(Invitrogen) following the manufacturer's instructions. Selective silencing of FAK was confirmed by western blot analysis.

Generation of FYN knockdown cells

To generate stable FYN knockdown cell lines, GIST-T1 cells were transfected with a commercial plasmid containing an anti-FYN short hairpin RNA (shRNA Fyn, plasmid KH00147N; SABiosciences [Qiagen], Frederick, MD) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The correctly transfected and expressing cells were selected with 600 μg/mL G418 (Invitrogen). Stable clones were maintained in 250 μg/mL G418. Three stable GIST-T1-FYN shRNA cell lines were established, designated B1, B2 and B3 cells. We also established a control cell line of GIST-T1 stably transfected with the empty vector, which we designated GIST-T1-C.

Measurement of IC_{50} after imatinib treatment

Cells were seeded in 96-well plates at 2000 cells/well (Costar; Corning, Corning, NY) for 24 hr and then exposed to various concentrations (0–40 μM) of imatinib for 72 hr. Cell proliferation was evaluated with the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-SF; Nacalai Tesque) at the indicated post-treatment times. A microplate reader Model 680 (Bio-Rad Laboratories, Hercules, CA) was used to measure WST-8 absorption at a wavelength of 450 nm with a reference wavelength of 630 nm. Growth rate was expressed as the percentage of absorbance for treated cells vs. control cells. Experiments were performed in triplicate in two independent experiments, and the presented values are the averages of all six wells.

Apoptosis assay

GIST-T1 cells were seeded in 6-well plates at a density of 3×10^5 cells per well and treated with imatinib and/or TAG372 for 2 days. The cells were then washed with PBS, and caspase-3 activity was detected using the caspase-3 fluorometric assay kit (R&D systems, Minneapolis, MN) following the manufacturer's instructions. The presented values are the means of three independent experiments.

Statistical analysis

Statistical analyses were performed using the Mann–Whitney *U*-test or one-way analysis of variance (ANOVA), followed by Scheffe's test. One-way ANOVA followed by Dunnett's test was used for multiple comparisons.

Results

Quantitative phosphotyrosine proteomic analysis identifies upregulation of FYN and FAK in imatinib-exposed GIST-T1 cells

GIST-T1 cells that possessed the activating mutation in exon 11 of *KIT* are sensitive to imatinib, with a K_i value for imatinib of 20 nM. Time-dependent decreases in the tyrosine phosphorylation of KIT were observed when GIST-T1 cells were treated with 400 nM imatinib for 0, 1, 6 and 24 hr (Fig. 1*a*). These time-points were used for subsequent MS analysis, with 0 hr used as a control.

By utilizing immunoaffinity enrichment of phosphotyrosine peptides with quantitative phosphoproteomic analysis using iTRAQ technology combined with nano LC-MS/MS analysis, we identified 171 tyrosine phosphorylation sites spanning 134 proteins (Supporting Information Table S1). After imatinib treatment, a total of 11 phosphotyrosine sites spanning 11 proteins exhibited increases of >1.5-fold and 21 phosphotyrosine sites spanning 15 proteins showed decreases of <0.3-fold (Table 1). As a representative protein, we confirmed a dramatic decrease in the tyrosine phosphorylation levels of the KIT protein (Y609, Y703, Y747, Y823 and Y936). In contrast, imatinib induced increased phosphorylation of FYN (Y420) and FAK (Y576). Phosphorylation of FYN (Y420) upregulates tyrosine kinase activities, and phosphorylation of FAK (Y576) is critical for its maximal catalytic activity.

To validate these results obtained from iTRAQ analysis, we further examined the tyrosine phosphorylation of FYN and FAK using western blotting. As shown in iTRAQ analysis, western blotting confirmed that FYN (Y420) and FAK (Y576) were time dependently phosphorylated (Figs. 1*b* and 2*c*). When tyrosine phosphorylation in the activation loop was measured for other Src family kinases, we found that imatinib treatment did not increase tyrosine phosphorylation of SRC, LYN, LCK or YES (Fig. 1*b*).

Inhibition of FYN or FAK enhances imatinib sensitivity of GIST-T1 cells

To examine the functions of FYN in GIST-T1 cells exposed to imatinib, FYN expression was stably suppressed using a FYN shRNA plasmid. We cloned and established GIST-T1 B1, B2 and B3 cells, as well as GIST-T1 C cells transfected with empty vector as a control (Fig. 1*d*). Compared with GIST-T1 C cells, the FYN knockdown cell lines showed significantly decreased IC_{50} values for imatinib ($p < 0.05$; Fig. 1*d*).

We also examined the role of FAK activation in imatinib treatment. We repressed FAK expression using siRNA, and we used a FAK inhibitor (TAG372) to inhibit FAK phosphorylation. Transfection of FAK siRNA reduced IC_{50} values for imatinib ($p < 0.05$; Fig. 1*e*). When used with imatinib, TAG372 further decreased cell survival in a dose-dependent manner (Fig. 1*f*).

Inhibition of FAK improves imatinib sensitivity in GIST-T1-R cells

Next, we examined FAK activation in imatinib-resistant cell lines established from GIST-T1 cells. Eight imatinib-resistant cell lines were established by incubation of GIST-T1 cells with imatinib of gradually increased concentrations. Constitutive phosphorylation of FAK was observed in two imatinib-resistant

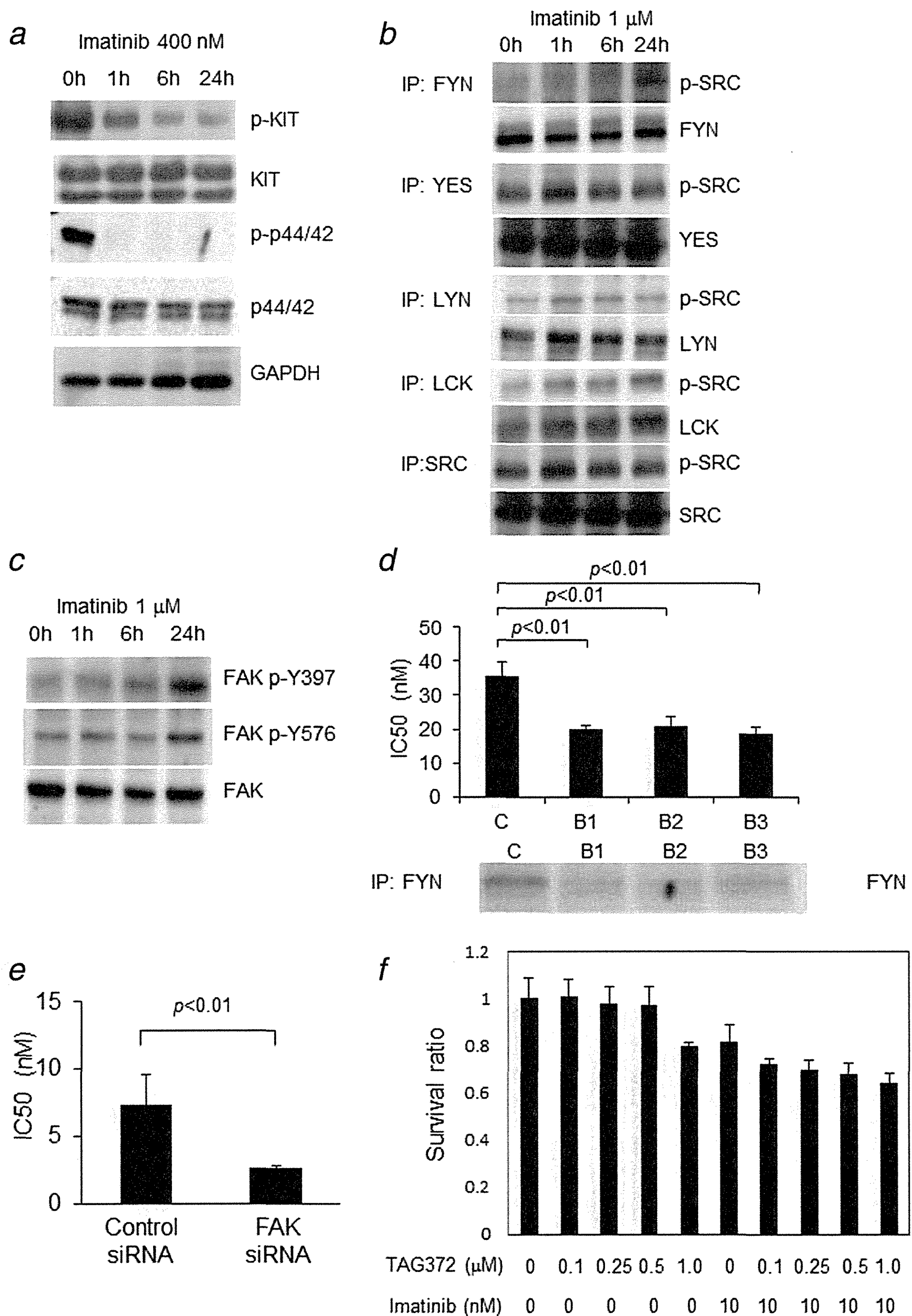

Figure 1. (*a*) Altered tyrosine phosphorylation levels detected by iTRAQ analysis were confirmed by western blotting. Imatinib treatment led to reduced phosphotyrosine levels. (*b*) Imatinib treatment changed SRC family kinase phosphorylation levels. (*c*) iTRAQ analysis showed that FYN (Y420) and FAK (Y576) exhibited similar tyrosine phosphorylation. (*d*) Compared with in GIST-T1 C cells, the IC_{50} for imatinib was significantly reduced in all FYN knockdown GIST-T1 cells (B1, B2 and B3). (*e*) The IC_{50} for imatinib was reduced in GIST-T1 cells that were transfected with FAK siRNA. (*f*) WST-8 assay showed the cell viability with imatinib and TAG372 treatment. Data are presented as means ± SD.

Table 1. Phosphotyrosine peptides that were increased or decreased by imatinib treatment, as quantified by iTRAQ analysis[1]

Accession Number	Sequence	Description	Site	1 hr/0 hr[2]	6 hr/0 hr[2]	24 hr/0 hr[2]
Greater than 1.5-fold increase of phosphorylated peptide at 24 hr compared with 0 hr						
P06241	LIEDNEYTAR	Tyrosine-protein kinase Fyn	Y420	0.845	2.365	3.641
P15880	AFVAIGDYNGHVGLGVK	40S ribosomal protein S2	Y133	2.138	1.807	2.443
O00401	VIYDFIEK	Neural Wiskott-Aldrich syndrome protein	Y256	0.768	1.491	1.978
Q99623	MLGEALSKNPGYIK	Prohibitin-2	Y248	1.446	1.778	1.726
P62829	NLYIISVK	60S ribosomal protein L23	Y38	2.138	1.348	1.679
P18433	VVQEYIDAFSDYANFK	Receptor-type tyrosine-protein phosphatase alpha	Y791	1.245	1.098	1.667
P30040	FDTQYPYGEK	Endoplasmic reticulum resident protein 29	Y64	1.804	2.558	1.640
Q05397	YMEDSTYYK	Focal adhesion kinase 1	Y570	1.043	0.893	1.606
A6NI28	LDTASSNGYQRPGSVVAAK	Rho GTPase-activating protein 42	Y792	0.599	0.980	1.594
P08758	LYDAYELK	Annexin A5	Y94	1.043	1.399	1.581
P18669	HYGGLTGLNK	Phosphoglycerate mutase 1	Y92	0.930	2.583	1.532
Less than 0.3-fold reduction of phosphorylated peptide at 24 hr compared with 0 hr						
P10721	IGSYIER	Mast/stem cell growth factor receptor Kit	Y747	0.649	0.244	0.291
O95490	SENEDIYYK	Latrophilin-2	Y1350	0.431	0.318	0.256
Q92569	LQEYHSQYQEK	Phosphatidylinositol 3-kinase regulatory subunit gamma	Y184	0.433	0.271	0.255
P10721	QEDHAEAALYK	Mast/stem cell growth factor receptor Kit	Y703	0.560	0.207	0.254
P10721	QISESTNHIYSNLANCSPNR	Mast/stem cell growth factor receptor Kit	Y936	0.445	0.212	0.244
Q92796	RDNEVDGQDYHFVVSR	Disks large homolog 3	Y673	0.556	0.427	0.241
Q5XXA6	STIVYEILKR	Anoctamin-1	Y251	0.508	0.720	0.235
Q00535	IGEGTYGTVFK	Cyclin-dependent kinase 5	Y15	1.877	0.821	0.232
Q06481	MQNHGYENPTYK	Amyloid-like protein 2	Y755	0.474	–[4]	0.220
O95297	INKSESVVYADIR	Myelin protein zero-like protein 1	Y263	0.785	0.249	0.211
Q969M3	QYAGYDYSQQGR	Protein YIPF5	Y42	0.145	–[4]	0.195
O14964	VVQDTYQIMK	Hepatocyte growth factor-regulated tyrosine kinase substrate	Y132	1.097	0.464	0.173
O95297	SESVVYADIR	Myelin protein zero-like protein 1	Y263	0.563	0.169	0.166
Q92569	VQAEDLLYGKPDGAFLIR	Phosphatidylinositol 3-kinase regulatory subunit gamma	Y373	1.186	0.176	0.130
O96000	YQDLGAYSSAR	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	Y143	0.864	0.393	0.122
P16333	LYDLNMPAYVK	Cytoplasmic protein NCK1	Y112	0.651	0.257	0.103
Q12846	NILSSADYVER	Syntaxin-4	Y251	0.179	0.131	0.072
Q8N128	YQYAIDEYYR	Protein FAM177A1	Y162	0.196	0.090	0.055
P10721	VVEATAYGLIK	Mast/stem cell growth factor receptor Kit	Y609	0.723	–[4]	0.023
P10721	DIKNDSNYVVK	Mast/stem cell growth factor receptor Kit	Y823	0.164	–[4]	–[4]
P53778	QADSEMTGYVVTR	Mitogen-activated protein kinase 12	Y185	1.582	0.888	–[4]

[1]The full list is provided in Supporting Information Table 1.
[2]The ratio of peptide derived from the iTRAQ reporter ion, as determined by iTRAQ analysis; 1 hr/0 hr, 6 hr/0 hr and 24 hr/0 hr refer, respectively, to the value of each peptide at 1, 6 and 24 hr divided by the value of that peptide at 0 hr.
[3]A 0-hr value of 0 (*i.e.* below background) and a positive value at 1, 6 or 24 hr.
[4]A 0-hr positive value and a value of 0 (*i.e.* below background) at 1, 6 or 24 hr.

GIST-T1 cell lines (GIST-T1-R2 and GIST-T1-R8), which had imatinib IC_{50} values of 30 μM, ~1000 times that of the GIST-T1 parent cells (Fig. 2*a*). TAG372 dose dependently inhibited FAK phosphorylation in GIST-T1-R2 (Fig. 2*b*), and significantly reduced the imatinib IC_{50} values when both drugs were used (Fig. 2*c*). Moreover, TAG372 induced apoptosis in GIST-T1-R2 (Fig. 2*d*). These results indicate that imatinib treatment induced activation of FYN and FAK in persistent GIST-T1 cells and was associated with imatinib insensitivity and resistance.

Discussion

Imatinib is a selective tyrosine kinase inhibitor of KIT, PDGFRA, ABL/BCR-ABL and CSF-1R, which was first used to treat GIST in 2000. Since then, it has been a standard treatment for advanced and/or recurrent GISTs.[6] Patients with advanced GIST usually respond to imatinib; however, most patients eventually experience disease progression with the reactivation of KIT tyrosine kinase and its downstream signaling pathways.[14,15] Although imatinib has high activity against GISTs, it cannot achieve complete eradication of tumor cells *in vivo* or *in vitro*. Results of the BFR14 trial showed that stopping imatinib treatment, even after complete response, resulted in disease progression or recurrence.[7,8] In chronic myelogenous leukemia (CML), mathematical models have indicated that secondary mutations might emerge after imatinib therapy;[16] therefore, residual tumor cells may also be a predisposing factor for acquired resistance to imatinib in GIST.

To quantitate the alteration of tyrosine phosphorylation levels induced by imatinib, we established quantitative tyrosine phosphoproteomic analysis. Using this technology, we identified 171 different tyrosine phosphorylation sites in 134 proteins of GIST-T1 cells. As we were searching for alternative pathways that were activated after inhibition of KIT signaling by imatinib, we pursued tyrosine kinases with increased tyrosine phosphorylation. Our comprehensive measures indicated that 11 tyrosine kinases exhibited tyrosine phosphorylation increases of greater than 1.5-fold (Table 1). The findings of the phosphoproteomic analysis were confirmed by western blotting showing the tyrosine-phosphorylation of KIT, FAK and other src-family kinases. SRC and LYN are reportedly phosphorylated and activated in GIST after imatinib treatment;[18] however, in the phosphoproteomic and western blotting analyses in the present study, we did not detect activation of SRC, LYN, YES, Lck or any other Src family kinases, except for FYN and FAK. Activation of FYN and FAK is reportedly involved in tumor proliferation and malignant transformation.[17–20]

FYN appears to participate in cell growth and survival, acting downstream of integrin and PI3K.[17,21] Although the

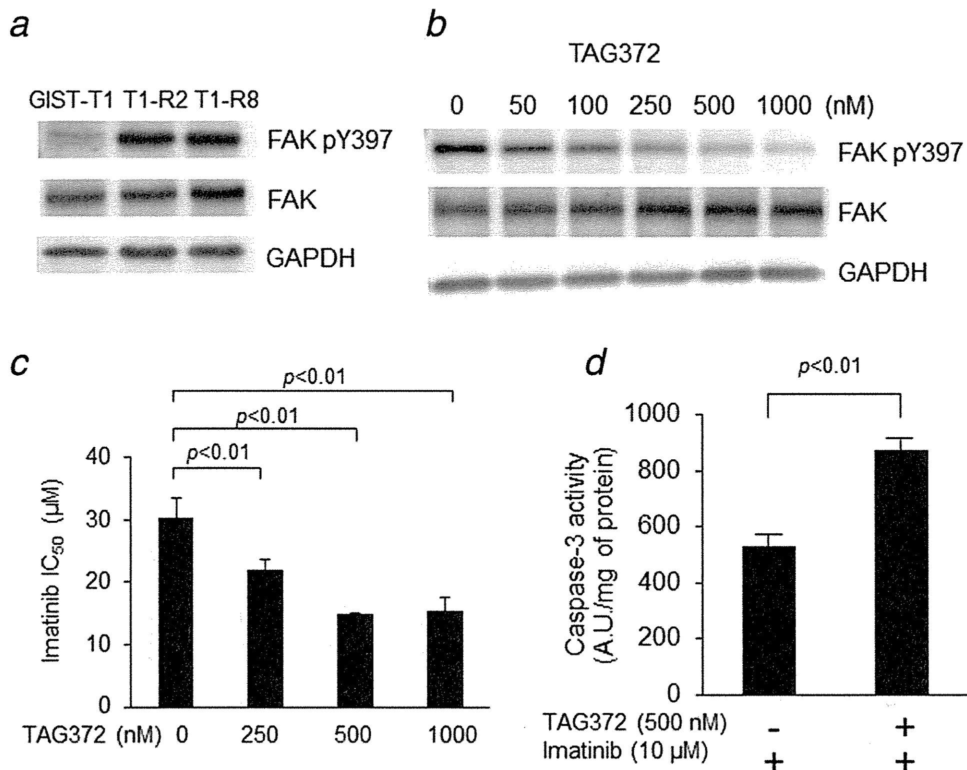

Figure 2. (*a*) Constitutive phosphorylation of FAK was observed in GIST-T1-R cells (GIST-T1-R2 and GIST-T1-R8). (*b*) Constitutive phosphorylation of FAK was observed in imatinib-resistant GIST-T1 cells (GIST-T1-R2), and TAG372 dose dependently inhibited this phosphorylation. (*c*) TAG372 reduced the IC_{50} of imatinib and induced apoptosis in GIST-T1-R2 cells. (*d*) TAG372 induced apoptosis in GIST-T1-R2 cells. Data are presented as means ± SD.

shRNA silencing of FYN induced additional cell death in GIST-T1 cells during imatinib treatment, dasatinib (a SRC family kinase inhibitor) had no effect in combination with imatinib (data not shown). These results suggest the involvement of FYN in alternative survival signaling pathways.

FAK is also a nonreceptor tyrosine kinase that is activated through autophosphorylation at Tyr^{397} by integrin and growth factor receptors; this is followed by subsequent activation of other functional phosphorylation sites to transduce the signals to downstream pathways.[22,23] FAK is reportedly overexpressed in malignant GISTs and correlated with recurrence.[19] Furthermore, FAK phosphorylation is associated with imatinib-resistance of a KIT exon 17 mutation, but not exon 11 mutation.[20] This imatinib-resistance was diminished by TAE226, which inhibits FAK and insulin-like growth factor-1 receptor. Our findings showed that imatinib induced time-dependent FAK activation in GIST-T1 cells with an imatinib-sensitive mutation of KIT exon 11. Moreover, FAK inhibition using either a FAK-specific TAG372 inhibitor or siRNA decreased the viability of GIST-T1 cells under imatinib treatment. TAG372 also induced apoptosis in imatinib-resistant cell lines with FAK activation. Taken together, it appears that FAK activation may be a critical survival signal of GIST cells under imatinib treatment, and targeting FAK with imatinib may be a promising therapeutic approach.

When imatinib was removed, KIT was quickly rephosphorylated, and FYN and FAK were simultaneously dephosphorylated (data not shown), suggesting that alternative pathway activation is functional during imatinib treatment, and may not be accompanied with qualitative changes, such as new mutations. FYN inhibition was accompanied with a subsequent lack of FAK activation, while FAK inhibition resulted in increased FYN phosphorylation, thus indicating that FYN acted upstream of FAK. However, the critical changes that induce FYN activation and subsequent FAK phosphorylation remain unknown. Additional studies are required to elucidate the FYN activation mechanisms and the alternative pathways induced by imatinib, as well as their association with acquired drug resistance.

In summary, here we found that imatinib induced increased tyrosine phosphorylation of FYN and FAK in GIST-T1 cells. Blockade of these tyrosine kinases might be a potential target to overcome imatinib resistance. Additionally, iTRAQ-based quantitative phosphotyrosine-focused proteomic analysis appears to be a useful approach to screening for phosphoproteins associated with drug resistance.

Acknowledgments

This study was partly supported by grants from Novartis Pharmaceuticals. **Conflict of interest**: N.T. has honoraria to disclose from the Novatis, Pfizer and Bayer within past 2 years.

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Subcuticular sutures versus staples for skin closure after open gastrointestinal surgery: a phase 3, multicentre, open-label, randomised controlled trial

CrossMark

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Summary

Background Staples have been widely used for skin closure after open gastrointestinal surgery. The potential advantages of subcuticular sutures compared with staples have not been assessed. We assessed the differences in the frequency of wound complications, including superficial incisional surgical site infection and hypertrophic scar formation, depending on whether subcuticular sutures or staples are used.

Methods We did a multicentre, open-label, randomised controlled trial at 24 institutions between June 1, 2009, and Feb 28, 2012. Eligible patients aged 20 years or older, with adequate organ function and undergoing elective open upper or lower gastrointestinal surgery, were randomly assigned preoperatively to either staples or subcuticular sutures for skin closure. Randomisation was done via a computer-generated permuted-block sequence, and was stratified by institution, sex, and type of surgery (ie, upper or lower gastrointestinal surgery). Our primary endpoint was the incidence of wound complications within 30 days of surgery. Analysis was done by intention to treat. This study is registered with UMIN-CTR, UMIN000002480.

Findings 1080 patients were enrolled and randomly assigned in a one to one ratio: 562 to subcuticular sutures and 518 to staples. 1072 were eligible for the primary endpoint and 1058 for the secondary endpoint. Of the 558 patients who received subcuticular sutures, 382 underwent upper gastrointestinal surgery and 176 underwent lower gastrointestinal surgery. Wound complications occurred in 47 of 558 patients (8·4%, 95% CI 6·3–11·0). Of the 514 who received staples, 413 underwent upper gastrointestinal surgery and 101 underwent lower gastrointestinal surgery. Wound complications occurred in 59 of 514 (11·5%, 95% CI 8·9–14·6). Overall, the rate of wound complications did not differ significantly between the subcuticular sutures and staples groups (odds ratio 0·709, 95% CI 0·474–1·062; p=0·12).

Interpretation The efficacy of subcuticular sutures was not validated as an improvement over a standard procedure for skin closure to reduce the incidence of wound complications after open gastrointestinal surgery.

Funding Johnson & Johnson.

Lancet 2013; 382: 1105–12

See Comment page 1076

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Introduction

Wound complications are among the most common issues reported after surgery, and are often very problematic for patients in terms of cosmetic appearance, decreased quality of life, prolonged hospital stays, and increased health-care costs.[1,2] Several publications have addressed ways to reduce the risk of wound complications associated with surgery,[3–6] such as intraoperative administration of antimicrobial prophylaxis,[4,5] skin preparation, barrier retractional wound protection,[7] use of absorbable sutures during intraperitoneal procedures,[8,9] and pulsatile lavage irrigation of wounds before closure.[10,11] Triclosan-coated sutures significantly reduced the rate of surgical site infections compared with conventional uncoated sutures in various types of surgery.[12]

Because of the increase in the number of patients with preoperative comorbidities that are risk factors for wound complications, such as malnutrition,[13] diabetes mellitus,[14] and obesity,[15] new, innovative approaches will be necessary to decrease the risk of wound complications after surgery.

Subcuticular suturing for skin closure is an attractive alternative for skin approximation in most types of surgery. It is often used in plastic surgery because of the low incidence of wound complications and good cosmetic appearance.[16–18] Compared with staples, several clinical trials have shown that subcuticular sutures are associated with a significantly lower incidence of wound complications and better cosmetic results after orthopaedic surgery,[19] cardiovascular surgery,[20,21] and caesarean section.[22,23]

In 242 patients undergoing coronary artery bypass graft surgery, Johnson and colleagues[24] prospectively closed half of each sternal and saphenous vein harvest wound with staples and half with intradermal sutures. The incidence of wound infection was similar with both methods, but significantly fewer wound complications were noted with subcuticular sutures than with staples. Additionally, patients who expressed a preference preferred sutures to staples. Basha and investigators[25] randomly assigned 435 patients undergoing caesarean delivery to stainless steel staples or subcuticular 4-0 monocryl sutures. They

reported that staple closure was associated with a four-times increased risk of wound separation (adjusted odds ratio [OR] 4·66, 95% CI 2·07–10·52; p<0·001) and poor patient satisfaction.

These trials had been done for class 1 surgical procedures—ie, clean surgery. However, the benefit of subcuticular sutures in gastrointestinal surgery, a class 2 (clean-contaminated) surgery that is associated with a high incidence of wound complications,[15,26,27] has not been fully examined.[28] Staples are the most commonly used technique for skin closure during gastrointestinal surgery because of convenience and speed. Because no consensus has been reached about how to apply findings from class 1 surgery to class 2 surgery, an optimum method of skin for gastrointestinal surgery remains to be established.

We investigated differences in prevention of wound complications between subcuticular sutures and staples after elective upper and lower gastrointestinal open surgery.

Methods

Study design and participants

We did a large-scale, multicentre, open-label, phase 3 randomised controlled trial at 24 institutions in Japan from June 1, 2009, to Feb 28, 2012. The study was organised by the Clinical Study Group of Osaka University on Risk Management (OSGO-RM), which is composed of hospitals affiliated from the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University.

Eligible patients were undergoing elective upper or lower gastrointestinal surgery, aged 20 years or older, and had adequate organ function. Patients undergoing abdomino-perineal resection for rectal cancer were also eligible, but we only assessed abdominal wounds for outcomes. We excluded patients needing emergency or laparoscopic surgery, with a history of laparotomy with a midline incision, or with long-term corticosteroid use; active infection such as peritonitis, pneumonia, or urinary tract infection; massive ascites; coagulopathy or other disorders that would preclude study participation; uncontrolled or insulin-treated diabetes; mental illness, poor general condition; severe cardiopulmonary disease; or who were deemed by surgeons to be inappropriate for participation in a randomised trial. The institutional review board of each hospital approved the protocol. All patients provided written informed consent before randomisation. We did not collect data on the number of patients approached and assessed for eligibility.

Randomisation and masking

Patients were recruited by the investigators and treatment allocation was made preoperatively after confirming eligibity.

Enrolment was done through a web-based system established for this trial and randomisation by a computer-generated permuted-block sequence. The size of the blocks used for randomisation was four. Patients were randomly assigned (1:1) to either subcuticular sutures or staples for skin closure and balanced according to institution, sex, and type of surgery (ie, upper or lower gastrointestinal open surgery). Investigator surgeons were informed of the treatment allocation via the internet and did the procedures. Patients and investigators were not masked to group assignment. The data centre, based at the Multicenter Clinical Study Group at Osaka University was responsible for treatment allocation, central monitoring, and statistical analyses under the supervision of the statistician in charge.

Procedures

In the subcuticular suture group, surgeons used interrupted subcuticular sutures with 3-0 or 4-0 monofilament absorbable suture (polydioxanone; PDS-II Ethicon, Tokyo, Japan). The interval of the sucuticular sutures was 15–25 mm and the length of the bite of sutures was 15–25 mm from the edge of the skin. Under this condition, the skin could be closed tightly. Use of sterile strips or skin glue for epidermal approximation in addition to subcuticular sutures was an institutional choice. In the staples group, metallic skin staples, which were the choice of individual institutions, 10–15 mm apart were used. Approximation of the fat layer was not allowed in the either group. Before the trial, investigators from participating institutions were instructed on how to do subcuticular sutures during the trial. A video in which a plastic surgeon used the subcuticular suturing technique (adopted as the standard) was provided to each participating institution. The standard procedure was also demonstrated at each investigator meeting. Investigators and physicians in training met yearly to examine how subcuticular sutures were done.

All participating institutions were asked to follow the guidelines about prevention of surgical site infections issued by the US Centers for Disease Control and Prevention (CDC).[29] Surgical gloves and instruments were changed before wound closure. Absorbable monofilament sutures were used for approximation of the fascia, and the subcutaneous space was irrigated with saline without added antibiotics. Intra-abdominal drain placement through a separate incision away from the operative incision was permitted but drainage of the wound was not allowed. Skin preparation techniques, prophylactic antibiotic administration, the volume of saline used for intra-abdominal irrigation, dressing methods, and timing of postoperative staple removal, perioperative care, and wound management were according to each participating institution's respective standards.

Our primary outcome was incidence of wound complications within 30 days of surgery. The secondary outcome was the incidence of hypertrophic scar formation 6 months after surgery. Wound complications were defined as the presence of at least one of several signs or symptoms necessitating treatment: wound disruption,

stitch abscess, abscess caused by metal allergy, seroma or haematoma, or superficial incisional surgical site infections. Superficial incisional surgical site infections are defined by the CDC[29] as infections occurring within 30 days of surgery that implicate only the skin or subcutaneous tissue of the incision. Diagnosis of superficial incisional surgical site infection must satisfy one or more of several criteria: purulent drainage (with or without laboratory confirmation) from the superficial incision, organisms isolated from an aseptically obtained culture of fluid or tissue from the superficial incision, or at least one of the signs or symptoms of infection (pain or tenderness, localised swelling, redness or heat, and superficial incision deliberately opened by the surgeon, unless the incision is culture-negative). Infection control personnel monitored and detected surgical site infections during patients' hospital stays. Changes noted in the wound were not defined as wound complications if they did not necessitate treatment. When superficial incisional surgical site infections and other wound complications coexisted in the same patient, we defined the complication as superficial incisional surgical site infections. We defined hypertrophic scar as a widened or elevated unsightly scar with erythema or pigmentation.

Responsible surgeons checked for the presence or absence of wound complications every day during the hospital stay and at every outpatient visit until 30 days after surgery. They were also responsible for checking for the presence or absence of hypertrophic scar formation at 6 months after surgery, and measured the width and length of detected hypertrophic scars. Before starting the trial, the principal investigator showed typical cases of various wound complications and hypertrophic scars, and consensus about all types of wound complications was reached by the investigators.

Statistical analysis

We planned a sample size of 530 patients per treatment group when we designed the trial. Such a sample size would provide power of 80% with a two-sided significance level of 0·05 to detect superiority in the reduction of the frequency of wound complications. Wound complications were anticipated in 11% of patients in the staples group and 6% in the subcuticular sutures group, allowing for a loss to follow-up of roughly 10%. The projected accrual period was 2 years and no interim analyses were planned.

We did the analysis on a modified intention-to-treat basis. We expressed continuous numerical data as medians and IQRs or means and SDs, when appropriate, and distribution of dichotomous data in percentages with 95% CIs. We used Fisher's exact test to compare binary variables and the Mann-Whitney U test to compare continuous variables. All p values of less than 0·05 were deemed significant.

The primary outcome was analysed with Fisher's exact test, and we used the Mantel-Haenszel test to adjust for the type of surgery, a potential confounding factor, which was not prespecified in the protocol. We used Fisher's exact test to analyse the secondary outcome and to calculate and compare outcomes as a post-hoc analysis on the basis of type of surgery.

We analysed thickness of subcutaneous fat (objectively classified by the surgeon as either thin, normal, or thick), American Society of Anesthesiologists (ASA) physical status classification,[30] operative time, intraoperative blood loss volume, duration of prophylactic antibiotics, presence of drainage tube and duration of drainage, and use of postoperative anticoagulant therapy as variables. Subgroups were analysed with logistic regression to assess for statistical interactions between treatments in various subgroups. Because of the exploratory nature of subgroup comparisons, we report test results without multiplicity adjustments for type I error. This study is registered with UMIN-CTR, UMIN000002480. UMIN-CTR is one of the network members of the Japan Primary Registries Network, which meets WHO registry criteria.

Role of the funding source

The sponsor had no roles in the study design; data collection, analysis, or interpretation; or writing of the Article. The corresponding author had full access to all

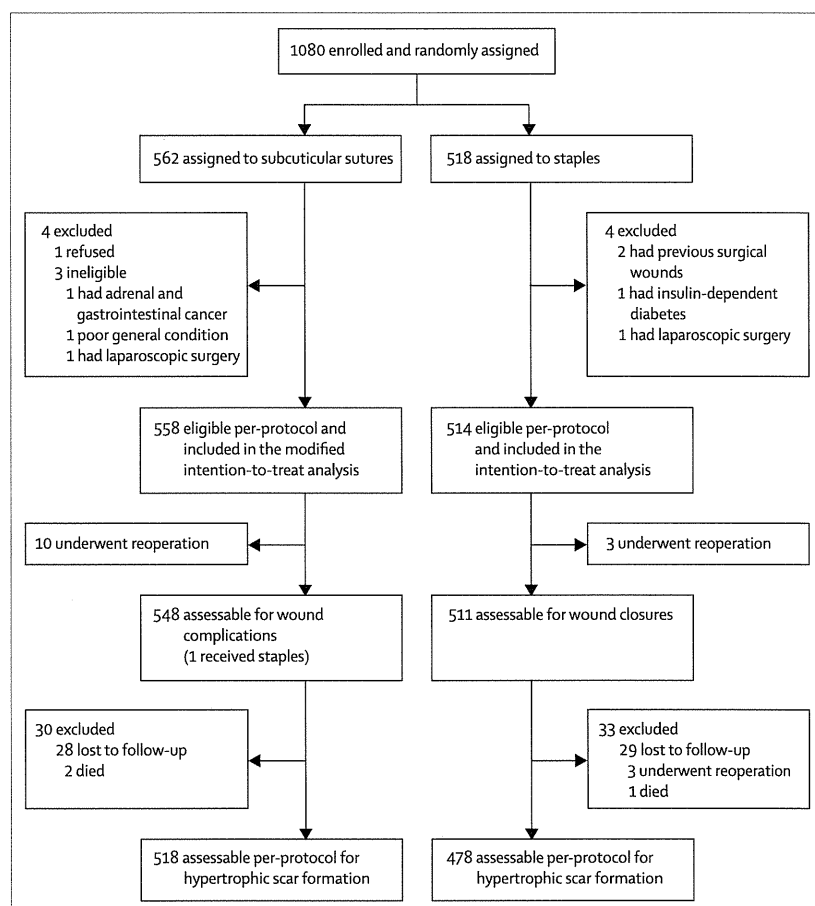

Figure 1: Trial profile

	Subcuticular sutures (n=562)	Staples (n=518)
Age (years)	68 (61–75)	68 (61–74)
Sex		
Male	388 (69·0%)	365 (70·5%)
Female	174 (31·0%)	153 (29·5%)
Surgery		
Upper gastrointestinal	385 (68·5%)	417 (80·5%)
Lower gastrointestinal	177 (31·5%)	101 (19·5%)
Thickness of subcutaneous fat*		
Thick	143 (25·6%)	109 (21·2%)
Standard	328 (58·8%)	317 (61·6%)
Thin	87 (15·6%)	89 (17·3%)
ASA physical status classification*		
1	201 (36·0%)	201 (39·0%)
2	313 (56·1%)	273 (53·0%)
3	44 (7·9%)	41 (8·0%)
Operative time (min)*	228 (180–270)	218 (175–264)
Blood loss (mL)	230 (100–430)	244 (120–450)
Wound protection†		
Surgical drape	535 (95·9%)	497 (96·3%)
Gauze	10 (1·8%)	6 (1·2%)
None	13 (2·3%)	13 (2·5%)
Duration of antibiotic prophylaxis (days)*		
1	379 (67·9%)	373 (72·4%)
2	33 (5·9%)	22 (4·3%)
3	100 (17·9%)	86 (16·7%)
≥4	46 (8·2%)	34 (6·6%)
Duration of drain insertion (days)*		
0 (ie, no drain)	118 (21·1%)	108 (21·0%)
1–3	50 (9·0%)	34 (6·6%)
≥4	390 (69·9%)	373 (72·4%)
Duration of hospital stay after surgery (days)	14 (11–21)	15 (12–21)
Anticoagulation therapy‡		
Yes	130 (23·3%)	96 (18·6%)
No	429 (76·7%)	420 (81·4%)

Data are n (%) or median (IQR). ASA=American Society of Anesthesiologists.[30] *Data missing for four patients in the subcuticular sutures group and three patients in the staples group. †Data missing for four patients in the subcuticular sutures group and two patients in the staples group. ‡Data missing for three patients in the subcuticular sutures group and two patients in the staples group.

Table 1: **Baseline demographic and clinical characteristics**

	Subcuticular sutures (n=385)	Staples (n=417)
Diseases		
Gastric cancer	375 (97·4%)	403 (96·6%)
Gastric submucosal tumour	6 (1·6%)	9 (2·2%)
Other	4 (1·0%)	5 (1·2%)
Procedures		
Total gastrectomy	149 (38·7%)	143 (34·3%)
Distal gastrectomy	186 (48·3%)	219 (52·5%)
Proximal gastrectomy	19 (4·9%)	16 (3·8%)
Exploratory laparotomy	4 (1·0%)	4 (1·0%)
Other	27 (7·0%)	35 (8·4%)

Data are n (%).

Table 2: **Types of diseases and surgical procedures in patients undergoing upper gastrointestinal surgery**

	Subcuticular sutures (n=177)	Staples (n=101)
Diseases		
Colon cancer	98 (55·4%)	51 (50·5%)
Rectal cancer	71 (40·1%)	48 (47·5%)
Anal cancer	2 (1·1%)	1 (1·0%)
Other	6 (3·4%)	1 (1·0%)
Procedures		
Right hemicolectomy	41 (23·2%)	28 (27·7%)
Left hemicolectomy	44 (24·9%)	8 (7·9%)
Low anterior resection	61 (34·5%)	38 (37·6%)
Abdominoperineal resection	11 (6·2%)	10 (9·9%)
Partial resection of colon	9 (5·1%)	10 (9·9%)
Other	11 (6·2%)	7 (6·9%)

Data are n (%).

Table 3: **Types of diseases and surgical procedures in patients undergoing lower gastrointestinal surgery**

the data and was responsible for the decision to submit for publication.

Results

Figure 1 shows the trial profile. 1080 patients from 24 institutions were enrolled and randomly assigned—562 to subcuticular sutures and 518 to staples. Assessment of case report forms showed that four patients in each group were ineligible for inclusion, and thus the modified intention-to-treat population comprised 558 patients in the subcuticular sutures group and 514 in the staples group (figure 1). Ten patients in the subcuticular sutures group and three in the staples group needed reoperation within 30 days, which met the exclusion criterion, a history of laparotomy, and thus were not assessed for wound complications.

Distribution of most demographic and clinical characteristics of enrolled patients was balanced between groups except type of surgery (table 1). Tables 2 and 3 show details of the diseases and surgical procedures in the two groups. 417 patients who underwent upper gastrointestinal surgery were allocated to the staples group and 385 to the subcuticular sutures group, and 177 patients who underwent lower gastrointestinal surgery were allocated to the subcuticular sutures group and 101 to the staples group.

In the subcuticular sutures group, wound complications occurred in 47 of 558 (8·4%, 95% CI 6·3–11·0) patients, including 36 (6·4%, 4·6–8·8) patients with superficial incisional surgical site infections. In the staples group, wound complications occurred in 59 of 514 patients

	All patients				Upper gastrointestinal surgery				Lower gastrointestinal surgery			
	Subcuticular suture (n=558)	Staples (n=514)	Odds ratio (95% CI)	p	Subcuticular sutures (n=382)	Staples (n=413)	Odds ratio (95% CI)	p	Subcuticular sutures (n=176)	Staples (n=101)	Odds ratio (95% CI)	p
Primary outcome												
Wound complication rate*	47 (8·4%)	59 (11·5%)	0·709 (0·474–1·062)	0·12	29 (7·6%)	39 (9·4%)	0·788 (0·459–1·339)	0·38	18 (10·2%)	20 (19·8%)	0·463 (0·217–0·978)	0·0301
Component outcomes												
Surgical site infection (superficial incisional)	36 (6·4%)	36 (7·0%)	0·928 (0·558–1·543)	0·81	23 (6·0%)	20 (4·8%)	1·259 (0·649–2·461)	0·53	13 (7·4%)	16 (15·8%)	0·425 (0·179–0·992)	0·0399
Non-surgical-site infection	11 (2·0%)	23 (4·5%)	0·435 (0·189–0·940)	0·0238	6 (1·6%)	19 (4·6%)	0·331 (0·107–0·875)	0·0149	5 (2·8%)	4 (4·0%)	0·710 (0·149–3·666)	0·73
Wound separation	3 (0·5%)	8 (1·6%)	0·346 (0·059–1·453)	0·13	1 (0·3%)	6 (1·5%)	0·178 (0·004–1·480)	0·13	2 (1·1%)	2 (2·0%)	0·570 (0·041–7·979)	0·62
Seroma	5 (0·9%)	12 (2·3%)	0·383 (0·105–1·179)	0·09	3 (0·8%)	11 (2·7%)	0·290 (0·052–1·108)	0·06	2 (1·1%)	1 (1·0%)	1·149 (0·059–68·457)	1·00
Haematoma	1 (0·2%)	2 (0·4%)	0·466 (0·008–8·969)	0·61	0 (0·0%)	1 (0·2%)	..	..	1 (0·6%)	1 (1·0%)	0·573 (0·007–45·300)	1·00
Other	2 (0·4%)	1 (0·2%)	1·867 (0·097–110·358)	1·00	2 (0·5%)	1 (0·2%)	2·166 (0·112–128·141)	0·61	0 (0·0%)	0 (0·0%)	..	..

Significance was calculated with Fisher's exact test. *Adjusted odds ratio 0·658 (95% CI 0·438–0·988; p=0·0438 [calculated with Mantel-Haenszel test]).

Table 4: **Primary outcome and its components in modified intention-to-treat population**

(11·5%, 8·9–14·6), including 36 (7·0%, 5·0–9·6) with superficial incisional surgical site infections (table 4). As a primary outcome, the number of wound complications did not differ significantly between the two groups (OR 0·709, 95% CI 0·474–1·062; p=0·12). Since we identified confounding with the stratified factor, type of surgery, adjustment was done to show a significant difference (0·658, 0·438–0·988; p=0·0438), although this was not prespecified.

Post-hoc exploratory analyses showed that wound complications excepting surgical site infections occurred significantly less often in the subcuticular suture group than in the staples group overall (OR 0·435, 95% CI 0·189–0·940; p=0·0238) and in patients who underwent upper gastrointestinal surgery (0·331, 0·107–0·875; p=0·0149). In patients who underwent lower gastrointestinal surgery, significantly fewer wound complications (0·463, 0·217–0·978; p=0·0301) and superficial incisional surgical site infections (0·425, 0·179–0·992; p=0·0399) were noted in the subcuticular sutures than in the staples group (table 4).

Table 5 summarises secondary outcomes. Significantly fewer hypertrophic scars formed in the subcuticular sutures group than in the staples group overall (OR 0·726, 0·528–0·998; p=0·0429) and specifically in patients who underwent upper gastrointestinal surgery (0·672, 0·465–0·965; 0·0282).

We did a post-hoc subset analysis to identify potential interactions between wound complications and background factors (figure 2). Significant risk reduction for wound complications was noted with subcuticular sutures compared with staples in male patients (*vs* female patients), lower gastrointestinal surgery (*vs* upper gastrointestinal surgery), cases with operative time of 220 min or greater (*vs* those with operative times <220 min), and patients receiving postoperative anticoagulant therapy (*vs* those not receiving such therapy). We did not identify any important treatment-related adverse events for stapling or subcuticular sutures.

	n	Hypertrophic scar formation	Odds ratio (95% CI)	p
All patients			0·726 (0·528–0·998)	0·0429
Subcuticular sutures	558	93 (16·7%)		
Staples	514	111 (21·6%)		
Upper gastrointestinal surgery			0·672 (0·465–0·965)	0·0282
Subcuticular sutures	382	66 (17·3%)		
Staples	413	98 (23·7%)		
Lower gastrointestinal surgery			1·226 (0·576–2·729)	0·72
Subcuticular sutures	176	27 (15·3%)		
Staples	101	13 (12·9%)		

Data for hypertrophic scar formation are n (%). Significance was calculated with Fisher's exact test.

Table 5: **Secondary outcomes in the modified intention-to-treat population**

Discussion

Subcuticular sutures for skin closure have been advocated instead of staples in clean (class 1) surgery, including cardiovascular surgery,[24] orthopaedic surgery,[19] and caesarean delivery,[25] on the basis of the results of randomised studies. Whether these results can be applied to class 2 surgery, as represented by gastrointestinal surgery, is of concern. Classification of the types of surgery is described in panel 1. Our results show that subcuticular sutures did not significantly reduce the frequency of

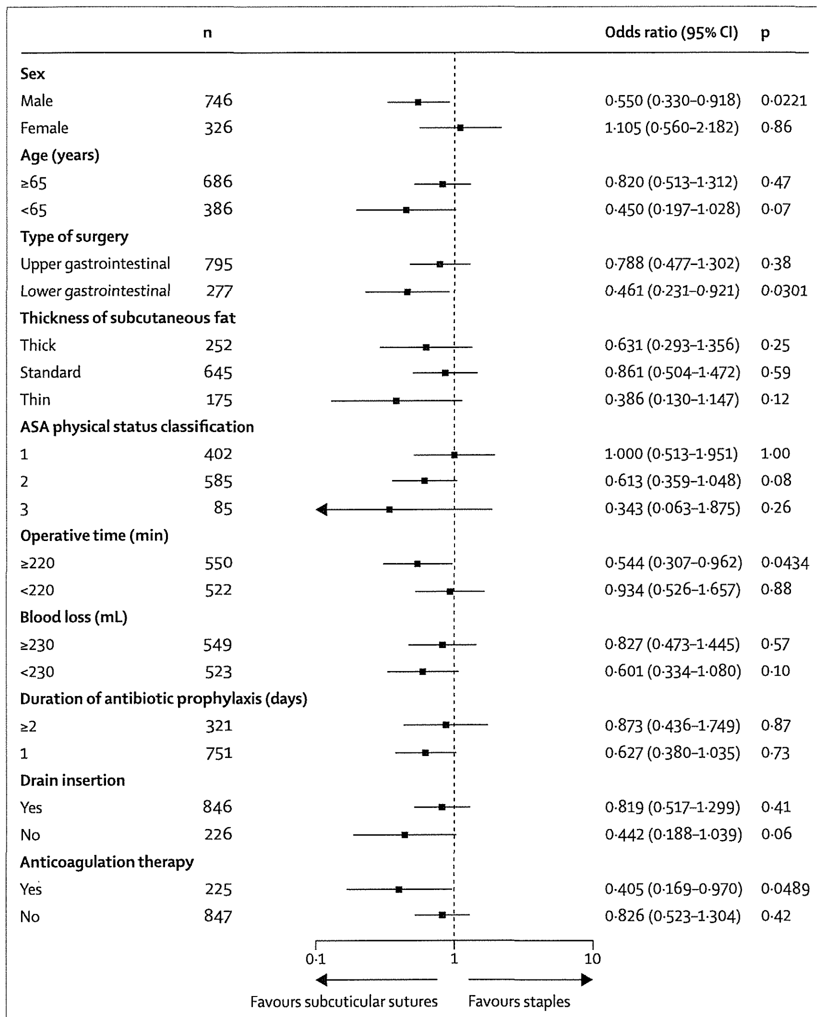

***Figure 2*: Subset analysis of wound complications in the modified intention-to-treat population**
Significance was calculated with Fisher's exact test. ASA=American Society of Anesthesiologists.

wound complications as a primary outcome and therefore subcuticular sutures are not validated as a new standard procedure for skin closure after gastrointestinal surgery (panel 2). As a secondary outcome, we noted fewer hypertrophic scars formed when subcuticular sutures were used than when staples were used.

Our sample size calculation was done on the assumption that the incidence of wound complications was 7·5% with upper gastrointestinal surgery and 15% with lower gastrointestinal surgery when staples were used and the expected number of patients receiving the respective surgery was equal (1:1), which gave the incidence of wound complications as 11%. We postulated that a 5% reduction of the incidence of wound complications by subcuticular sutures was necessary to be a new standard procedure for skin closure. There are several reasons why we did not obtain the results we expected. We showed that the incidences of wound complications were 8·4% in the subcuticular sutures group and 11·5% in the staples group (p=0·12) in all patients, 7·6% and 9·4% (p=0·38) in upper gastrointestinal surgery, and 10·2% and 19·8% (p=0·0301) in lower gastrointestinal surgery (table 4). Subcuticular sutures were more effective in lower gastrointestinal surgery, whereas enrolment of patients receiving open lower gastrointestinal surgery was substantially lower than that of patients receiving open upper gastrointestinal surgery (278 *vs* 802) because laparoscopic surgery has become more prevalent in lower gastrointestinal surgery. Although we included type of surgery as one of our stratification variables, more patients who underwent lower gastrointestinal surgery received subcuticular sutures than staples (177 *vs* 101) and more patients who underwent upper gastrointestinal surgery received staples than subcuticular sutures (417 *vs* 385) as a result of the unexpected unbalanced allocation (tables 2, 3), which might be caused by participation of many institutions and the presence of three stratification factors. These factors attenuated the postulated effect of subcuticular sutures and the analysis of the primary outcome did not reach significance. When adjusting for the type of surgery, subcuticular sutures seemed to confer a benefit, although this result is not conclusive. Thus, preferential use of subcuticular sutures might be supported in some circumstances. Although we did not analyse outcomes of individual institutions, there was possibility of heterogeneity with regard to the effect of subcuticular sutures caused by as many as 24 institutions.

Before this trial, few data for potential differences in the rate of wound complications and hypertrophic scar formation between upper and lower gastrointestinal surgery were available. That the incidence of superficial incisional surgical site infections was higher with lower gastrointestinal surgery than with upper gastrointestinal surgery had been previously reported,[15] which was the reason why we used type of surgery as a stratification factor. We showed that the incidence of total wound complications and superficial incisional surgical site infections was significantly higher in lower than in upper gastrointestinal surgery, whereas the incidence of

***Panel 1*: Classification of types of surgery (class 1 and 2)[29]**

Class 1 (clean)
An uninfected operative wound in which no inflammation is encountered and the respiratory, alimentary, genital, or uninfected urinary tract is not entered. For example, skin procedures (ie, biopsies), simple orthopaedic surgery, vascular surgery, and elective caesarean section.

Class 2 (clean-contaminated)
An operative wound in which the respiratory, alimentary, genital, or urinary tracts are entered under controlled conditions and without unusual contamination. For example, gastrointestinal surgery, thoracic procedures, gynaecological procedures, and emergency caesarean section.

hypertrophic scar formation was higher in upper than in lower gastrointestinal surgery. Subcuticular sutures reduced the incidence of wound complications compared with staples in lower gastrointestinal surgery and the formation of hypertrophic scars in upper gastrointestinal surgery, possibly because of the higher number of events of those types in these types of surgery, respectively.

Subset analysis showed that subcuticular sutures resulted in significantly fewer wound complications in some subgroups, such as lower gastrointestinal surgery, longer operative time, and postoperative anticoagulant therapy, and the frequency of wound complications in almost all subsets of patients was lower in the subcuticular sutures group than in the staples group.

It is reasonable to employ subcuticular sutures in other types of gastrointestinal surgery, especially hepatobiliary or pancreatic surgery, which exert extensive surgical stress and are associated with large volumes of blood loss, long operative times, and a high incidence of surgical site infections.[41,42] We did not include hepatobiliary or pancreatic surgery in this trial because they contain a wide variety of surgical procedures and different levels of surgical site infection rates. The results of our subset analysis imply that subcuticular sutures could be applied to other types of gastrointestinal surgery and might reduce wound complications.[43]

We persuaded investigators to follow the US national surgical infection prevention guidelines, which recommend that antibiotic prophylaxis should be discontinued within 24 h of surgery.[4] As a result, 67·9% in the subcuticular sutures group and 72·4% in the staples group received prophylaxis with antibiotics for 1 day in this trial. Compared with the result of a national cohort study in the USA,[44] reporting that about 60% of patients who had major surgery were still receiving antimicrobial prophylaxis at 24 h after surgery, our results were acceptable. We did not find an imbalance between the groups.

Our study had several limitations. First, the absence of masking could have biased the detection of wound complications. However, assessment of surgical site infections was done by infection control personnel at the participating institutions who did not have roles in trial design or conduct. Detection of other wound complications was based on whether some treatment (dressing or surgical intervention) for wound management was documented in the medical record, which could minimise bias. However, it was possible that the open nature of our trial might have affected the findings. The Japanese insurance system and common clinical practice permitted examination of patients by responsible surgeons at outpatient clinics 1 month and 6 months after surgery, which allowed for accurate assessment of the wound even though allocation was not masked.

Second, it has been reported that subcuticular sutures for skin closure have advantages compared with staples with regard to cosmetic considerations,[16–18] patient satisfaction,[24,25] and wound handling.[24,25] Nevertheless, we did not assess patients' satisfaction, patients' preference, or potential overall effects on the health-care system, and we did not use a validated scale to assess scars. We did not directly compare costs either, but the price of one stapling device and that of two packs of PDS-II sutures were roughly the same and median operative time was 10 min longer in the subcuticular sutures group (table 1).

In conclusion, the efficacy of subcuticular sutures was not validated as an improvement over a standard procedure for skin closure after gastrointestinal surgery.

Panel 2: Research in context

Systematic review

We searched Medline and the Cochrane Database of Systematic Reviews with the terms "subcuticular suture, cutaneous closure, or dermal closure", "staple or staple closure", and "randomised controlled trial or phase 3 trial". We identified 11 randomised trials: four for caesarean delivery,[25,31–33] three for cardiovascular surgery,[24,34,35] two for orthopaedic surgery,[19,36] one for gynaecological surgery,[41] and one for laparotomy.[28] All these surgical procedures are class 1 (clean) surgery except for laparotomy, for which the details of the specific surgical procedures were not specified in the report. Six trials recommended subcuticular sutures[19,24,25,28,32,34] and four[31,35–37] showed equivalent results for sutures and staples. Only one trial recommended staples.[33] Most were small-scale trials (n=48–435). The number of patients in the trials with equivalent results ranged from 77 to 187. Three[23,38,39] of the four meta-analyses about caesarean delivery recommended subcuticular sutures; the other showed similar outcomes with sutures and staples.[40] A meta-analysis[21] of cardiovascular surgery recommended subcuticular sutures to reduce the number of wound complications. We identified no randomised trials in gastrointestinal surgery.

Interpretation

To our knowledge, our trial is the first done in gastrointestinal surgery (a class 2 surgery). Although the results of most randomised trials done in class 1 surgery support the use of subcuticular sutures to reduce wound complications and improve cosmetic outcomes, the benefits of subcuticular sutures in clean-contaminated surgeries remain unclear. This trial failed to prove subcuticular sutures were a new standard procedure for skin closure after gastrointestinal surgery; however, the formation of hypertrophic scars was significantly reduced with subcuticular sutures compared with staples.

Contributors

TT and KY drafted the paper. TT designed the protocol. YD and MM supervised the design of the trial and assisted with doing the trial. SK and TS obtained and analysed the data. TT, KU, and TI were the main investigators. All other authors participated in study conduct and recruitment of patients.

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Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments
This study was partly funded by Johnson & Johnson. We thank Satoshi Morita (Yokohama City University) for his valuable advice on statistical analysis and interpretation. Data collection was supported by Media Planning).

For more on **Media Planning** see http://www.mediapng.co.jp

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BJC

FULL PAPER

British Journal of Cancer (2013) 109, 502–511 | doi: 10.1038/bjc.2013.320

Keywords: microRNA; miR-320c; pancreatic cancer; gemcitabine resistance; SMARCC1

miR-320c regulates gemcitabine-resistance in pancreatic cancer via SMARCC1

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Background: Gemcitabine-based chemotherapy is the standard treatment for pancreatic cancer. However, the issue of resistance remains unresolved. The aim of this study was to identify microRNAs (miRNAs) that govern the resistance to gemcitabine in pancreatic cancer.

Methods: miRNA microarray analysis using gemcitabine-resistant clones of MiaPaCa2 (MiaPaCa2-RGs), PSN1 (PSN1-RGs), and their parental cells (MiaPaCa2-P, PSN1-P) was conducted. Changes in the anti-cancer effects of gemcitabine were studied after gain/loss-of-function analysis of the candidate miRNA. Further assessment of the putative target gene was performed *in vitro* and in 66 pancreatic cancer clinical samples.

Results: miR-320c expression was significantly higher in MiaPaCa2-RGs and PSN1-RGs than in their parental cells. miR-320c induced resistance to gemcitabine in MiaPaCa2. Further experiments showed that miR-320c-related resistance to gemcitabine was mediated through SMARCC1, a core subunit of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex. In addition, clinical examination revealed that only SMARCC1-positive patients benefited from gemcitabine therapy with regard to survival after recurrence ($P = 0.0463$).

Conclusion: The results indicate that miR-320c regulates the resistance of pancreatic cancer cells to gemcitabine through SMARCC1, suggesting that miR-320c/SMARCC1 could be suitable for prediction of the clinical response and potential therapeutic target in pancreatic cancer patients on gemcitabine-based therapy.

Pancreatic cancer is one of the leading causes of tumour-related mortalities. The prognosis of patients after complete resection is poor, and >50% of patients develop tumour recurrence at distant or locoregional sites, with an estimated 5-year survival of only 20% (Li *et al*, 2004). Treatment modalities for pancreatic cancer include surgery, chemotherapy, radiation therapy, and combination therapy. Gemcitabine-based chemotherapy has formed the core of the multimodal therapy and improved the prognosis of patients with pancreatic cancer (Oettle *et al*, 2007), but its effect is modest because of high drug resistance. The selection of patients who derive a true benefit from gemcitabine could be an important stepping stone toward improvement of outcome of pancreatic cancer.

Several molecular mechanisms in drug resistance in pancreatic cancer have been elucidated. We have previously described that RRM1, the gene that encodes the regulatory subunit of ribonucleotide reductase, is strongly associated with gemcitabine resistance in pancreatic cancer (Nakahira *et al*, 2007; Akita *et al*, 2009). However, the development of drug resistance appears to be a multifactorial process, so our understanding is still fragmentary.

Recently, several studies have indicated that microRNAs (miRNAs) regulate this drug resistance (Tomimaru *et al*, 2010; Tomokuni *et al*, 2011). miRNAs are endogenous, single-stranded, non-coding RNAs and modulators of gene expression in the post-transcriptional phase, composed of 18–25 nucleotides. Currently, 1600 human miRNAs have been identified (miRBase 19, http://www.mirbase.org/). miRNAs are predicted to control the activity of approximately 30% of all protein-coding genes in mammals, and each miRNA can regulate up to 100 different messenger RNAs. Currently, the most promising miRNAs in association with

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Received 10 March 2013; revised 2 June 2013; accepted 4 June 2013; published online 25 June 2013

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pancreatic cancer drug resistance against gemcitabine are miR-15a (Zhang *et al*, 2010), miR-21 (Park *et al*, 2009; Ali *et al*, 2010; Giovannetti *et al*, 2010; Hwang *et al*, 2010), miR-34 (Ji *et al*, 2009), miR-200b and miR-200c (Li *et al*, 2009; Ali *et al*, 2010), miR-214 (Zhang *et al*, 2010), miR-221 (Park *et al*, 2009), and members of the let7 family (Li *et al*, 2009). However, only selected miRNAs have been investigated for their role in drug resistance in pancreatic cancer.

In this study, we developed gemcitabine-resistant cell clones from human pancreatic cancer cell lines and performed comprehensive expression profiling of miRNAs. The results indicate that miR-320c confers resistance to gemcitabine in pancreatic cancer cells through SMARCC1.

MATERIALS AND METHODS

Cell lines. The human pancreatic cell lines MiaPaCa2 and PSN1 were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, $100\,U\,ml^{-1}$ penicillin, and $100\,mg\,ml^{-1}$ streptomycin at 37 °C in a humidified incubator with 5% CO_2 in air.

Establishment of gemcitabine-resistant cell clones. Gemcitabine-resistant cells were generated by exposure to gradually increasing concentrations of the drug for 2 months as described previously (Goan *et al*, 1999; Davidson *et al*, 2004). Parental MiaPaCa2 cells (MiaPaCa2-P) and parental PSN1 cells (PSN1-P) were exposed to gemcitabine at an initial concentration of $1\,ng\,ml^{-1}$. When cells adapted to the drug, the gemcitabine concentration was increased. The final concentrations were $20\,ng\,ml^{-1}$ gemcitabine for MiaPaCa2 and $10\,ng\,ml^{-1}$ gemcitabine for PSN1. Through this process, we successfully established gemcitabine-resistant cells (Nakahira *et al*, 2007). Limiting the dilution of the established cells allowed the production of MiaPaCa2 cell clones and PSN1 cell clones that were resistant to gemcitabine. Four clones (MiaPaCa2-RGs: MiaPaCa2-RG1, MiaPaCa2-RG2, MiaPaCa2-RG3, and MiaPaCa2-RG4) and three clones (PSN1-RGs: PSN1-RG1, PSN1-RG2, and PSN1-RG3) from each line were used in these experiments.

Drugs and reagents. Gemcitabine was kindly supplied by Eli Lilly Pharmaceuticals (Indianapolis, IN, USA). In reference to previous studies, polyclonal rabbit anti-human SMARCC1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for western blot analysis (DelBove *et al*, 2011) and monoclonal mouse anti-human SMARCC1 antibody (Santa Cruz Biotechnology Inc.) for immunohistochemistry (Andersen *et al*, 2009) were used as primary antibodies. Monoclonal rabbit anti-human actin for western blot analysis was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Transfection. The precursor oligonucleotide of hsa-miR-320c (pre-miR-320c), antisense oligonucleotide inhibitor of hsa-miR-320c (anti-miR-320c), SMARCC1 small interfering (si) RNA oligonucleotide (siSMARCC1), and their scrambled oligonucleotides were obtained from Ambion Inc. (Austin, TX, USA). Pre/anti-miR-320c and siSMARCC1 were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Each scrambled oligonucleotide was transfected in the same way as a matched negative control.

RNA extraction. Total RNA, including the small RNA fraction, was isolated from cell lines with Trizol reagent (Invitrogen) as previously described (Yang *et al*, 2009). The quality of the RNA was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 and 280 nm ($A_{260/280}$) wavelengths.

Real-time quantitative reverse-transcription-PCR for miRNA expression. The reverse transcription (RT) reaction was performed with the TaqMan MicroRNA RT Kit (Applied Biosystems, Foster City, CA, USA), and real-time quantitative (q) PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) using the ABI7900HT system (Applied Biosystems). The expression of the target miRNA was normalised relative to that of the endogenous control, RNU48. Data were analysed according to the comparative Ct method (Schmittgen *et al*, 2004).

Real-time qRT-PCR for messenger RNA expression. Complementary DNA was synthesised from 8.0 μg total RNA using the SuperScript first-strand synthesis system (Invitrogen), according to the instructions supplied by the manufacturer. Real-time quantitative PCR was performed using designed oligonucleotide primers and the LightCycler 480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). For detection of the amplification products, the LightCycler-DNA master SYBR green I (Roche Diagnostics) was used as described previously (Yamamoto *et al*, 2004), and the amount of target gene expression was calculated. The expression of the target gene was normalised relative to the expression of GAPDH, which was used as an endogenous control. The designed PCR primers were as follows: GAPDH forward primer 5′-GTCGGAGTCAACGGATTTGGT-3′ and GAPDH reverse primer 5′-GCCATGGGTGGAATCATATTGG-3′; and SMARCC1 forward primer 5′-TCATGCGGATGCTCCTACCA-3′ and SMARCC1 reverse primer 5′-AAACCTCCGCCATCCCT GTT-3′.

MiRNA microarray experiments. The purified RNAs obtained from MiaPaCa2-P, MiaPaCa2-RGs (MiaPaCa2-RG1, MiaPaCa2-RG2, MiaPaCa2-RG3, and MiaPaCa2-RG4), PSN1-P, and PSN1-RGs (PSN1-RG1, PSN1-RG2, and PSN1-RG3) were used as samples and assessed as being of high quality by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and NanoDrop (NanoDrop Technologies). Next, 500 ng of extracted total RNA was labeled with Hy5 using the miRCURY LNA Array microRNA Power Labeling kit (Exiqon, Vedbaek, Denmark). The labeled RNAs were hybridised onto 3D-Gene Human miRNA Oligo chips containing 1011 anti-sense probes printed in duplicate spots (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase miRNA database (http://microrna.sanger.ac.uk/sequences/). After stringent washes, the fluorescent signals were scanned with the ScanArray Express Scanner (Perkin Elmer, Waltham, MA, USA) and analysed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA, USA). The raw data for each spot were normalised by substitution with the mean intensity of the background signal determined by the signal intensities of all blank spots with 95% confidence intervals. Measurements of both duplicate spots with signal intensities >2 s.ds. of the background signal intensity were considered to be valid. The relative expression level of a given miRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments after normalisation by their median values adjusted equivalently.

Construction of reporter plasmids and evaluation of luciferase reporter activity. To construct a luciferase reporter plasmid, a SMARCC1-3′UTR fragment containing the miR-320c target site was subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) located 3′ to the firefly luciferase gene. Nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analysis. For luciferase reporter assays, MiaPaCa2-P were seeded in 96-well plates and then cotransfected with the pmirGLO-SMARCC1-3′UTR construct and either pre-miR-320c or scrambled oligonucleotide for negative control (Ambion) using Lipofectamine 2000

(Invitrogen). Assays were conducted 48 h after transfection using Dual-Glo Luciferase Assay System (Promega). Firefly luciferase signals were normalised to renilla luciferase signals. All transfection experiments were conducted in triplicate.

Western blot analysis. Western blot analysis was performed as described previously (Yamamoto *et al*, 2003). Briefly, total protein was extracted from cells grown to semiconfluence in radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Aliquots of total protein (12 μg) were electrophoresed on sodium dodecyl sulfate polyacrylamide, 10% Tris-HCl gels (Bio-Rad Laboratories Inc., Hercules, CA, USA). The separated proteins were transferred to polyvinylidene difluoride membranes (Millipore Co., Billerica, MA, USA) and incubated with primary antibodies for 1 h.

Growth inhibitory assay. The growth inhibitory assay was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich Co.) assay as described previously (Eguchi *et al*, 2000). In brief, cells were incubated for 72 h under several concentrations of gemcitabine. After reincubation for 4 h with MTT solution, an acid–isopropanol mixture was added to dissolve the resulting formazan crystals. The absorbance of the plate was measured in a microplate reader at a wavelength of 550 nm with a 650-nm reference, and the results were expressed as a percentage of absorbance relative to that of untreated controls.

Patients and specimens. The study subjects were 66 patients with pancreatic cancer, recruited as described previously (Akita *et al*, 2009). Between January 1992 and March 2008, 166 patients underwent surgery for pancreatic cancer at Osaka University Hospital, Osaka, Japan. We excluded 100 patients for the following reasons: tumours were not resectable in 26 patients because of liver metastases or peritoneal carcinomatosis; surgery resulted in R1 (residual microscopic cancer) or R2 (residual macroscopic cancer) resections in 21 patients; chemotherapy or chemoradiotherapy was provided preoperatively to 37 patients; lack of neutral-buffered formalin-fixed and paraffin-embedded tumour blocks or/and clinical follow-up information for study purposes in 14 cases; and radiotherapy or immunotherapy was provided postoperatively to 2 patients. Because the natural history of variant pancreatic neoplasms differs from that of the usual pancreatic ductal adenocarcinoma, patients with intraductal papillary mucinous neoplasms, mucinous cystic adenocarcinomas, and medullary adenocarcinomas were excluded from this study. Supplementary Table S1 summarises the characteristics of the 66 patients who were enrolled. They included 31 men and 35 women with a mean age of 65.3 $\pm$ 7.6 years ($\pm$ s.d.). All patients had R0 (no residual cancer) resections by pancreaticoduodenectomy in 53 patients, distal pancreatectomy in 11 patients, and other resections in 2 patients. The histopathological grading showed well, moderately, and poorly differentiated adenocarcinoma in 26, 30, and 10 patients, respectively. The UICC-TNM classification was 2, 1, and 63 patients with pT1, pT2, and pT3; 28, 32, and 6 patients with pN0, pN1, and pM1lym; and 1, 1, 26, 32, and 6 patients with stage IA, IB, IIA, IIB, and IV, respectively. None of the patients had received neoadjuvant therapy preoperatively. All 66 patients were followed until disease recurrence and/or death. The median follow-up period was 17.0 months (3.5–147.7), the 5-year survival rate was 25.0%, and recurrence of disease was observed in 51 patients. Treatment with gemcitabine was carried out in 26 patients; 3 patients received it as adjuvant chemotherapy, and 23 patients received it after disease recurrence. Radiation therapy was not carried out during all the follow-up period.

Immunohistochemical staining. Immunohistochemical staining for SMARCC1 in 66 pancreatic cancer samples was performed using the method described previously (Kondo *et al*, 1999). Briefly, formalin-fixed, paraffin-embedded 4-μm-thick sections were deparaffinised in xylene, then treated with an antigen-retrieval procedure, and incubated in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase. After incubation with normal protein block serum, the sections were incubated overnight at 4 °C with an anti-SMARCC1 antibody as the primary antibody. Thereafter, the sections were detected with avidin–biotin complex reagents (Vector Laboratory Inc., Burlingame, CA, USA) and diaminobenzidine. All sections were counterstained with haematoxylin. The positivity for SMARCC1 staining was defined as detectable nuclear staining of $>10\%$ of cancer cells.

Statistical analysis. Data are expressed as means $\pm$ s.d. Clinicopathological parameters were compared using the χ^2 test, and continuous variables were compared using the Student's *t*-test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. A P value <0.05 denoted the presence of a statistically significant difference. Statistical analysis was performed using JMP software version 8.0.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Characteristics of established gemcitabine-resistant cells. The morphology of MiaPaCa2-RGs resembled that of MiaPaCa2-P. Although MiaPaCa2-RGs showed similar growth curves compared with MiaPaCa2-P in the absence of gemcitabine (data not shown), MiaPaCa2-RGs were significantly resistant to gemcitabine compared with MiaPaCa2-P, which MTT assays confirmed (Figure 1A). On the other hand, the morphology of PSN1-RGs was slightly spindle-like in comparison with that of PSN1-P, and the growth rate of PSN1-RGs was slightly slower than that of PSN1-P in the absence of gemcitabine (data not shown). MTT assays showed significant resistance to gemcitabine in PSN1-RGs compared with PSN1-P (Figure 1B).

miR-320c expression was significantly higher in gemcitabine-resistant clones than in parental cells. To identify the candidate miRNAs related to resistance to gemcitabine, miRNA microarray analysis was performed using a MiaPaCa2 combination (MiaPaCa2-P and MiaPaCa2-RGs) and PSN1 combination (PSN1-P and PSN1-RGs). The analysis showed that, among the 1011 miRNAs, the miRNA expression levels of 20 (2.0%) in MiaPaCa2-RGs (Supplementary Table S2) and 74 (7.3%) in PSN1-RGs (Supplementary Table S3), respectively, were altered by >1.5 average fold relative to parental, keeping adequate expression quantities and excluding miRNA*s. Furthermore, eight miRNAs were identified in common both in MiaPaCa2-RGs and PSN1-RGs (Figure 1C). These miRNAs are listed with average fold change and P values of MiaPaCa2-RGs relative to MiaPaCa2-P and PSN1-RGs relative to PSN1-P in Table 1. Among them, miR-320c showed the highest alteration (1.97 average fold change; 2.20-fold increase in MiaPaCa2-RGs, and 1.73-fold increase in PSN1-RGs) and was statistically significant ($P=0.0033$ in MiaPaCa2-RGs, $P=0.0366$ in PSN1-RGs). The results of real-time qRT-PCR for miR-320c confirmed the upregulation in MiaPaCa2-RGs (Figure 1D). Therefore, miR-320c was selected for further analysis.

Gain-of-function and loss-of-function of miR-320c alters the resistance of MiaPaCa2 cells to gemcitabine. To evaluate the effect of miR-320c on the response to gemcitabine in MiaPaCa2 cells, pre-miR-320c was first transfected into MiaPaCa2-P. Real-time qRT-PCR showed that transfection of pre-miR-320c markedly increased the miR-320c expression level for over 72 h (Figure 2A). The MTT assay demonstrated that transfection of pre-miR-320c into MiaPaCa2-P induced resistance to gemcitabine treatment

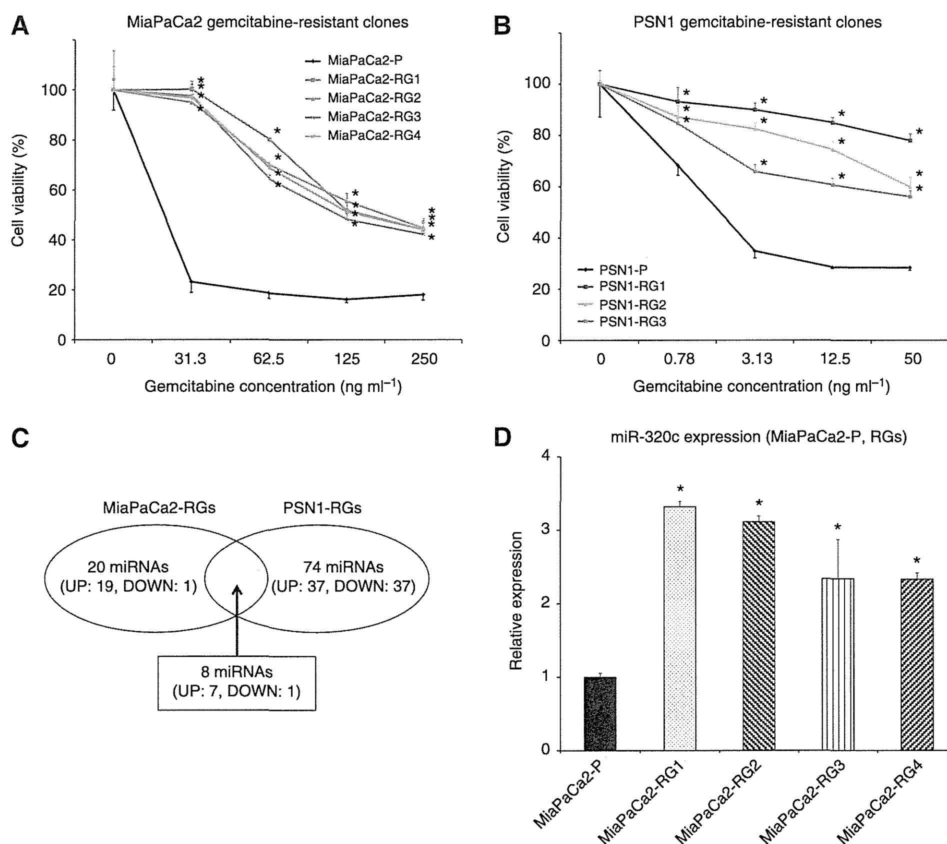

Figure 1. Characteristics of gemcitabine-resistant MiaPaCa2 cell clones (MiaPaCa2-RGs) and PSN1 cell clones (PSN1-RGs). (**A**, **B**) MTT assay showed significantly lower antitumour effect of gemcitabine in MiaPaCa2-RGs than in parental MiaPaCa2 cells (MiaPaCa2-P) and in PSN1-RGs than in parental PSN1 cells (PSN1-P). Data are mean ± s.d. of triplicate independent experiments. *$P<0.05$ compared with parental cells. (**C**) Schematic diagram of the results of microarray analysis. The protocol identified eight miRNAs in common with >1.5 average fold relative to parental, keeping adequate expression quantities and excluding miRNA*s both in MiaPaCa2-RGs and PSN1-RGs. (**D**) Real-time qRT–PCR demonstrated significantly higher miR-320c expression in MiaPaCa2-RGs than in MiaPaca2-P. Data are mean ± s.d. of triplicate independent experiments. *$P<0.05$.

Table 1. Common up- or downregulated miRNAs both in MiaPaCa2-RGs and PSN1-RGs

		Fold change (relative to MiaPaCa2-P)		Fold change (relative to PSN1-P)		
miR no.	**Fold change (average)**	**MiaPaCa2-RGs (average)**	**P-value**	**PSN1-RGs (average)**	***P*-value**	**Ref sequence ID**
Common upregulated miRNAs both in MiaPaCa2-RGs and PSN1-RGs						
hsa-miR-320c	1.97	2.20	0.0033	1.73	0.0366	MIMAT0005793
hsa-miR-29a	1.85	2.13	0.0097	1.57	0.2751	MIMAT0000086
hsa-miR-10a	1.69	1.64	0.0202	1.73	0.0606	MIMAT0000253
hsa-miR-30c	1.68	1.54	0.0222	1.81	0.0384	MIMAT0000244
hsa-miR-30a	1.65	1.51	0.0243	1.79	0.2634	MIMAT0000087
hsa-miR-29b	1.58	1.53	0.0026	1.63	0.3311	MIMAT0000100
hsa-miR-320a	1.56	1.51	0.0142	1.61	0.0556	MIMAT0000510
Common downregulated miRNAs both in MiaPaCa2-RGs and PSN1-RGs						
hsa-miR-1246	3.65	1.97	0.0085	5.32	0.1209	MIMAT0005898

Abbreviations: MiaPaCa-P = parental MiaPaCa2 cells; MiaPaCa2-RGs = gemcitabine-resistant clones of MiaPaCa2; miR and miRNA = microRNA; PSN1-P = parental PSN1 cells; PSN1-RGs = gemcitabine-resistant clones of PSN1.

(Figure 2C). To further assess the effect of miR-320c on the gemcitabine resistance, anti-miR-320c was transfected into MiaPaCa2-RG1. Real-time qRT-PCR showed sufficient inhibition of miR-320c expression for over 72 h (Figure 2B), and the MTT assay demonstrated significant reduction of viability of anti-miR-320c-transfected cells compared with the control cells (Figure 2D). These results indicate that, at least partially, miR-320c induces gemcitabine resistance in MiaPaCa2 cells.

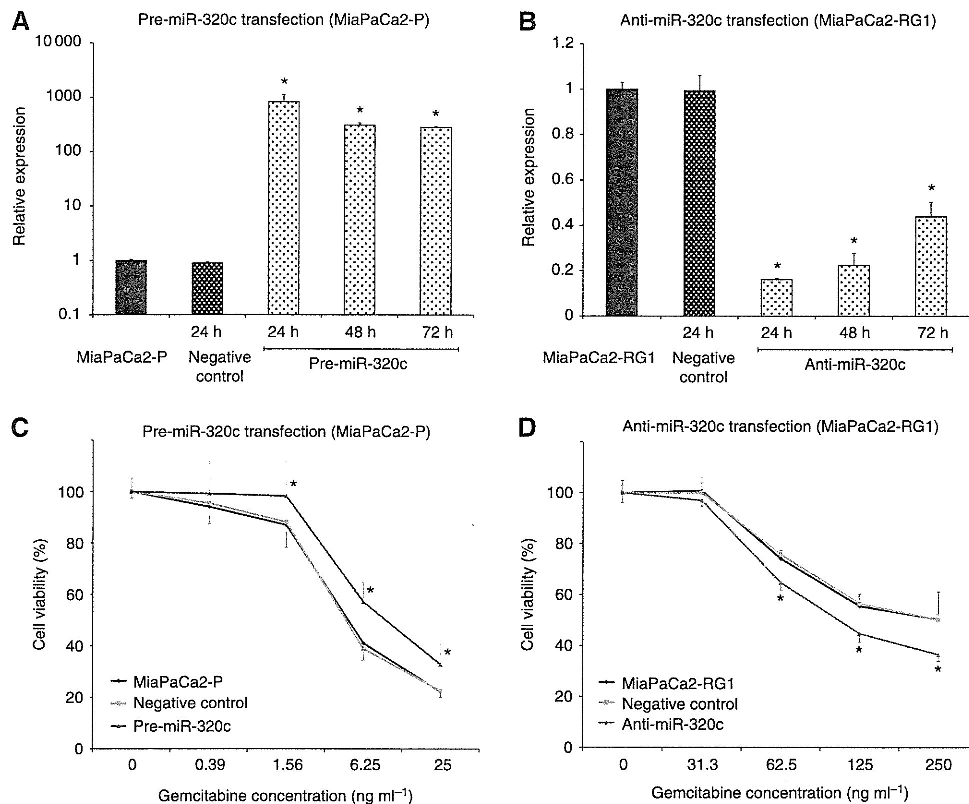

Figure 2. miR-320c induced gemcitabine resistance in MiaPaCa2 cells. (**A**, **B**) Real-time qRT-PCR confirmed overexpression (MiaPaCa2-P transfected with pre-miR-320c) and suppression (MiaPaCa2-RG1 transfected with anti-miR-320c) of miR-320c for >72 h. (**C**, **D**) MTT assay showed significant changes in resistance to gemcitabine by gain-of-function of miR-320c in MiaPaCa2-P and loss-of-function in MiaPaCa2-RG1. Data are mean ± s.d. of triplicate independent experiments. $^{*}P<0.05$.

miR-320c inhibits the response to gemcitabine by targeting SMARCC1. Few studies have reported miR-320 expression in gastrointestinal cancers, and little is known about the function of this miRNA. As putative targets of miR-320c, 539 genes were predicted by TargetScan. Among them, SMARCC1, a component of the chromatin remodeling complex, also known as a tumour suppressor, was selected for further analysis. SMARCC1 expression was lower in MiaPaCa2-RG1 than in MiaPaCa2-P (Figure 3A). We investigated direct binding of miR-320c and the SMARCC1 gene by luciferase assay in MiaPaCa2-P and observed reduction of the luciferase activity in the pre-miR-320c-treated cells in comparison with negative control (Supplementary Figure S1). Pre-miR-320c transfection decreased SMARCC1 expression, and anti-miR-320c transfection increased it (Figures 3B and C), suggesting that SMARCC1 is one of the target genes of miR-320c in MiaPaCa2 cells. Next, we used siRNA for SMARCC1 to validate its involvement in the resistance to gemcitabine. Knockdown of SMARCC1 was confirmed by western blot analysis (Figure 3D). The MTT assay demonstrated that transfection of siSMARCC1 enhanced the resistance of MiaPaCa2-P to gemcitabine (Figure 3E). These results suggest that SMARCC1 mediates, at least in part, the miR-320c-related resistance to gemcitabine.

SMARCC1 expression in clinical pancreatic cancer samples. Pancreatic cancer samples of 66 patients who underwent R0 resection were immunohistochemically stained for SMARCC1 expression. Whereas the expression of SMARCC1 in pancreatic cancer lesions varied among the patients, a homogeneous staining for SMARCC1 was observed in the nucleus in normal pancreatic duct cells (Figure 4A). Although SMARCC1 has shown to appear not only in the nucleus but also in the cytoplasm in the previous study (Andersen *et al*, 2009), functional SMARCC1 protein is considered to localise in the nucleus, therefore we defined SMARCC1-positive samples as having the spotted granular nuclear pattern (Figure 4B) and SMARCC1-negative samples as having the cytoplasmic pattern (not stained in the nucleus) (Figure 4C) or the negative pattern (not stained in the nucleus or the cytoplasm) (Figure 4D) in pancreatic cancer lesions. Among the 66 patients examined, 31 (47.0%) showed positive staining whereas 35 (53.0%) patients were negative for SMARCC1.

SMARCC1 expression was not associated with overall and disease-free survival. Of all the 66 patients, the median overall survival was 17.0 months (3.5–147.7), and the median disease-free survival was 11.1 months (2.0–147.7). There were no significant differences between the groups who were SMARCC1 expression positive and negative with respect to age, sex, histopathological type (well/mod/poor), tumour size, tumour location (head/body/tail), pathological depth of tumour (pT1/T2/T3), and whether or not gemcitabine was used as chemotherapy. However, pathological lymph node metastasis and pathological stage were significantly different in the two groups ($P=0.0383$, $P=0.0383$, respectively) (Supplementary Table S4). The Kaplan–Meier overall survival estimates were not significantly different for patients who were SMARCC1 positive compared with those with SMARCC1-negative expression (median overall survival: 1.693 *vs* 2.189 years, $P=0.5585$; Supplementary Figure S2A). With regard to disease-free survival, there was no significant difference between the SMARCC1-positive and -negative groups (median disease-free survival, 0.956 *vs* 1.334 years, $P=0.5633$; Supplementary Figure S2B).

SMARCC1 was a useful predictor of clinical response to gemcitabine therapy. Of the 66 patients, 26 received therapy with single-agent gemcitabine. In 23 patients, this treatment was

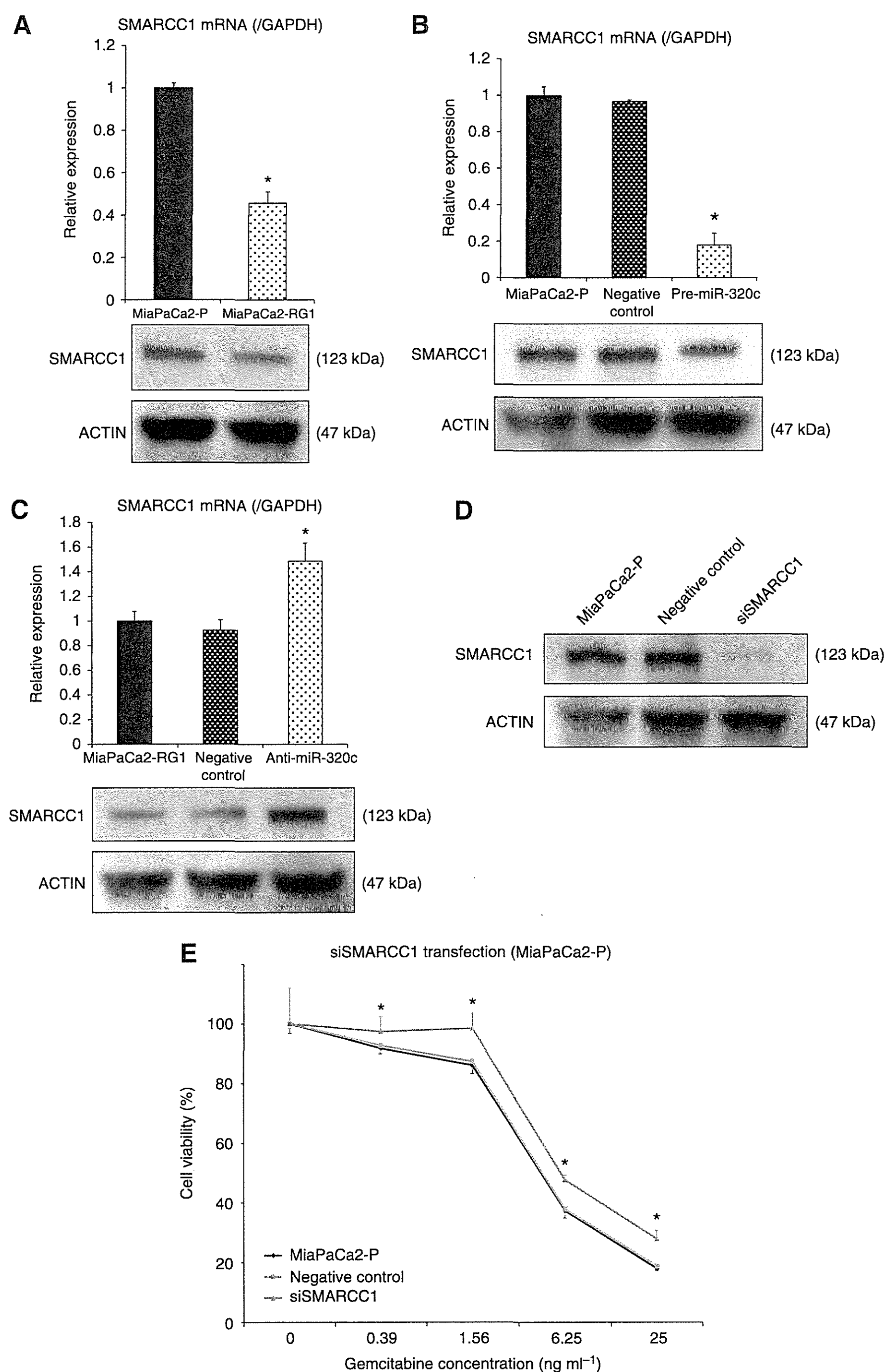

Figure 3. The miR-320c–related resistance to gemcitabine treatment is mediated through SMARCC1. (**A**) Real-time qRT-PCR and western blot analysis confirmed the significantly low SMARCC1 expression in MiaPaCa2-RG1 compared with MiaPaCa2-P. (**B**) SMARCC1 gene and protein expression was confirmed to be significantly suppressed by pre-miR-320c transfection in real-time qRT-PCR and western blot analysis. (**C**) real-time qRT-PCR and western blot analysis showed enhancement of SMARCC1 gene and protein expression levels by anti-miR-320c transfection. (**D**) Knockdown of SMARCC1 in MiaPaCa2-P was confirmed in western blot analysis. (**E**) MTT assay showed that knockdown of SMARCC1 induced resistance to gemcitabine treatment. Data are mean ± s.d. of triplicate independent experiments. *$P<0.05$.

initiated at the time of tumour recurrence. To elucidate the relationship between SMARCC1 expression and gemcitabine therapy, we used survival after recurrence, which represented the period from starting gemcitabine therapy or other therapies in 51 patients with relapse, until death. There were no significant differences between patients with and without gemcitabine therapy in clinicopathological factors (Table 2). First, we examined the survival benefit of gemcitabine. The 23 patients who were treated with gemcitabine had a significantly better survival than those who did not ($P=0.0046$; Supplementary Figure S3). After dividing patients who were treated with gemcitabine into SMARCC1-positive and -negative groups, only patients who were SMARCC1 positive benefited from gemcitabine therapy ($P=0.0463$). The relationship between SMARCC1 and survival after recurrence was not significant in patients treated without gemcitabine therapy ($P=0.9095$; Figure 5).

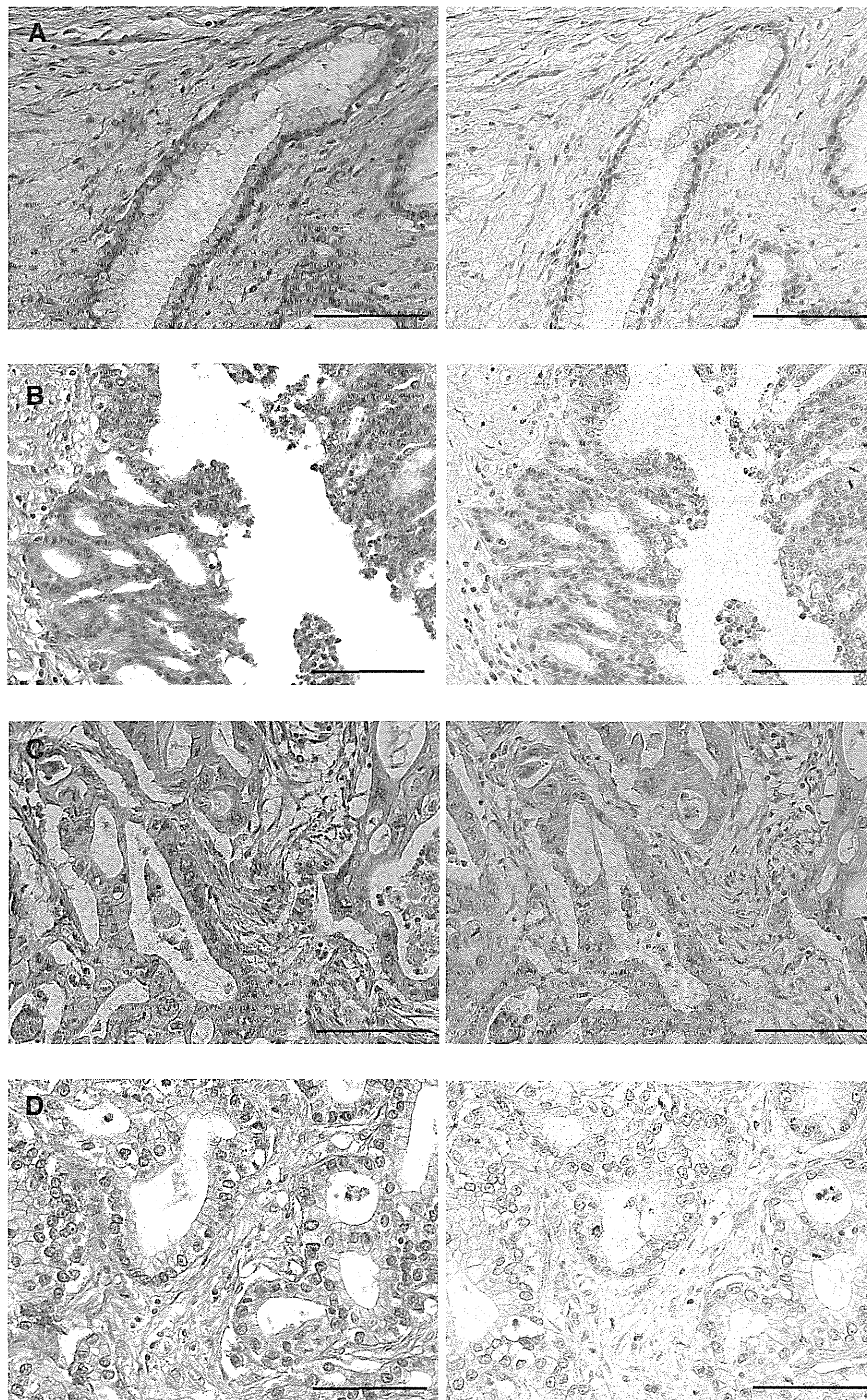

Figure 4. Immunohistochemistry for SMARCC1 in clinical samples. (A–D) Haematoxylin and eosin staining on the left side and SMARCC1 staining on the right side. (A) A normal pancreatic duct sample. SMARCC1 expression was identified in the nucleus homogeneously in normal pancreatic duct cells. (B) A representative SMARCC1-positive sample. SMARCC1 staining was in the spotted granular nuclear pattern in pancreatic carcinoma cells. (C, D) Representative SMARCC1-negative samples. SMARCC1 staining was in the cytoplasmic pattern (not stained in the nucleus) or in the negative pattern (not stained in the nucleus and the cytoplasm) in pancreatic carcinoma cells. Bar = 100 μm.

DISCUSSION

Several studies have examined the involvement of miR-320c in various types of cancer. It has been reported that miR-320c is upregulated in breast cancer (Yan *et al*, 2008), retinoblastoma (Zhao *et al*, 2009), and malignant transformed bronchial epithelial cells (Shen *et al*, 2009; Duan *et al*, 2010), whereas it is downregulated in lung cancer (Gao *et al*, 2011) and in cholangiocarcinoma (Chen *et al*, 2009). It has also been reported that miR-320 is regulated by PTEN in mammary stromal fibroblasts (Bronisz *et al*, 2012), correlates with recurrence-free survival in colon cancer (Schepeler *et al*, 2008), and inhibits proliferation in leukaemia (Schaar *et al*, 2009). Regarding the association of miR-320 and drug resistance, it has recently been reported that miR-320 facilitates chemotherapeutic drug-triggered apoptosis in cholangiocarcinoma (Chen *et al*, 2009). The present study identified miR-320c as one of the common upregulated

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