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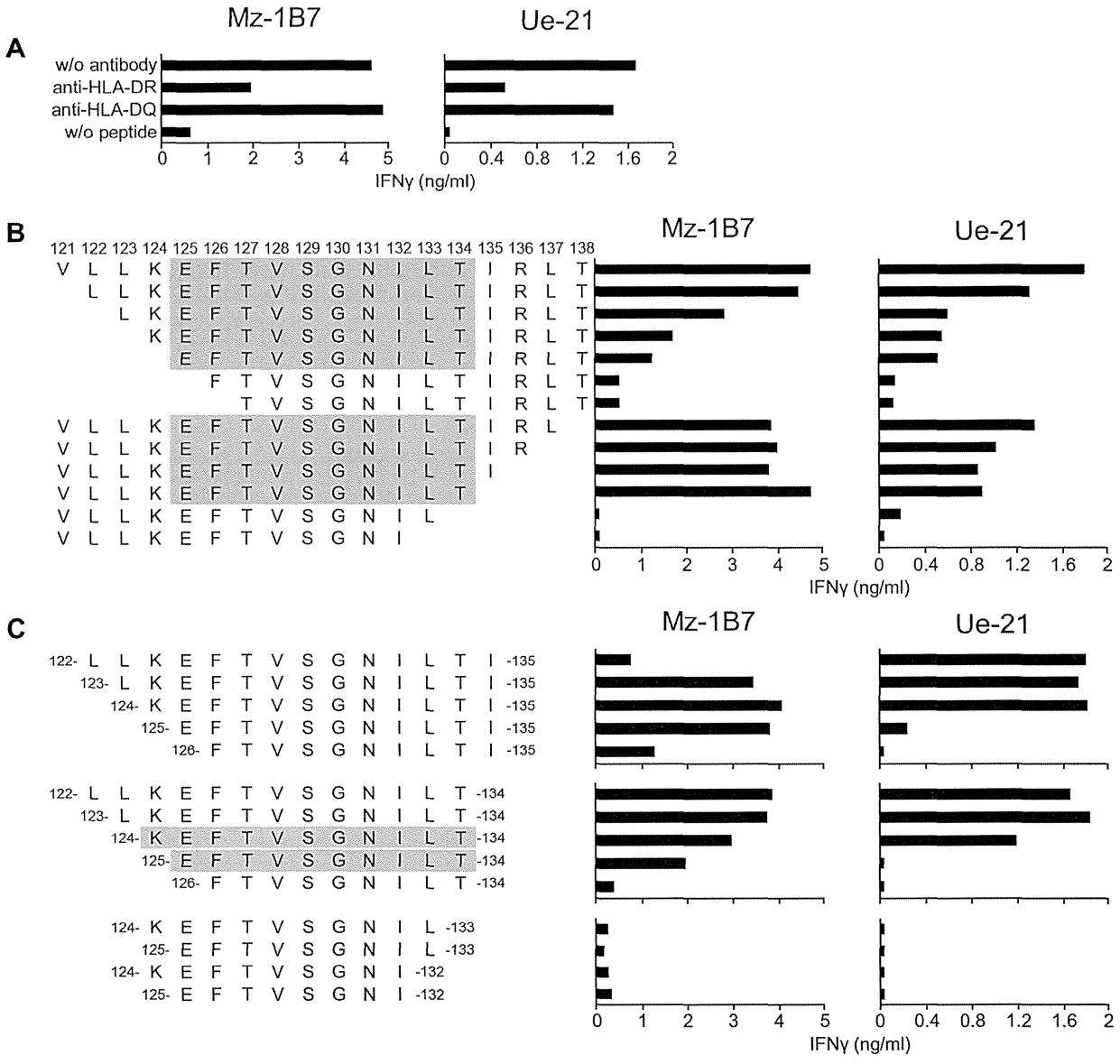

Fig. 1. Antibody blocking (A) and determination of NY-ESO-1 minimal epitopes ((B) and (C)) recognized by E-2 CD4 T-cell clones Mz-1B7 and Ue-21. In (A), CD4 T-cell clones (5×10^3) were stimulated for 18 h with autologous EBV-B cells (5×10^3) in the presence of NY-ESO-1 121–138 (VLLKEFTVSGNILTIRLT) peptide (100 nM), and anti-HLA-DR or anti-HLA-DQ mAb (5 μg/ml) in the culture. IFNγ in the culture supernatants was determined by ELISA. In B and C, CD4 T-cell clones (5×10^3) were stimulated for 18 h with autologous EBV-B cells (5×10^3) in the presence of truncated NY-ESO-1 121–138 peptides (100 nM). The core peptide region and each minimal epitopes recognized by CD4 T-cell clones are shown in gray boxes. IFNγ in the culture supernatants was determined by ELISA.

closely related, but different, minimal NY-ESO-1 peptides in restriction to the same DRB1*08:03. Recognition of closely related, but different, peptides by these CD4 T-cell clones was further confirmed with responses to other peptides. Peptide 122–135 was recognized by Ue-21, but not Mz-1B7. On the other hand, peptide 125–135 and peptide 126–135 were recognized by Mz-1B7, but not Ue-21.

3.2. *Differential recognition by clone Mz-1B7 and clone Ue-21 of the longer peptide 122–135, including minimal epitopes recognized by either clone*

To confirm that the longer peptide 122–135 was recognized by only clone Ue-21, but not clone Mz-1B7, irrespective of including epitopes recognized by either clone, an IFNγ capture assay together with ELISA was performed examining IFNγ in the same culture stimulated with peptide 122–135 and five other related peptides using autologous EBV-B cells as APC as above. As shown in Fig. 2A, a response of clone Mz-1B7 was observed against the peptides 123–135, 124–135, 122–134, 123–134 and 124–134, but not 122–135 in either the IFNγ capture assay or ELISA. No response against peptide 122–135 was observed up to a peptide concentration of 100 nM in ELISA. On the other hand, a response of clone Ue-21 was observed against all of the peptides used. These results were consistent with the results shown in Fig. 1.

3.3. *Tetramer binding*

We produced tetramers using the longer peptide 122–135, and five other related peptides 123–135, 124–135, 122–134, 123–134 and 124–134. The DR molecule was constructed by combining the DRA*01:01 and DRB1*08:03 chains that fused the leucine zipper motif at the C-terminal ends [8]. In the DRA locus, seven alleles DRA*01:01:01:01, DRA*01:01:01:02,

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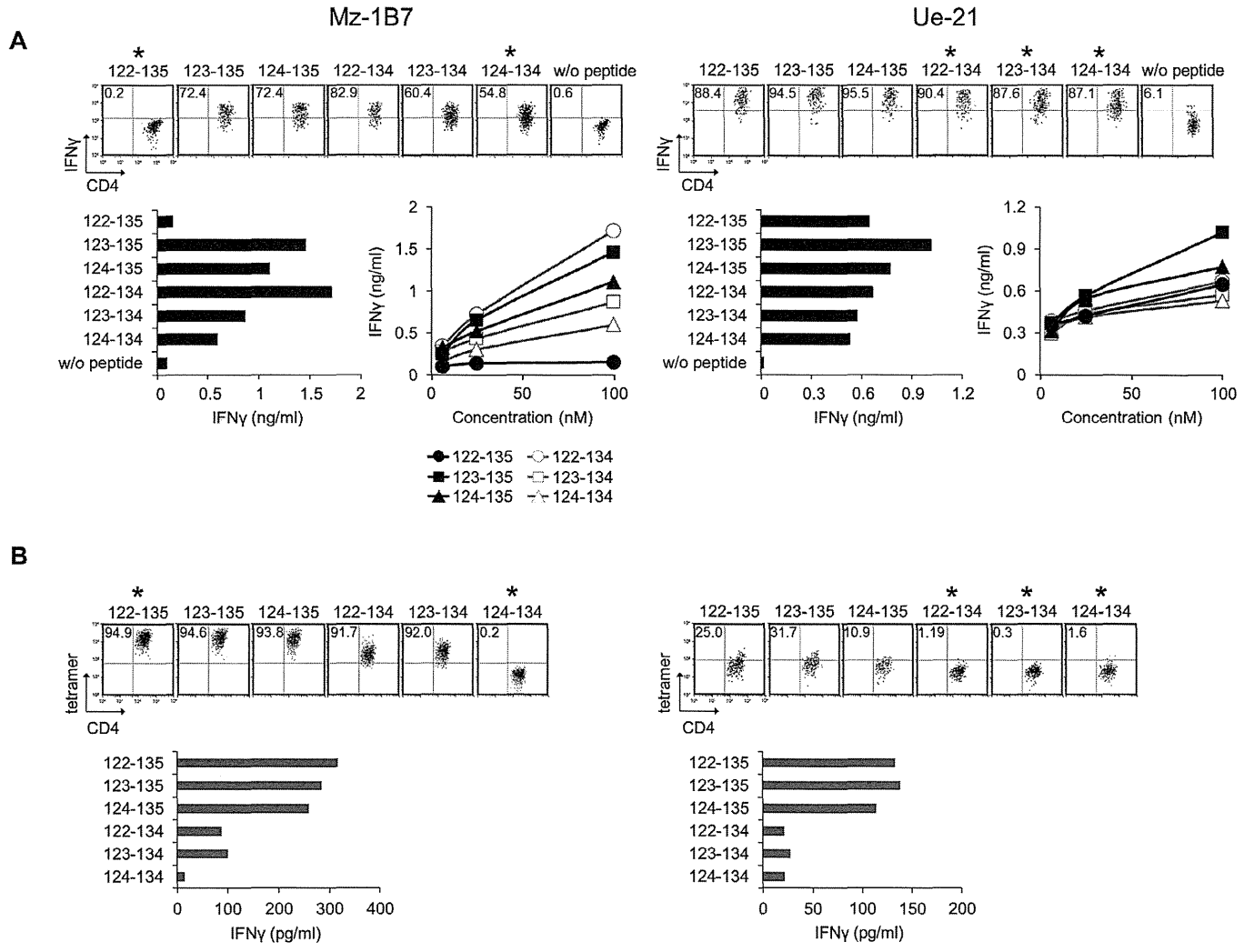

Fig. 2. Discrepancy between peptide recognition (A) and tetramer binding (B) in E-2 CD4 T-cell clones Mz-1B7 and Ue-21. In A top, CD4 T-cell clones (1×10^4) were stimulated for 4 h with the indicated peptides (1 μM) using autologous EBV-B cells (1×10^4) as APC. IFNγ-secreting CD4 T-cells were determined by an IFNγ capture assay using FACS Canto II. In A bottom, CD4 T-cell clones (5×10^3) were stimulated for 18 h with autologous EBV-B cells (5×10^3) pre-pulsed for 30 min with the indicated peptides (100 nM) (left) or with graded concentrations (6.25, 25 or 100 nM) of the indicated peptides (right). IFNγ in the culture supernatant was determined by ELISA. In B top, CD4 T-cell clones were stained with the indicated peptide/HLA-DRB1*08:03 tetramers (5 μg/ml) at 37 °C for 1 h followed by staining with an anti-CD4 mAb, and analyzed using FACS Canto II. In B bottom, CD4 T-cell clones (5×10^3) were stimulated for 18 h with the indicated peptide/HLA-DRB1*08:03 tetramers coated on wells in microculture plates. IFNγ in the culture supernatant was determined by ELISA. The peptides that show a discrepancy between recognition (A) and tetramer binding (B) are marked by *.

DRA*01:01:01:03, DRA*01:01:02, DRA*01:02:01, DRA*01:02:02 and DRA*01:02:03 have been identified. These alleles differ only at amino acid 217 in the cytoplasmic domain, which is included in the region replaced by a leucin zipper motif from amino acid residue 152 in the α2 domain. Therefore, any DRA allele can be used for tetramer production.

With these six peptide/DR tetramers, we examined binding to clones Mz-1B7 and Ue-21. As shown in Fig. 2B, to clone Mz-1B7, binding of tetramers with peptide 122–135, 123–135, 124–135, 122–134 and 123–134, but not 124–134, was observed. The peptide 122–135 including the minimal epitope 125–134 was not recognized by Mz-1B7, but a tetramer constructed using the same peptide bound to Mz-1B7. Furthermore, peptide 124–134 that also included the minimal epitope 125–134 was recognized by Mz-1B7, but a tetramer constructed using the same peptide did not bind to the same clone.

On the other hand, to clone Ue-21, weak binding of tetramers with peptides 122–135, 123–135 and 124–135, but only marginal binding of tetramers with 122–134, 123–134 or 124–134, was observed. The peptides 122–134 and 123–134, including the minimal epitope 124–134 and the peptide124–134 itself, were recognized by Ue-21, but the tetramers constructed using the same peptides bound to the same clone only marginally. IFNγ production by CD4 T-cell clones in stimulation with the tetramers was consistent with tetramer binding (Fig. 2B bottom).

We further examined the only marginal binding of a tetramer constructed using the peptide 124–134 to Mz-1B7 and Ue-21 under different culture conditions. As shown in Fig. 3A and B, efficient binding of the tetramer constructed using the peptide 123–135 to clone Mz-1B7 was observed at 25–37 °C after incubation for 10–120 min. However, only marginal binding was observed with the tetramer constructed using the peptide 124–134, even at 37 °C after incubation for 120 min. Only marginal binding of the tetramer with the peptide 124–134 to Mz-1B7 or Ue-21 was observed up to a concentration of 10 μg/ml (Fig. 3C and D).

3.4. Expression of CD4 and TCR on CD4 T-cell clones

Expression of CD4, CD3 and TCRαβ was analyzed by FACS. As shown in Fig. 4A, expression of CD4 was observed similarly on clones Mz-1B7 and Ue-21. On the other hand, expression of CD3 and TCRαβ was observed on Ue-21 strongly, but on Mz-1B7

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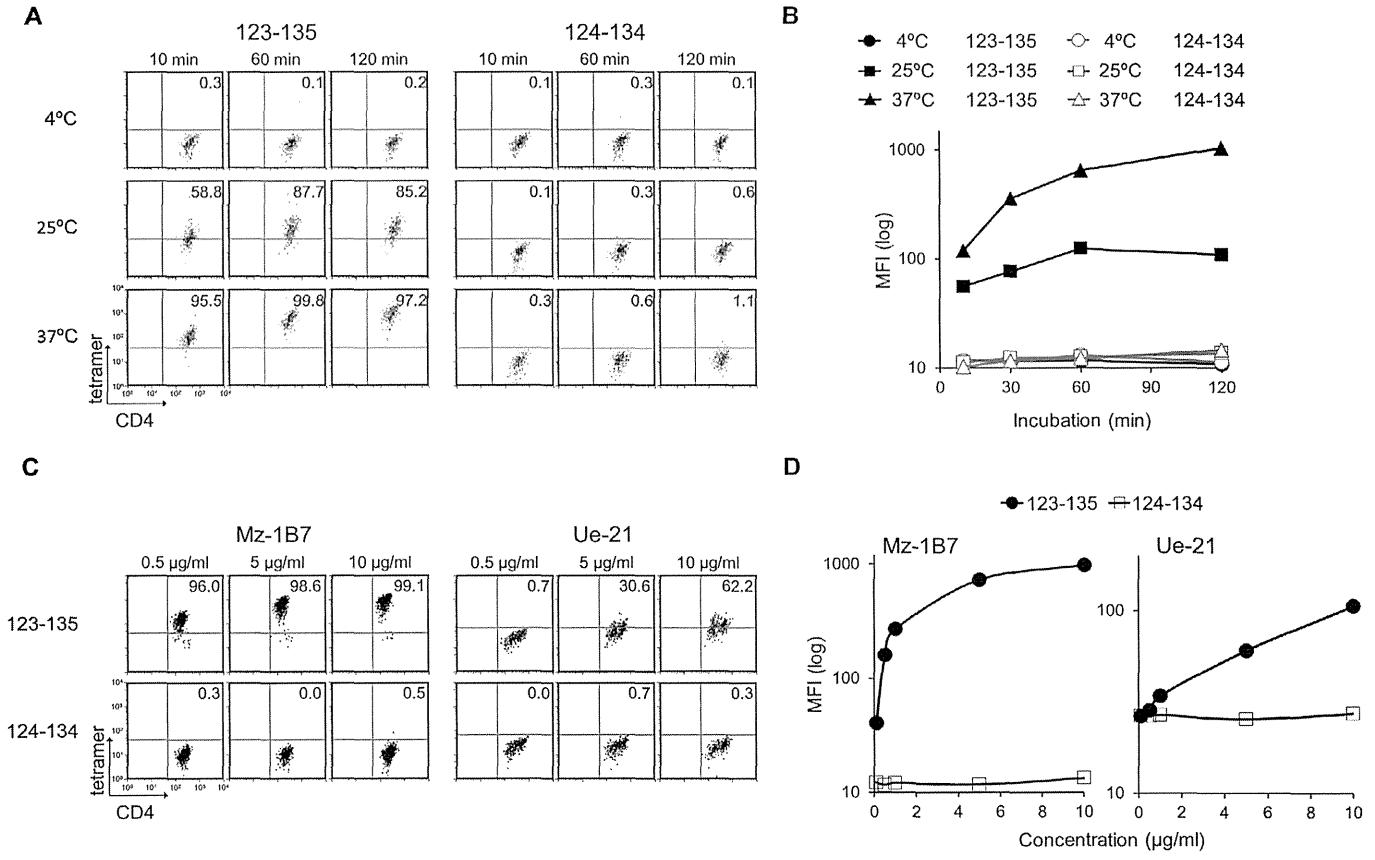

Fig. 3. Effect of temperature, incubation time and dose in tetramer staining. In (A) and (B), the E-2 CD4 T-cell clone Mz-1B7 was stained with NY-ESO-1 123–135 (LKEFTVSGNILTI) or NY-ESO-1 124–134 (KEFTVSGNILT) peptide/HLA-DRB1*08:03 tetramers (5 μg/ml) at 4, 25 or 37 °C for 10, 30, 60 or 120 min followed by staining with anti-CD4 mAb. In C and D, E-2 CD4 T-cell clones Mz-1B7 and Ue-21 were stained with NY-ESO-1 123–135 (LKEFTVSGNILTI) or NY-ESO-1 124–134 (KEFTVSGNILT) peptide/HLA-DRB1*08:03 tetramers (0.5, 1, 5 or 10 μg/ml) at 37 °C for 1 h followed by staining with an anti-CD4 mAb. Analysis was done using FACS Canto II. Dot plots (A and C) and the mean fluorescence intensity (MFI) (B and D) of tetramer staining are shown.

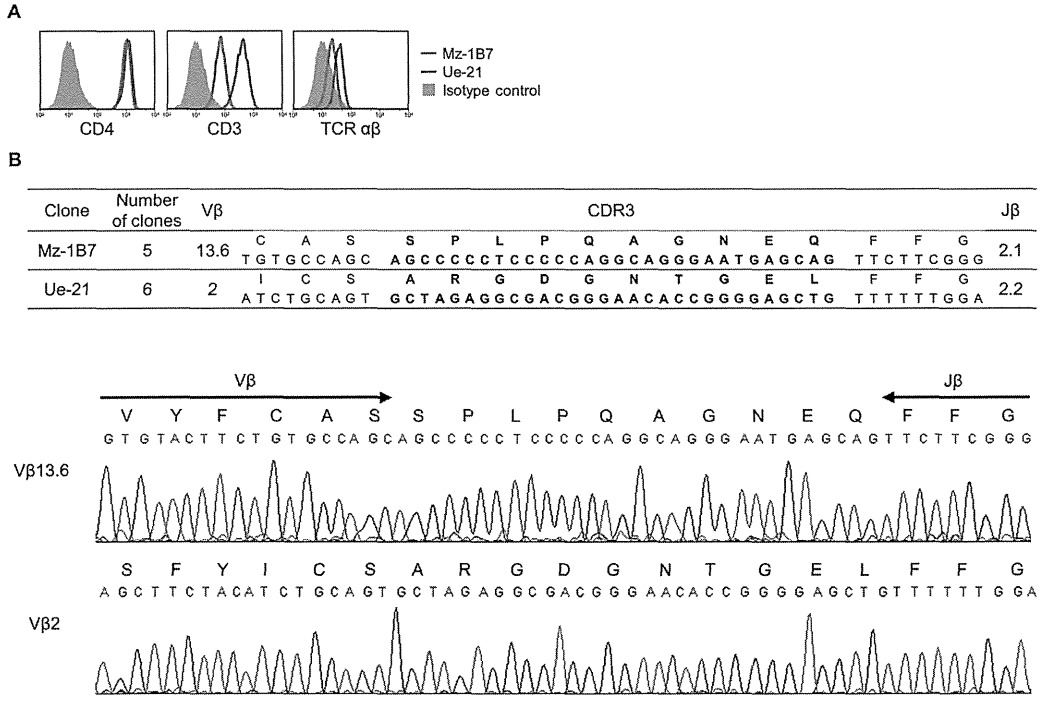

Fig. 4. Surface expression of the molecules on CD4 T-cell clones (A) and analysis of CDR3 sequences (B). In A, CD4 T-cell clones Mz-1B7 and Ue-21 stained with anti-CD4, CD3 and TCRαβ mAb were analyzed using FACS Canto II. In B, the nucleotide sequence and deduced amino acid sequences of the V–D–J junctional region of TCR β chain from the E-2 CD4 T-cell clones are shown.

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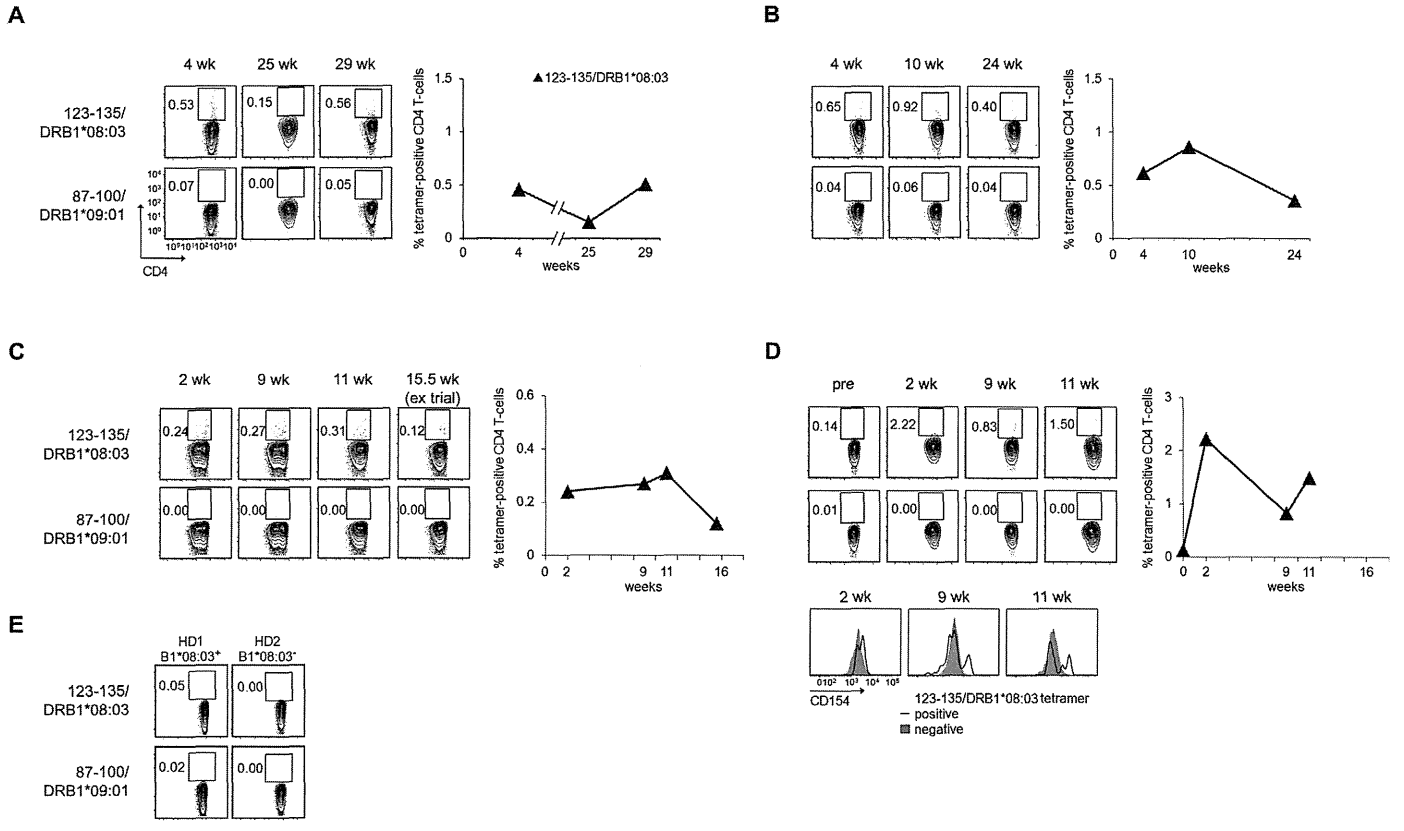

Fig. 5. Immunomonitoring of CD4 T-cell responses by the tetramer in cancer patients immunized with NY-ESO-1. CD4 T-cells from prostate cancer patient P-3 (A) and esophageal cancer patient E-1 (B) who were immunized with CHP-NY-ESO-1, and a lung cancer patient TK-OLP-01 (C) who was immunized with NY-ESO-1 OLP were stained ex vivo with the NY-ESO-1 123–135/HLA-DRB1*08:03 tetramer or a control NY-ESO-1 87–100/HLA-DRB1*09:01 tetramer (5 μg/ml) at 37 °C for 1 h followed by staining with an anti-CD4 mAb. In D, TK-OLP01 CD4 T-cells after in vitro stimulation twice were stained with the NY-ESO-1 123–135/HLA-DRB1*08:03 tetramer or a control NY-ESO-1 87–100/HLA-DRB1*09:01 tetramer and anti-CD154 mAb at 37 °C for 2 h followed by staining with an anti-CD4 mAb. The histogram shows CD154 expression on NY-ESO-1 123–135/DRB1*08:03 tetramer-positive (open) and negative (filled) CD4 T-cells. CD4 T-cells from two HDs were stained with tetramers as a negative control (E). HD1 and HD2 are DRB1*08:03-positive and -negative individuals, respectively. Analysis was done using FACS Canto II.

moderately. As shown in Fig. 4B, analysis of CDR3 sequences revealed that clone Mz-1B7 utilizes the Vβ13.6, SPLPQAGNEQ sequence for CDR3 and Jβ2.1. On the other hand, clone Ue-21 utilizes the Vβ2, ARGDGNTGEL sequence for CDR3 and Jβ2.2.

By cloning bulk CD4 T-cells from the E-2 patient, we obtained 58 DRB1*08:03-restricted clones. Within these, 5 clones utilized Vβ13.6 and 53 clones Vβ2. 5 clones with Vβ13.6 and 6 clones with Vβ2 were sequenced for CDR3. A combination of the same CDR3 sequence and Jβ was utilized by clones with each Vβ, respectively.

3.5. Monitoring of CD4 T-cell response by a tetramer constructed using the peptide 123–135 in cancer patients immunized with NY-ESO-1

Tetramers constructed using the peptide 123–135 (NY-ESO-1 123–135/DRB1*08:03) were used to monitor CD4 T-cell responses in DRB1*08:03-expressing cancer patients immunized with CHP-NY-ESO-1, or a mixture of NY-ESO-1 OLPs (NY-ESO-1 79–108, 100–129, 121–150 and 142–173) with Picibanil and Montanide. As shown in Fig. 5, the tetramer detected positive cells ex vivo in CD4 T-cells from PBMCs of a prostate cancer patient (P-3) (Fig. 5A) and an esophageal cancer patient (E-1) (Fig. 5B) who expressed DRB1*08:03 after immunization with CHP-NY-ESO-1. The tetramer also detected positive cells in CD4 T-cells from PBMCs of a lung cancer patient (TK-OLP-01) immunized with NY-ESO-1 OLP ex vivo (Fig. 5C) and after in vitro stimulation (Fig. 5D). Predominant detection of tetramer NY-ESO-1 123–135/DRB1*08:03-positive cells was observed after in vitro stimulation. Induction of CD154 (CD40L) expression on tetramer-positive cells was examined. At 9 and 11 weeks (3 and 4 vaccinations) after immunization, CD154 (CD40L)-positive cells were detected in tetramer NY-ESO-1 123–135/DRB1*08:03-positive, but not negative, cells suggesting their activation. No tetramer-positive cells were detected in CD4 T-cells from DRB1*08:03-positive or negative healthy donors (HD) (Fig. 5E). No clonal analysis of CD4 T-cells was possible because PBMCs from these patients were not available for further study.

4. Discussion

In this study, we demonstrated that HLA class II tetramers produced using minimal epitope peptides efficiently recognized by CD4 T-cell clones did not bind to cognate CD4 T-cell clones. Furthermore, we showed that a tetramer produced using a peptide which included the epitope sequence, but was not recognized by the cognate CD4 T-cell clone, could bind to the same CD4 T-cell clone.

It has long been observed that production of HLA class II tetramers is extremely difficult when compared to the production of MHC class I tetramers [5,6]. HLA class II tetramers produced using minimal epitope peptides and HLA class II molecules dimerized by a leucine zipper motif incorporated in the molecule generally failed to bind cognate CD4 T-cell clones. There have been only a few reports of successful binding of MHC class II tetramers to CD4 T-cells in which long peptides which were recognized by those T-cells were used for tetramer production [9–11].

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The reason for the difficulty in producing MHC class II tetramers has generally been considered to be due to inappropriate accommodation of the peptide in the groove of the MHC class II molecule, resulting in unnatural conformation. One of the constraints for MHC class II tetramer production is derived from the ambiguity of determining epitopes for CD4 T-cells. Peptides with the addition of various lengths of N- and C-terminal ends to the minimal core sequence are recognized by CD4 T-cells. Moreover, it is difficult to determine whether the minimal peptide is a naturally presenting epitope or not [18,19]. Lack of accurate information about natural HLA class II epitopes appears to be one of the reasons for the difficulty in HLA class II tetramer production.

Moreover, low binding affinity/avidity of the peptide to MHC class II molecules may also be involved. In this study, we confirmed successful tetramer production with differential retention time by HPLC. For example, the prolongation of the retention time was 0.554 min with the addition of the 12-mer NY-ESO-1 123–134 peptide (LKEFTVSGNILT) to the DRB1*08:03 monomer, but was 0.039 min with the addition of a negative control peptide to DRB1*08:03. The prolongation of the retention time was 0.246 min with the positive control 15-mer CLIP peptide (PVSKMRMATPLLMQA). However, the possibilities discussed above were also considered for the failure to produce a tetramer using the minimal epitope peptides. First, the use of an inappropriate epitope may have been involved. Defining the precise length of natural epitopes bound to class II molecules is extremely difficult as described above. Second, the epitope peptide may have weak binding affinity for the MHC class II molecules used for tetramer production (see below). With the core 9-mer peptides bound to HLA-DRB1*08:03, hydrophobic residues at P1 as phenylalanine (F) or tyrosine (Y) and residues at P6 as proline (P), serine (S), arginine (R) or asparagine (N) are relevant as anchor residues [20,21]. F at position 126 and N at position 131 in NY-ESO-1 121–138 may contribute to binding. Addition of isoleucine (I) at position 135 strongly stabilized tetramer production. Third, binding instability of the peptide to class II molecules may also be involved.

In addition to the failure to produce MHC class II tetramers using the epitope peptides, this study showed unexpected binding of the tetramer with a peptide not recognized by CD4 T-cells. The clone Mz-1B7 did not recognize the free peptide 122–135 on autologous EBV-B cells as APC, but the peptide 122–135/DRB1*08:03 tetramer bound to the TCR on those cells. The possibility of a lack of binding of the free peptide 122–135 to the DRB1*08:03 molecule on autologous APC is unlikely because clone Ue-21 recognized it efficiently. Rather, the tetramer binding could be due to a subtly modified structure of the 122–135 peptide/DRB1*08:03 tetramer from the structure of the free 122–135 peptide/DRB1*08:03 molecule. This could result from structural modification of either the peptide or the DR molecule, or both, during preparation of the peptide/DR tetramer, or simply be due to a subtle conformational change in the DR molecule itself due to fusion of the leucine zipper motif [8]. In the latter, it is possible that association of DRα and DRβ chains by the leucine zipper motif on each chain caused a subtle difference in the conformation of the natural DR molecule, although there was no convincing evidence to support this idea in this study.

Here, we also demonstrated that the NY-ESO-1 123–135/DRB1*08:03 tetramer detected ex vivo CD4 T-cell responses in PBMCs from a prostate cancer patient P-3 and an esophageal cancer patient E-1 after CHP-NY-ESO-1 vaccination, and a lung cancer patient TK-OLP-01 after NY-ESO-1 OLP vaccination. These patients possessed the DRB1*08:03 allele. Patient P-3 was positive for the NY-ESO-1 antibody before vaccination (sero-positive) and patients E-1 and TK-OLP-01 were sero-negative [15]. In these patients, tetramer-positive CD4 T-cells were detected after vaccination.

Based on the discussion above, a possible difference in CD4 T-cell clones recognizing the epitope peptides from those detected by the respective peptide/HLA class II tetramer should be taken into consideration in HLA class II tetramer analysis.

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Conflict of interest: There is no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.12.042.

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FULL PAPER

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NY-ESO-1 antibody as a novel tumour marker of gastric cancer

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Uncorrected Proof

Background: NY-ESO-1 antibodies are specifically observed in patients with NY-ESO-1-expressing tumours. We analysed whether the NY-ESO-1 humoral immune response is a useful tumour marker of gastric cancer.

Methods: Sera from 363 gastric cancer patients were screened by enzyme-linked immunosorbent assay (ELISA) to detect NY-ESO-1 antibodies. Serial serum samples were obtained from 25 NY-ESO-1 antibody-positive patients, including 16 patients with curative resection and 9 patients who received chemotherapy alone.

Results: NY-ESO-1 antibodies were detected in 3.4% of stage I, 4.4% of stage II, 25.3% of stage III, and 20.0% of stage IV patients. The frequency of antibody positivity increased with disease progression. When the NY-ESO-1 antibody was used in combination with carcinoembryonic antigen and CA19-9 to detect gastric cancer, information gains of 11.2% in stages III and IV, and 5.8% in all patients were observed. The NY-ESO-1 immune response levels of the patients without recurrence fell below the cutoff level after surgery. Two of the patients with recurrence displayed incomplete decreases. The nine patients who received chemotherapy alone continued to display NY-ESO-1 immune responses.

Conclusion: When combined with conventional tumour markers, the NY-ESO-1 humoral immune response could be a useful tumour marker for detecting advanced gastric cancer and inferring the post-treatment tumour load in seropositive patients.

Gastric cancer is the second most common cause of cancer-related death worldwide (Statistics and Information Department, 2006; Katanoda and Yako-Suketomo, 2009). Although complete removal of the tumour by surgical resection is an ideal treatment option for patients with gastric cancer, many patients with advanced-stage gastric cancer need to be treated with intensive chemotherapy. Gastric cancer patients exhibit high relapse rates even after curative surgery and unresponsiveness to chemotherapy, resulting in dismal survival rates (Sasako *et al*, 2011). Several methods for the prediction and early detection of subclinical 'minimal residual cancer' after surgery (Austrup *et al*, 2000; Klein *et al*, 2002) or relapse have been developed, for example, peritoneal lavage, positron emission tomography, gene profiling, and so on. (Motoori *et al*, 2006; Makino *et al*, 2010; Graziosi *et al*, 2011), reliable markers that can specifically reflect gastric cancer disease status have not been determined.

Analysing serum level of tumour markers is employed for cancer detection, monitoring patients' disease status, and prognosis prediction. Several organ-specific tumour markers are used in the clinic, for example, prostate-specific antigen and prostatic acid

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phosphatase for prostate cancer (Seamonds *et al*, 1986; Ferro *et al*, 1987) and protein induced by vitamin K absence-II for liver cancer (Fujiyama *et al*, 1986). As no gastric cancer-specific markers have been determined, a combination of several nonspecific tumour markers, for example, carcinoembryonic antigen (CEA), CA19-9,and so on, is merely applicable for monitoring treatment efficacy, but not the diagnosis of gastric cancer (Takahashi *et al*, 1995, 2003). Carcinoembryonic antigen and CA19-9 are found in the sera of 20–60% of gastric cancer patients, and their expression levels in gastric cancer are related to clinical events, such as relapse (Kodera *et al*, 1996). Carcinoembryonic antigen value, in particular, is indicative of the formation of a large tumour, liver or peritoneal metastasis, and/or a high risk of relapse and poor prognosis (Ikeda *et al*, 1993; Yamamoto *et al*, 2004). However, as CEA, a cell surface-anchored glycoprotein, is expressed in normal cell membranes, 5% of CEA-positive cases are pseudopositives, that is, caused by heavy smoking, endometriosis, and ageing, and so on. (Alexander *et al*, 1976), suggesting the importance of novel markers for gastric cancer.

NY-ESO-1 antigen, a cancer/testis (CT) antigen, was originally identified in oesophageal cancer by serological expression cloning using autologous patient serum and has been shown to be strongly immunogenic. Spontaneous NY-ESO-1 antibody production is often observed in patients with NY-ESO-1-expressing tumours, for example, 9.4% of melanoma patients, 12.5% of ovarian cancer patients, 7.7–26.5% of breast cancer patients, 4.2–20.0% of lung cancer patients, and 52% of prostate cancer patients, but has not been detected in non-cancerous donors (Stockert *et al*, 1998; Nakada *et al*, 2003; Türeci *et al*, 2006; Chapman *et al*, 2007; Isobe *et al*, 2009; Gati *et al*, 2011). Thus, it is possible that the NY-ESO-1 humoral immune response could be used as a serological marker for detecting these cancers and to facilitate the clinical management of some patients with particular types of cancer (Gnjatic *et al*, 2006). Jäger *et al* (1999) found that the change in the NY-ESO-1 humoral immune response reflected the overall tumour load in 10 out of 12 patients with various cancers. However, there is ongoing controversy regarding the association between the NY-ESO-1 immune response and prognostic criteria (Yuan *et al*, 2011). To address these issues in gastric cancer, we investigated the clinical usefulness of the NY-ESO-1 humoral immune response for diagnosis, monitoring, and relapse prediction in gastric cancer patients.

MATERIALS AND METHODS

Serum sample and tissue specimen collection from gastric cancer patients. In all, 363 patients with histologically confirmed gastric cancer, who underwent surgical resection or chemotherapy at one of four institutions between 2004 and 2011, were included in this study after providing written informed consent. Serum samples were obtained from the 363 patients during their admission to hospital for surgical treatment and/or chemotherapy, and afterwards, serial serum samples were obtained at each follow-up visit from 25 patients who displayed NY-ESO-1 humoral immune responses. All serum samples were collected as surplus samples after routine blood tests and stored. Fixed and frozen gastric cancer tissue samples were obtained from 60 out of 363 patients during surgery and stored. The samples were subsequently subjected to expression analysis. Information regarding blood test results, tumour stage, histological type, depth of invasion, lymph node metastasis, and distant metastasis, which were obtained from pathological examinations and CT scans, were collected from the relevant patient databases. Serum samples obtained from 50 healthy donors were used as controls. This study was approved by the institutional review boards of Osaka University Hospital, Toyonaka Municipal Hospital, Ikeda City Hospital, and Minoh City Hospital.

Reverse transcription–polymerase chain reaction. Total cellular RNA was extracted from the frozen tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The total RNA (1 μg) was subjected to the reverse transcription (RT) in 20 μl buffer with oligo-$(dT)_{15}$ primer using a RT system (Promega, Madison, WI, USA). Conventional polymerase chain reaction (PCR) was performed in a 25-μl reaction mixture containing 1 μl of cDNA template, 500 nM of each primer, and 1 U of *Taq* DNA polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA, USA) in the following conditions: one cycle of 95 °C for 12 min; followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min; and then a final step of 72 °C for 10 min. The sequences of the primers for *NY-ESO-1* were as follows: ESO1-1, 5′-AGTTC TACCTCGCCATGCCT-3′; and ESO1-2, 5′-TCCTCCTCCAGC GACAAACAA-3′. The integrity of each RNA sample was verified by performing RT–PCR for *porphobilinogen deaminase* (*PBGD*). The PCR products were subjected to electrophoresis on a 2% agarose gel and visualised with ethidium bromide.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were used for the immunohistochemistry (IHC) analyses. Slides were incubated with the primary antibody overnight at 4 °C. The monoclonal antibody E978, which was previously generated by our group, was used to detect NY-ESO-1. The slides were then subjected to a heat-based antigen retrieval technique by immersing them in a preheated buffer solution (hipH solution; Dako, Carpinteria, CA, USA). A polymer-based antibody detection system (PowerVision; Leica Microsystems, Buffalo Grove, IL, USA) was used as the secondary reagent, and 3,3-diaminobenzidine tetrahydrochloride (Liquid DAB; Biogenex, San Ramon, CA, USA) was used as the chromogen. Normal adult testis tissue as a positive control and appropriate negative controls were included for each case.

Enzyme-linked immunosorbent assay. A measure of 100 μl of 1 μg ml^{-1} recombinant protein in coating buffer (pH 9.6) were added to each well of 96-well PolySorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The plates were then washed with PBS and blocked with 200 μl per well of 5% FCS/PBS for 1 h at room temperature. After being washed again, 100 μl of serially diluted serum were added to each well and incubated for 2 h at room temperature. Then, after extensive washing, goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells as a secondary antibody, and the plates were incubated for 1 h at room temperature. The plates were washed again, and the signals were developed with 100 μl per well of 0.03% *o*-phenylene diamine dihydrochloride, 0.02% hydrogen peroxide, and 0.15 M citrate buffer, and absorbance was read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). Ovalbumin (OVA; Sigma, St Louis, MO, USA) was used as the control protein. Levels of NY-ESO-1 humoral response were assessed using optical density (OD) values.

CEA and CA19-9. Serum CEA and CA19-9 levels were measured at each hospital's clinical laboratory department. Carcinoembryonic antigen and CA19-9 positivity were defined as serum levels of CEA and CA19-9 of >5.0 ng ml^{-1} and >37 U ml^{-1}, respectively.

Statistical analysis. Fisher's exact test was used to assess the associations between NY-ESO-1 antibody expression and clinicopathological parameters. Kaplan–Meier curves were plotted to assess the effect of the NY-ESO-1 antibody on overall survival. Survival curves were compared using the log-rank test.

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Table 1. Frequencies of NY-ESO-1 antibody, CEA, and CA19-9 in gastric cancer patients

Stage	NY-ESO-1 Ab	CEA	CA19-9	CEA and/or CA19-9	CEA and/or CA19-9 and/or NY-ESO-1 Ab
I	6/176 (3.4)	24/176 (13.6)	6/176 (3.4)	27/176 (15.3)	31/176 (17.6)
II	2/45 (4.4)	8/45 (17.8)	7/45 (15.6)	11/45 (24.4)	12/45 (26.6)
III	17/67 (25.3)	22/67 (32.9)	11/67 (16.4)	25/67 (37.3)	35/67 (52.2)
IV	16/75 (20.0)	23/75 (30.7)	30/75 (40.0)	40/75 (53.3)	46/75 (61.3)
I + II	8/221 (3.6)	32/221 (14.5)	13/221 (5.9)	38/221 (17.2)	43/221 (19.5)
III + IV	33/142 (23.2)	45/142 (31.7)	41/142 (28.9)	65/142 (45.8)	81/142 (57.0)
Total	41/363 (11.1)	77/363 (21.2)	54/363 (14.9)	103/363 (28.4)	124/363 (34.2)

Abbreviations: Ab = antibody; CA = carbohydrate antigen; CEA = carcinoembryonic antigen. Values within parentheses are percentages.

RESULTS

Determination of NY-ESO-1 humoral immune response positivity. We first determined the OD cutoff value for NY-ESO-1 humoral immune response positivity. When the serum samples from the 50 healthy donors were examined for reactivity to the NY-ESO-1 recombinant protein by ELISA, their OD values ranged from 0.08 to 0.20, and their mean and standard deviation values were 0.15 and 0.05, respectively, at a dilution of 1 : 200. Thus, NY-ESO-1 humoral immune response positivity was defined as an OD value of >0.25 at a dilution of 1 : 200 (95% accuracy level) and >3 times of the OD value against control protein (OVA).

NY-ESO-1 humoral immune responses of gastric cancer patients. Serum samples were obtained from 363 gastric cancer patients, including 176 stage I, 45 stage II, 67 stage III, and 75 stage IV patients at admission (Table 1). The NY-ESO-1 antibody was detected in 3.4% (6 of 176) of stage I, 4.4% (2 of 45) of stage II, 25.3% (17 of 67) of stage III, and 20.0% (16 of 75) of stage IV gastric cancer patients, resulting in an overall detection rate of 11.1% (41 of 363). An analysis of the gastric cancer patients' characteristics found that NY-ESO-1 antibody positivity was significantly correlated with gender (male > female) and tumour progression (Table 2). In particular, the patients with progressive gastric cancer involving deeper tumour invasion, positive lymph node metastasis, positive distant metastasis, or a higher clinical stage tended to produce the NY-ESO-1 antibody.

Analysis of NY-ESO-1 antigen expression. *NY-ESO-1* mRNA and NY-ESO-1 protein expression were analysed by RT–PCR and IHC, respectively, in gastric cancer tissues obtained from 60 patients for whom both frozen and formalin-fixed specimens were available, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients (Table 3). *NY-ESO-1* mRNA was detected in six specimens. NY-ESO-1 was immunohistochemically detected in 19 specimens, including 6 and 13 that were positive and negative for *NY-ESO-1* mRNA, respectively. Most of the specimens displayed a heterogeneous staining pattern (data not shown).

NY-ESO-1 antibody and antigen expression. We analysed the frequency of NY-ESO-1 antibody positivity in gastric cancer patients in whom NY-ESO-1 antigen expression was or was not detected by RT–PCR or IHC. As shown in Table 3, 9 out of the 60 gastric cancer patients whose specimens were available for expression analysis possessed the NY-ESO-1 antibody in their sera. The NY-ESO-1 antibody was detected in 8 of 19 (42.1%) patients with IHC-positive gastric cancer and 5 of 6 (83.3%) patients with RT–PCR (and IHC)-positive gastric cancer, whereas only 1 of 41 patients in whom both RT–PCR and IHC analysis produced negative results displayed an NY-ESO-1 humoral immune responses.

Table 2. Relationship between NY-ESO-1 antibody positivity and clinicopathological features in gastric cancer patients

Variable	NY-ESO-1 Ab		*P*-value*
	Negative	Positive	
Gender			
Male	223 (86.4)	35 (13.6)	0.04307
Female	99 (94.3)	6 (5.7)	
Age (years)			
>65	178 (88.6)	23 (11.4)	0.9209
<65	144 (88.9)	18 (11.1)	
Histological type			
Differentiated	143 (89.4)	17 (10.6)	0.5605
Undifferentiated	132 (87.4)	19 (12.6)	
Depth of tumour invasion			
cT1–T2	193 (92.8)	15 (7.2)	0.0044
cT3–T4	129 (83.2)	26 (16.8)	
Lymph node metastasis			
Negative	196 (97.0)	6 (3.0)	<0.001
Positive	126 (78.3)	35 (21.7)	
Distant metastasis			
Negative	277 (91.1)	27 (8.9)	<0.001
Positive	45 (76.3)	14 (23.7)	
Stage			
I–II	213 (96.4)	8 (3.6)	<0.001
III–IV	109 (76.8)	33 (23.2)	

Abbreviations: Ab = antibody. Fisher's exact test was used for the statistical analysis. Values within parentheses are percentages.

Frequencies of NY-ESO-1 humoral immune responses and conventional tumour markers in gastric cancer patients. The frequency of the NY-ESO-1 humoral immune response was compared with those of conventional tumour markers in gastric

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Table 3. Frequency of NY-ESO-1 antibody positives in gastric cancer patients in whom the NY-ESO-1 antigen was or was not detected by IHC or RT–PCR

	IHC		
	Positive	Negative	Total
mRNA			
Positive	5/6 (83.3)	0/0 (0.0)	5/6 (83.3)
Negative	3/13 (23.1)	1/41 (2.4)	4/54 (7.4)
Total	8/19 (42.1)	1/41 (2.4)	9/60 (15.0)

Abbreviations: IHC = immunohistochemistry; RT–PCR = reverse transcription–polymerase chain reaction. Frozen and formalin-fixed tissue specimens from 60 patients, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients, were analysed. All stage IV patients had previously undergone surgical treatment. Values within parentheses are percentages.

cancer patients. The serum CEA and CA19-9 levels of 363 gastric cancer patients were measured at admission (Table 1). Carcinoembryonic antigen and CA19-9 positivity were observed in 21.2% (77 of 363) and 14.9% (54 of 363) of the gastric cancer patients, respectively, and, except for CA19-9 in the stage III patients, they displayed higher frequencies than the NY-ESO-1 humoral immune response in all stages of the disease. We then analysed whether the addition of the NY-ESO-1 humoral immune response to CEA and CA19-9 increased the diagnostic frequency of gastric cancer. The combined use of CEA and CA19-9 tests produced positivity rates of 15.3% (27 of 176) in stage I, 24.4% (11 of 45) in stage II, 37.3% (25 of 67) in stage III, and 53.3% (40 of 75) in stage IV gastric cancer patients, resulting in an overall positivity rate of 28.4% (103 of 363). When the NY-ESO-1 humoral immune response was added to these two conventional tumour markers, the positivity rates of all stages increased, resulting in information gains of 14.9% (from 25 to 35 patients; 10 of 67) in stage III and 11.2% (from 65 to 81 patients; 16 of 142) in stage III and IV gastric cancer patients.

Changes in the NY-ESO-1 humoral immune responses of the patients during their clinical courses. Serial serum samples were obtained from 25 gastric cancer patients who displayed positive NY-ESO-1 antibody at admission, and the changes in their NY-ESO-1 humoral immune responses were examined throughout their clinical courses. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgical treatment, and 14 did not suffer recurrence. The NY-ESO-1 immune response levels of the patients who did not suffer recurrence decreased after treatment and had fallen below the cutoff level by 9 months after surgery in most cases and did not subsequently increase (Figure 1). The half-lives of their NY-ESO-1 humoral immune response levels were 1.5, 1.6, 2.1, 3.2, and 6.6 months in the stage I patients; 3.0 and 4.0 months in the stage II patients; and 1.6, 1.9, 2.3, 3.0, 3.2, 4.1, and 6.7 months in the stage III patients (mean: 3.0 months). On the other hand, the two patients who underwent curative surgery but subsequently suffered recurrence, M-2 (stage I) and M-11 (stage III), displayed not only incomplete decreases in their NY-ESO-1 humoral immune response levels but also their subsequent restoration to pretreatment levels (Figure 1 and Figure 2A and B). In a comparison between the patients' conventional tumour marker levels and their NY-ESO-1humoral immune response levels, we found that the changes in their CEA and CA19-9 levels were consistent with their NY-ESO-1 immune response levels in patient M-2, whereas patient M-11 was negative for both CEA and CA19-9 throughout their clinical course. Nine stage IV patients who received chemotherapy alone maintained high NY-ESO-1 humoral immune response levels throughout their clinical courses,

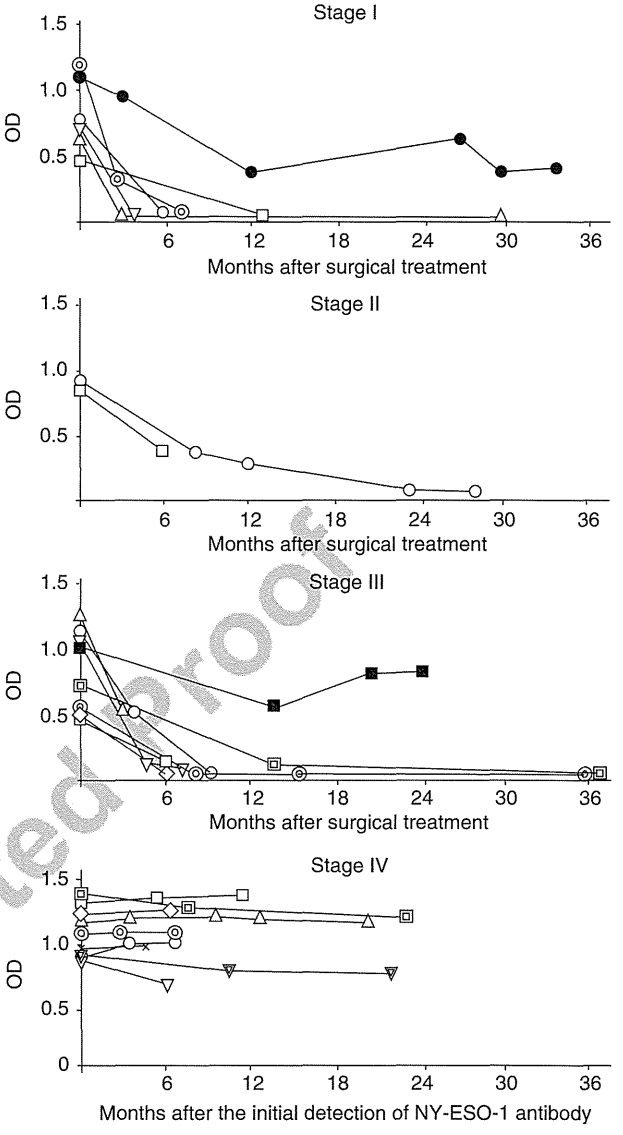

Figure 1. Change in the NY-ESO-1 humoral immune responses of gastric cancer patients after treatment. The serum NY-ESO-1 humoral immune responses of patients with stage I, II, III, or IV gastric cancer in whom NY-ESO-1 antibody production was detected before surgical treatment or chemotherapy were serially analysed. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgery, and only 2 patients (●, ■) suffered recurrence. Nine patients with stage IV gastric cancer received chemotherapy alone after the initial detection of NY-ESO-1 antibody. Each mark represents a patient. Optical density (OD) values were measured at a serum dilution of 1 : 200.

including some patients who achieved partial tumour responses after chemotherapy (Figure 1).

Prognostic value of the NY-ESO-1 humoral immune response in gastric cancer. The prognostic value of the NY-ESO-1 immune response was evaluated in gastric cancer patients. An analysis of the cumulative overall survival of the gastric cancer patients indicated that there was no difference in the survival rates of the patients who did and did not display positive NY-ESO-1 humoral immune responses (Figure 3A). However, among the patients with higher stage gastric cancer, overall survival was better in the patients in whom NY-ESO-1humoral immune responses were

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detected, although the difference was not significant (Figure 3B). NY-ESO-1 protein expression, as detected by IHC, did not affect the overall survival rate (data not shown).

DISCUSSION

NY-ESO-1 antibody was detected in 23.2% of stage III and IV gastric cancer patients, and the combinatorial use of the NY-ESO-1 antibody with CEA and CA19-9 as tumour markers increase the percentage of tumour detection from 45.8 to 57.0%. As the frequency of NY-ESO-1 humoral immune response was relatively low in the patients with early-stage gastric cancer, analysing serum NY-ESO-1 antibody levels alone might not be useful for screening for early-stage gastric cancer. Nevertheless, the expression of NY-ESO-1, a CT antigen, is restricted to tumour tissues and NY-ESO-1 antibody is only detectable in patients with NY-ESO-1-expressing tumours (Stockert *et al*, 1998), indicating the highly specific nature of NY-ESO-1 humoral immune responses in cancer patients. Given that NY-ESO-1 expression by malignant cells is required for antibody induction (Stockert *et al*, 1998), the detection of NY-ESO-1 antibody would be helpful for diagnosing malignancy, although extensive analysis of serum samples from patients with non-cancerous disease, for example, liver or renal disorders, autoimmune diseases, and so on, would be necessary to confirm. In our expression analysis, more NY-ESO-1-positive cases were detected by IHC (19 of 60) than by RT–PCR (6 of 60). This was probably due to the heterogeneous expression of NY-ESO-1 in gastric cancer and the fact that a limited number of biopsy samples were used for the RT–PCR, whereas multiple slices from whole tumour specimens were used for the IHC. Extensive IHC analysis should be used for NY-ESO-1 expression studies of gastric cancer.

We detected a correlation between the NY-ESO-1 humoral immune response levels and the clinical outcome after therapy in gastric cancer patients. The patients who underwent surgery and did not suffer a subsequent relapse displayed consistent decreases in their NY-ESO-1 humoral immune response levels or even the complete disappearance of the NY-ESO-1 antibody from their sera. It is generally accepted that constant immunological stimulation is necessary to maintain a strong humoral immune response (Jager *et al*, 1999). Thus, reduction of antigen doses by the removal of NY-ESO-1-expressing tumour is one possible reason for the observed decreases in these patients' NY-ESO-1 humoral immune response levels after surgery. Patients M-2 and M-11, in whom NY-ESO-1 humoral immune responses remained high for 1 year after surgery and increased thereafter, may have a subclinical residual disease of the so-called 'minimal residual cancer' (Austrup *et al*, 2000; Klein *et al*, 2002) after curative surgery. Local recurrent tumours of 23 and 25 mm in diameter subsequently developed in M-2 and M-11, respectively, suggesting that even a small tumour burden is sufficient to stimulate antibody production. Patient M-2 showed a partial decrease in their NY-ESO-1 humoral immune response levels after the resection of the relapsed tumour, and we are carefully observing the progression of this tumour.

Nine patients with stage IV gastric cancer received chemotherapy alone. Among them, six patients displayed stable disease, two

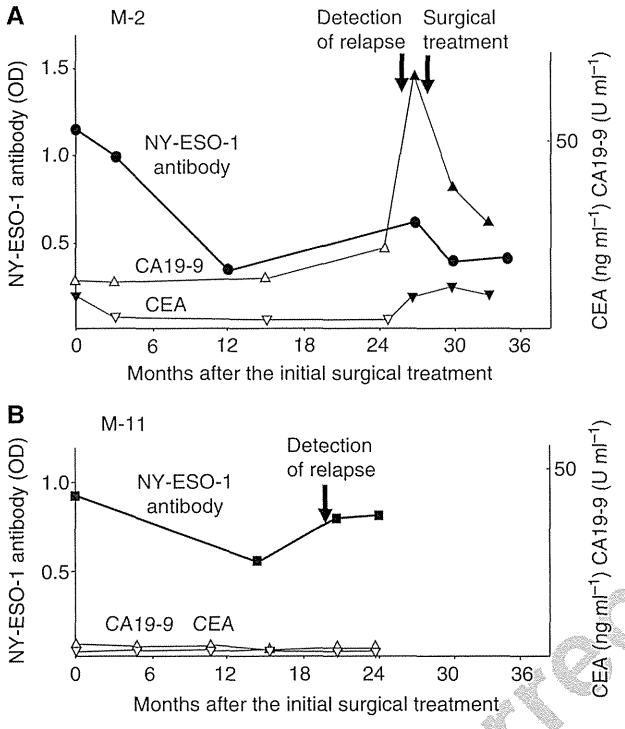

Figure 2. NY-ESO-1 humoral immune response, CEA, and carbohydrate antigen (CA)19-9 levels of patients who relapsed after curative surgery. The NY-ESO-1 humoral immune response (●, ■; Figure 1), ~~CEA, and CA19-9~~ levels of two patients, M-2 (stage I) (**A**) and M-11 (stage III) (**B**), who underwent curative surgery but subsequently suffered recurrence, were serially analysed. OD values were measured at a serum dilution of 1 : 200. The closed marks indicate CEA or CA19-9 positivity.

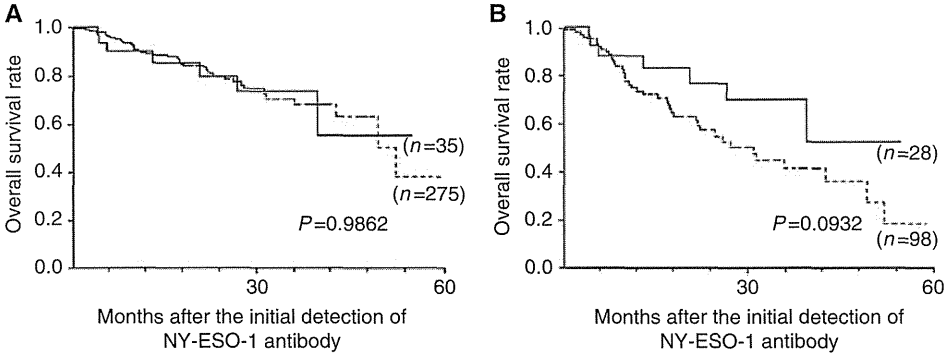

Figure 3. Prognostic role of NY-ESO-1 antibody in gastric cancer patients. The cumulative overall survival rate was analysed in all patients ($n=310$; **A**) and stage III and IV ($n=126$; **B**) gastric cancer patients in whom NY-ESO-1 antibodies were (continuous line) and were not detected (dotted line). The detection of NY-ESO-1 protein by IHC analysis did not affect the overall survival rate (data not shown). Survival curves were plotted using the Kaplan–Meier method. The log-rank test was used for comparisons between groups. *P*-values <0.05 were considered significant.

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patients displayed progressive disease, and one patient (M-19) achieved a partial response. Serial analysis of the NY-ESO-1 humoral immune responses of these nine patients including M-19 showed that they barely changed throughout their clinical courses, suggesting that even small tumours are enough to provoke strong NY-ESO-1 humoral immune responses. In this regard, the NY-ESO-1 humoral immune response might not be suitable as a clinical marker for palliative therapy.

We have performed serial cancer vaccine clinical trials with NY-ESO-1 because of its strong immunogenicity and high specificity (Uenaka *et al*, 2007; Wada *et al*, 2008; Kakimi *et al*, 2011). The NY-ESO-1humoral immune response could be a reliable marker of the induction of immune response, as well as for predicting clinical responses in these trials. Furthermore, antibody-based examinations detected both intra- and intermolecular antigen spreading in the sera of patients who had been vaccinated with NY-ESO-1 protein (Kawada *et al*, 2012), suggesting the possible correlation of NY-ESO-1 humoral immne responses and clinical status. In addition, we have started a phase I study of vaccination with NY-ESO-1 protein mixed with Hiltonol (Poly ICLC), Picibanil (OK-432), and Montanide (ISA-51) in patients with NY-ESO-1-expressing cancers (UMIN000007954). Furthermore, NY-ESO-1 vaccine involving modulators of immune checkpoints, for example, anti-CTLA4 antibody and anti-PD-1 antibody, and reagents that are antagonistic to regulatory T cells, for example, anti-CCR4 antibody (Pardoll, 2012) should be considered.

Recently, the antibody against p53, another tumour antigen, has been recognised as a useful tumour marker (Lubin *et al*, 1995). Shimada *et al* (2000)) reported that p53 antibody was detected in 35% of serum samples from patients with *in situ* oesophageal cancer and that it disappeared after endoscopic mucosal resection, proposing that p53 antibody is useful for the early detection and subsequent monitoring of oesophageal cancer. In addition, Müller *et al* (2006) reported that p53 antibody was found in 23.4% of serum samples from cancer patients with 100% accuracy and was correlated with poor prognosis in hepatocellular carcinoma and breast cancer.

Here, we have demonstrated that the NY-ESO-1 humoral immune response could also be valuable as a marker for detecting advanced gastric cancer and inferring whether residual tumour cells remain after treatment, although its frequency in gastric cancer is not very high. We have started a prospective multi-institutional clinical study of NY-ESO-1 humoral immune responses in higher stage gastric cancer patients. In this new study, the NY-ESO-1 humoral immune responses of approximately 100 patients who relapsed after curative surgery will be serially analysed and then followed up. This trial has been registered as UMIN000007925 in Japan.

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Uncorrected Proof

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

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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.

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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 abdominoperineal 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

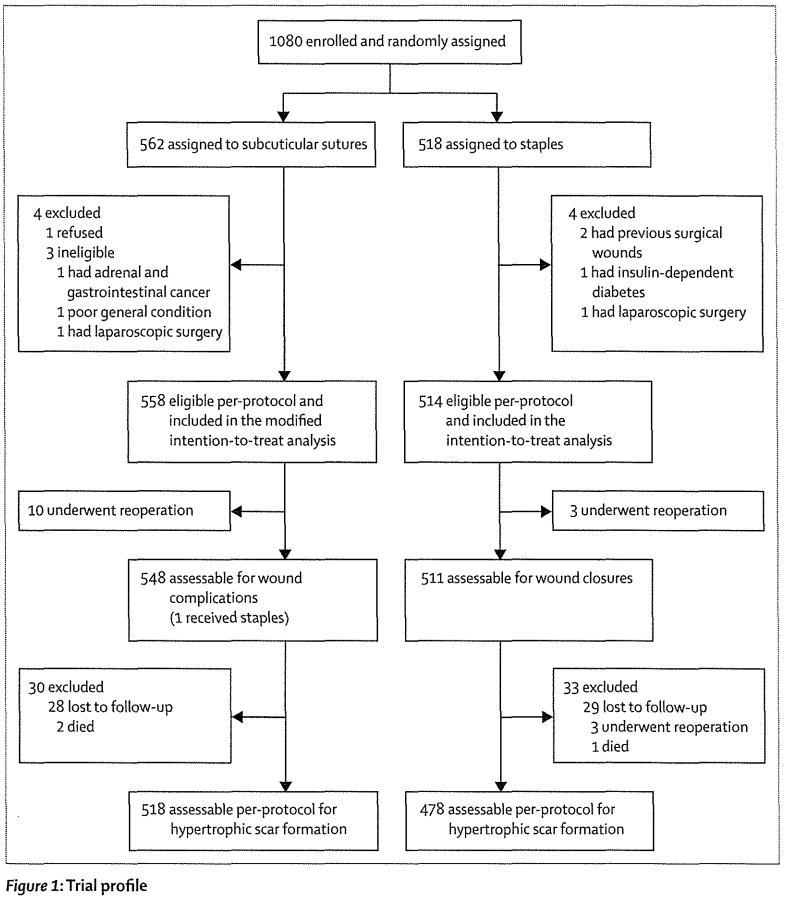

Figure 1: **Trial profile**

	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.[39] *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

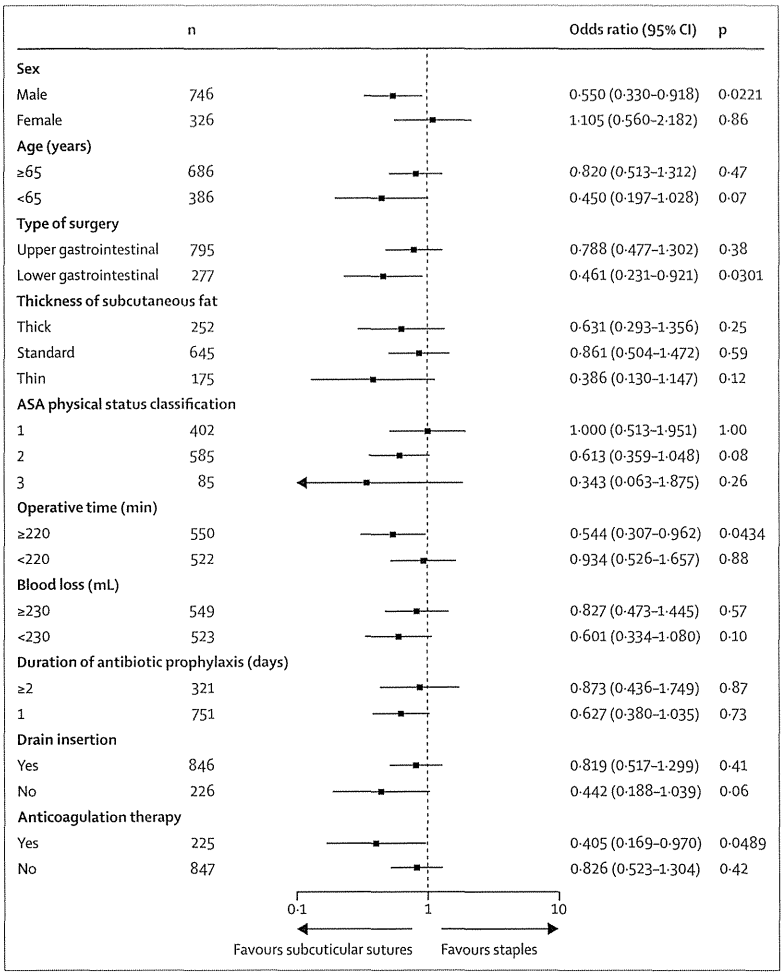

Figure 2: **Subset analysis of wound complications in the modified intention-to-treat population**
Significance was calculated with Fisher's exact test. ASA=American Society of Anesthesiologists.

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.