Reverse transcription-polymerase chain reaction; PARP: poly (ADP-ribose) polymerase; 5-FU: 5-fluorouracil; TNF-α: tumor necrosis factor-alpha; PBS: phosphate-buffered saline

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Authors' contributions

HA performed major experimental work. FS designed HA's original work and helped HA's work and completed manuscript. KH and HK corrected HA's manuscript. SM, YW, YL, JK and HO helped HA's work. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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