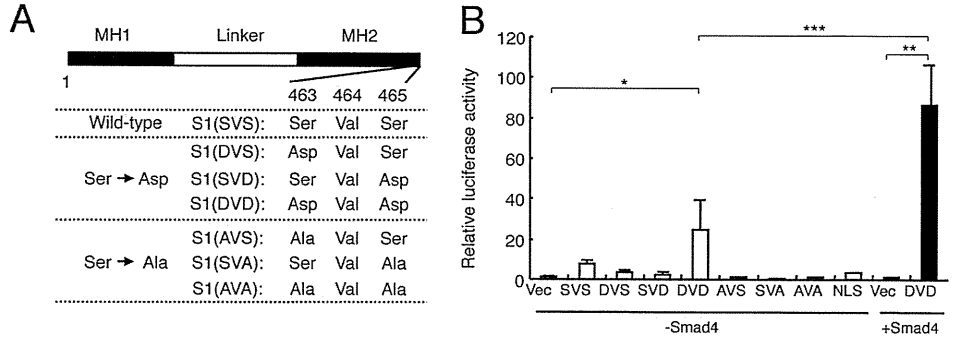

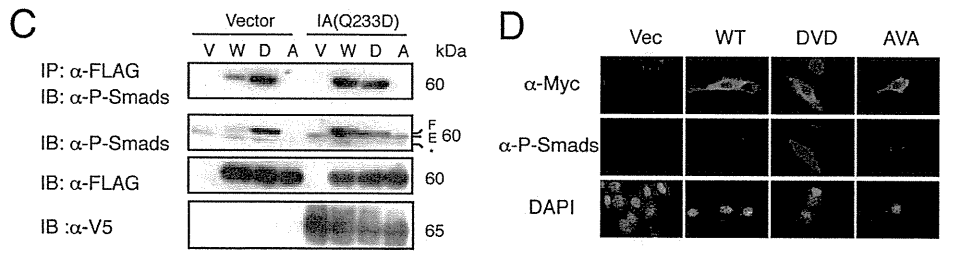

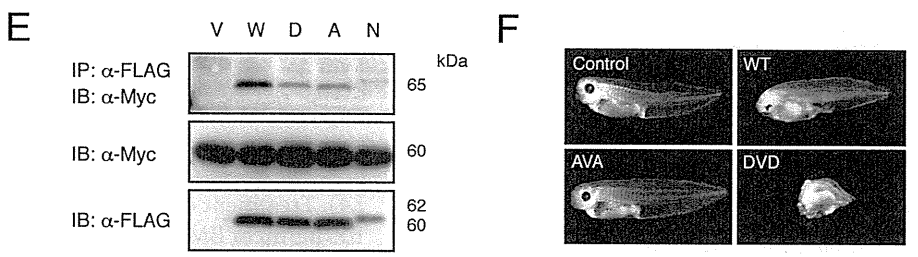

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	Viability	DAI (dorso-anterior index)					Average	Others
		5	4	3	2	1		
Control	34 (100)	33 (97.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5.00	1 (2.9)
Smad1 WT	34 (100)	1 (2.9)	22 (64.7)	7 (20.6)	3 (8.8)	0 (0.0)	3.64	1 (2.9)
Smad1(AVA)	34 (100)	29 (85.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5.00	5 (14.7)
Smad1(DVD)	34 (100)	0 (0.0)	0 (0.0)	9 (26.5)	9 (26.5)	16 (47.1)	1.79	0 (0.0)

FIGURE 2. **Establishment of a constitutively activated Smad1.** *A*, construction of mutant Smad1. Serine 463 and/or 465 at the carboxyl terminus of mouse Smad1 was substituted by aspartic acid or alanine. *B*, transcriptional activities of Smad1 mutants in luciferase assay in C2C12 cells. Wild-type and mutant *Smad1* were transfected with IdWT4F-luc in C2C12 cells in the presence or absence of *Smad4* ($n = 3$). Note that Smad1(DVD) activated the reporter activity even in the absence of *Smad4*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C* and *D*, Smad1(DVD) was recognized by the α-phospho-Smad1/5/8 antibody. C2C12 cells were co-transfected with empty vector (*Vector*) or V5-tagged *Bmpr-Ia(Q233D)* (*IA(Q233D)*) and an empty vector (*V*), FLAG-tagged wild-type *Smad1* (*W*), *Smad1(DVD)* (*D*), or *Smad1(AVA)* (*A*). Whole cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody followed by immunoblotting (*IB*) using α-P-Smads antibody to detect the mutant Smad1 and endogenous Smad1/5/8. *D*, C2C12 cells transfected with Myc-tagged wild-type or *Smad1(DVD)*, *Smad1(AVA)*, or *NLS-Smad1* were stained with α-P-Smads and α-Myc antibodies without BMP stimulation. Note that Smad1(DVD) was detected in the cytoplasm with α-P-Smads antibody. *E*, interaction with Smad1 and Smad4. C2C12 cells were co-transfected with Myc-tagged *Smad4* and an empty vector (*V*), FLAG-tagged wild-type *Smad1* (*W*), *Smad1(DVD)* (*D*), *Smad1(AVA)* (*A*), or *NLS-Smad1* (*N*). Whole cell lysates were immunoprecipitated with α-FLAG antibody followed by immunoblotting using α-Myc antibody to detect Smad4 in complex with Smad1. *F* and *G*, ventralization inducing activity of Smad1 in *Xenopus* embryos. *F*, *Xenopus* embryos at four-cell stage were injected with 500 pg of synthetic mRNA of wild-type, *Smad1(AVA)*, or *Smad1(DVD)* into the dorsal marginal region. *G*, dorsal-anterior index of *Xenopus* embryos induced by Smad1. Values smaller than 5 indicate degree of ventralization.

adenovirus expressing Cre recombinase (pAxCANCre) or a human histone 2B-GFP unit (pAxH2BGFP) under control of the CAG promoter (22, 23). After being cultured for an additional 48 h, MEFs were transfected with plasmids using Lipofectamine 2000 (Invitrogen) in combinations as described in each figure legend. The cells were further cultured with or without BMP-4 before being examined for BMP activities as described below. A cell line, clone 16, was established by a limiting dilution method from *Smad4*$^{floxed/floxed}$ MEFs maintained by more than 30-fold serial passage. Bone marrow stromal cells were prepared from tibiae of 8-week-old *Smad4*$^{floxed/floxed}$ mice.

Immunohistochemistry, Immunoprecipitation, and Western Blot Analysis—The following antibodies were used for immunohistochemistry, Western blot analysis, and immunoprecipitation: anti-MHC antibody (clone MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA), anti-myogenin (clone F5D, Santa Cruz, Santa Cruz, CA), anti-FLAG antibody (clone M2, Sigma), anti-Myc antibody (clone 9E10, Santa Cruz), anti-Myc polyclonal antibody (Medical & Biological Laboratories Co., Nagoya, Japan), anti-Smad4 antibody (clone B-8, SC-7966, Santa Cruz), anti-V5 antibody (P/N 46–0705, Invitrogen), anti-E4F1 antibody (Bethyl Laboratories, Montgomery, TX), anti-phospho-Smad1/5/8 polyclonal antibody (Cell Signaling, Beverly, MA), anti-Runx2/Cbfa-1 (Medical & Biological Laboratories Co.), and anti-β-actin antibody (I-19, SC-1616, Santa Cruz). For immunohistochemical analysis, target proteins were visualized using a Histofine SimpleStain Kit (Nichirei, Tokyo, Japan) or an Alexa 488- or Alexa 594-conjugated secondary antibody (Invitrogen). A BZ-9000 (Keyence, Tokyo, Japan) microscope was used for fluorescent analysis. Western blot analysis was performed as described (17). The target proteins were immunoprecipitated for 6 h at 4 °C using M2-agarose beads (Sigma). The target proteins were detected using a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (GE Healthcare).

ALP and Luciferase Assays—ALP activity was stained as a typical marker of osteoblastic differentiation (24). Enzyme

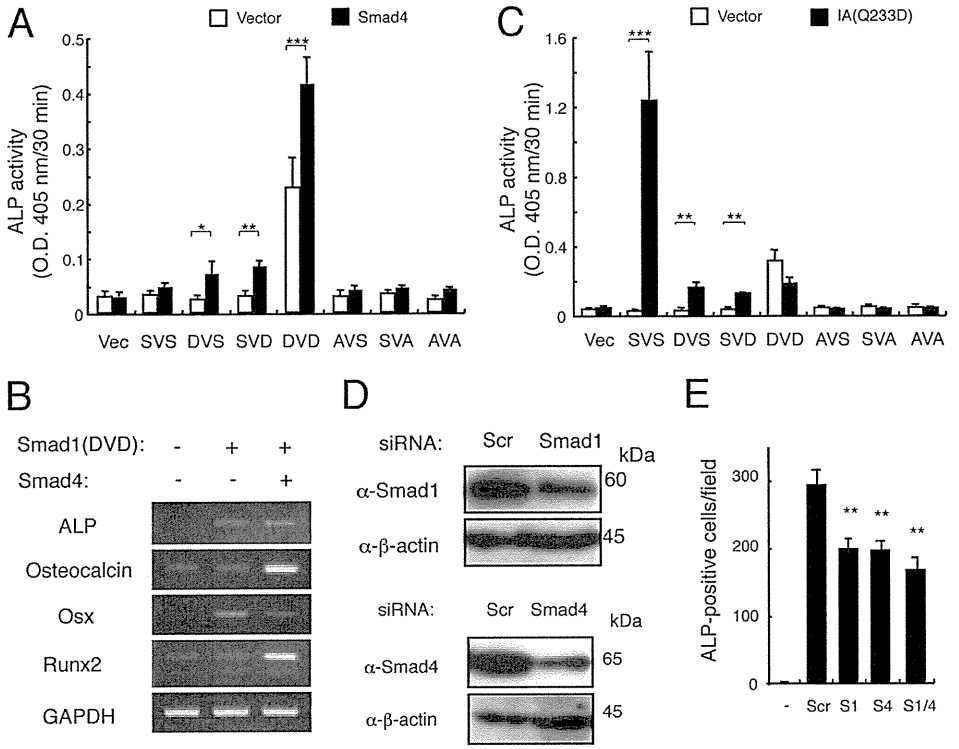

FIGURE 3. **Smad1(DVD) induces osteoblastic differentiation of C2C12 myoblasts.** *A*, Smad1(DVD) (*DVD*) induces ALP activity in C2C12 myoblasts. C2C12 cells were transfected with one of the *Smad1* constructs in the presence (*closed bars*) or absence (*open bars*) of *Smad4*, and ALP activity was measured on day 3. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, Smad4 enhances Smad1(DVD) activity. C2C12 cells were transfected with empty vector alone or with *Smad1(DVD)* in the absence or presence of *Smad4*. Reverse transcription-PCR was performed on day 3. *C*, synergism between Smad1 and a constitutively activated BMPR-IA receptor. C2C12 cells were transfected with one of the *Smad1* constructs in the absence (*open bars*) or presence of *Bmpr-Ia(Q233D)* (*closed bars*), and ALP activity was determined on day 3. Values are mean ± S.D. ($n = 3$). *D*, knockdown of Smad1 and Smad4 in C2C12 cells. C2C12 cells were transfected with 20 nM *Smad1* (*upper panels*) and *Smad4* (*lower panels*) siRNA. Protein levels were determined by Western blots at 24 h. *E*, knockdown of Smad1 or Smad4 reduced ALP activity induced by BMP-4. The siRNA-transfected C2C12 cells were treated for 3 days with 100 ng/ml of BMP-4 and ALP activity was determined. Values are mean ± S.D. ($n = 3$). **, $p < 0.01$ compared with a scrambled siRNA transfection group.

activity was measured using *p*-nitrophenyl phosphate as a substrate (24). Luciferase assays were performed using pGL3MG-185 (25) or IdWT4F-luc reporter plasmids and phRL-SV40 (Promega, Madison, WI) with the Dual-Glo Luciferase Assay System (Promega) as described previously (13).

Dorsoventral Assay in Xenopus Embryos—A dorsoventral assay in *Xenopus* embryos was performed essentially as described (26). The injected embryos were allowed to develop until stages 34–40 for observation of external appearance and then subjected to histological analysis. The activity of each Smad1 was expressed by a dorso-anterior index (26, 27).

Reverse Transcription-PCR Analysis—Total RNAs were extracted using TRIzol (Invitrogen) and reverse transcribed with SuperScript III (Invitrogen). The PCR was performed using Platinum Pfx DNA polymerase (Invitrogen) as described (28). The primer sets used were previously described (29).

Chromatin Immunoprecipitation Assay—Cells were lysed in ChIP buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and sonicated. The chromatin solution was subjected to immunoprecipitation using a OneDay ChIP Kit (Diagenode, Sparta, NJ) according to the manufacturer's instructions. The following antibodies were used: anti-E4F1 (Bethyl), anti-Smad4 (sc-7966, Santa Cruz Biotechnology), anti-MyoD (sc-760, Santa Cruz Biotechnology), and anti-histone H3 (Upstate, Lake Placid, NY). The *Myogenin* promoter was amplified by PCR using the following primers: 5′-TAATTGAAAGGAGCAGATGAGACGGGG-3′ and 5′-CCATCAGGTCGGAAAAGGCTTGTTC-3′.

Statistical Analysis—Comparisons were made using an unpaired Student's *t* test. Results are represented as mean ± S.D. Statistical significance is displayed as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

RESULTS

Induction of the Smad-dependent Pathway by BMP-4 Regulates Both Myogenic and Osteoblastic Differentiation of C2C12 Cells—First, we determined the minimal culture periods of C2C12 cells with BMP-4 required for the inhibition of myogenic differentiation and induction of osteoblastic differentiation (Fig. 1*A*). Treatment of C2C12 cells with 100 ng/ml of BMP-4 for the first 30 min of a 3-day culture markedly suppressed myogenic differentiation on day 3 but did not induce ALP activity (Fig. 1, *B–E*). ALP-positive cells were detected in cultures treated with BMP-4 for longer than 9 h (Fig. 1, *B* and *E*). The BMP-4-induced inhibition of myogenic differentiation and induction of osteoblastic differentiation were blocked by Dorsomorphin, a BMP-Smad specific inhibitor, but not by inhibitors of mitogen-activated protein kinases, suggesting that the Smad-dependent pathway regulates conversion to differentiation (Fig. 1, *F–H*, and supplemental Fig. S1).

Construction of Constitutively Activated Smad1—We generated a series of Smad1 mutants, in which one or two serine residues at the carboxyl termini were substituted with aspartic acid or alanine residues (Fig. 2*A*). Among these Smad1 mutants, Smad1(DVD) exhibited transcriptional activity in a luciferase assay using the ID1 reporter without the addition of BMPs, and this activity was further enhanced by co-transfection with Smad4 (Fig. 2*B*). NLS-Smad1, in which a nuclear localization signal (NLS) was added to the amino terminus of Smad1, failed to induce luciferase activity. Smad1(DVD) was recognized by the α-phospho-Smad1/5/8 antibody, even in the absence of a constitutively active BMP receptor, BMPR-IA(Q233D), without affecting endogenous phospho-Smad1/5/8 levels (Fig. 2, *C* and *D*). Smad1(DVD) did not exhibit changes in cellular localization or interaction with Smad4 that were distinguishable from wild-type or other Smad1 mutants (Fig. 2, *D* and *E*). Injection of synthetic *Smad1(DVD)* mRNA into the dorsal sides

of *Xenopus* embryos induced ventralization, although *Smad1(AVA)* did not exhibit this activity (Fig. 2*F*). The average dorso-anterior index values induced by *Smad1(DVD)* and *Smad1(AVA)* were 1.79 and 5.00, respectively, indicating that Smad1(DVD) is a constitutively activated Smad1 in *Xenopus* embryos as well (Fig. 2*G*).

Activated Smad1 and Runx2, but Not Smad4, Cooperatively Induce Osteoblastic Differentiation of C2C12 Cells—Transient transfection of *Smad1(DVD)* in C2C12 cells induced expression of osteoblastic differentiation markers such as *ALP, Osteocalcin, Runx2,* and *Osterix*; this was further enhanced by the presence of *Smad4* for every marker except *Osterix*, which might be peaked within 3 days before sample preparation (Fig. 3, *A* and *B*). BMPR-IA(Q233D) stimulated ALP activity in cooperation with wild-type Smad1, confirming that Smad1 is a critical substrate of the type I receptor for induction of osteoblastic differentiation (Fig. 3*C*). However, no synergism was observed between BMPR-IA(Q233D) and any Smad1 mutants, including Smad1(DVD), suggesting that Smad1 mutants are not recognized as substrates by the receptor (Figs. 2*C* and 3*C*). Transfection of siRNA against *Smad1, Smad4,* or a combination of the two reduced the ALP activity induced by BMP-4 or BMPR-IA(Q233D) (Fig. 3, *D* and *E*). Similar results were obtained using siRNA against *Smad5* (data not shown).

The role of Smad proteins in osteoblastic differentiation was further examined using MEFs prepared from *Smad4$^{floxed/floxed}$* mice. The MEFs had been infected with an adenovirus expressing Cre recombinase or EGFP *in vitro* before being treated with BMP-4. Western blot analysis revealed that the phosphorylation of Smad1/5/8 in response to BMP-4 was independent of Smad4 (Fig. 4*A*). In these MEF cultures, the ALP activity induced by BMP-4 was reduced but not eliminated in the Cre-expressing MEFs (Fig. 4*B*). We further examined the role of Smad4 in osteoblastic differentiation using bone marrow stromal/osteoblastic cells prepared from *Smad4$^{floxed/floxed}$* mice. Again, *Smad4* was deleted *in vitro* by infection with an adenovirus expressing Cre, but expression of ALP was not eliminated in these cells; in fact, ALP expression was still induced by BMP-4 in a dose-dependent fashion in Smad4-deleted cultures (Fig. 4, *C* and *D*). These results suggested that Smad4 is not essential for BMP-induced osteoblastic differentiation but that it may enhance BMP signaling.

Runx2 is essential for osteoblast differentiation and also interacts with R-Smads (30–32). Overexpression of Smad1(DVD) or Runx2 alone induced ALP activity in C2C12 cells, and co-expression of Smad1(DVD) and Runx2 further increased ALP activity (Fig. 5*A*). In contrast, a dominant-negative form of Runx2 blocked ALP induction by Smad1(DVD) (Fig. 5*A*). RNAi knockdown of Runx2 reduced numbers of ALP-positive cells in C2C12 cultures induced by BMPR-IA(Q233D) (Fig. 5, *B* and *C*). Taken together, these findings suggest that phosphorylated R-Smads and Runx2 may cooperatively induce osteoblast differentiation in response to BMPs.

Smad4 Is Involved in Inhibition of Myogenic Differentiation by BMPs—We next examined the roles that the Smad signaling pathway plays in the inhibition of myogenic differentiation by BMPs. Overexpression of Smad1 mutants, including Smad1(DVD), did not inhibit the myogenic differentiation

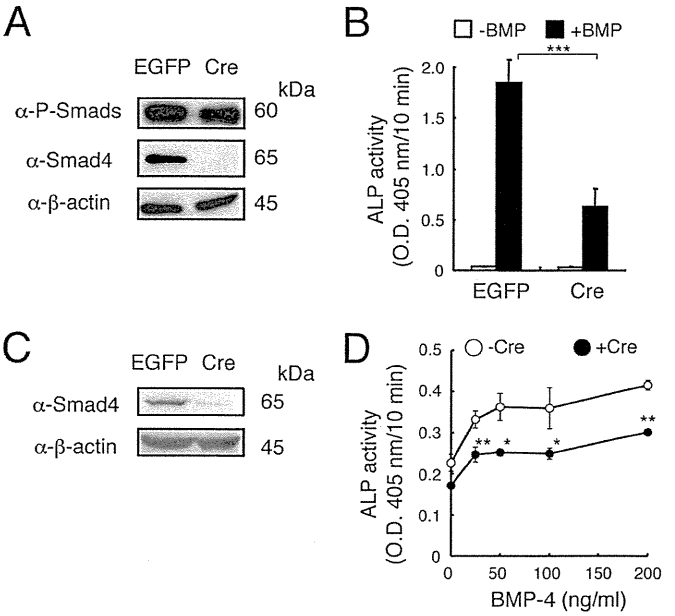

FIGURE 4. **Smad4 is not essential for the osteoblastic differentiation induced by BMP-4.** *A*, MEFs prepared from *Smad4$^{floxed/floxed}$* were infected with adenovirus expressing Cre recombinase or EGFP. The cells were stimulated for 30 min with 100 ng/ml of BMP-4 to induce phosphorylation of Smad1/5/8. *B*, adenovirus-infected MEFs were treated for 3 days with 100 ng/ml of BMP-4, and ALP activity was determined on day 3. Values are mean ± S.D. ($n = 5$). ***, $p < 0.001$. *C*, Western blot analysis of Smad4-ablated bone marrow stromal/osteoblastic cells prepared from *Smad4$^{floxed/floxed}$* mice. *D*, dose-dependent induction of ALP activity by BMP-4 in *Smad4$^{floxed/floxed}$*-derived bone marrow stromal/osteoblastic cells infected with Cre-expressing adenovirus or uninfected cells. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$.

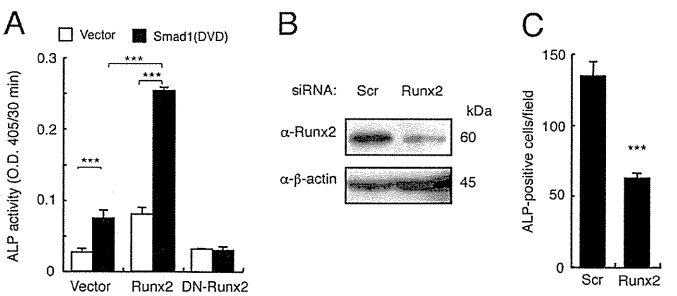

FIGURE 5. **Runx2 and Smad1(DVD) cooperatively induce osteoblastic differentiation of C2C12 cells.** *A*, C2C12 cells were co-transfected with *Smad1(DVD)* and *Runx2* or dominant-negative *Runx2*, and ALP activity was determined on day 3. Values are mean ± S.D. ($n = 3$). ***, $p < 0.001$. *B*, 20 nmol of *Runx2* siRNA or scrambled oligonucleotide was transfected in C2C12 cells, and protein levels were determined by Western blots at 24 h post-transfection. *C*, the siRNA-transfected C2C12 cells were co-transfected with *Bmpr-Ia(Q233D)* and *Smad1*, and ALP activity was determined on day 3. Values are mean ± S.D. ($n = 3$). ***, $p < 0.001$ compared with a scrambled siRNA transfection group.

induced by MyoD. Co-transfection of *Smad4* with *Smad1(DVD)* was required to reduce the number of MHC-positive cells (Fig. 6, *A* and *B*). This role of Smad4 in myogenic differentiation was confirmed in *Smad4$^{floxed/floxed}$* MEFs. The transcriptional activity of MyoD was suppressed by BMP-4 in MEF cultures infected with a control virus (Fig. 6*C*). In contrast, the basal transcriptional activity of MyoD was increased ~1.4-fold in MEFs infected with the Cre-expressing adenovirus. This increase was not suppressed by BMP-4, suggesting that Smad4 is essential for the suppression of myogenic differentiation by BMPs

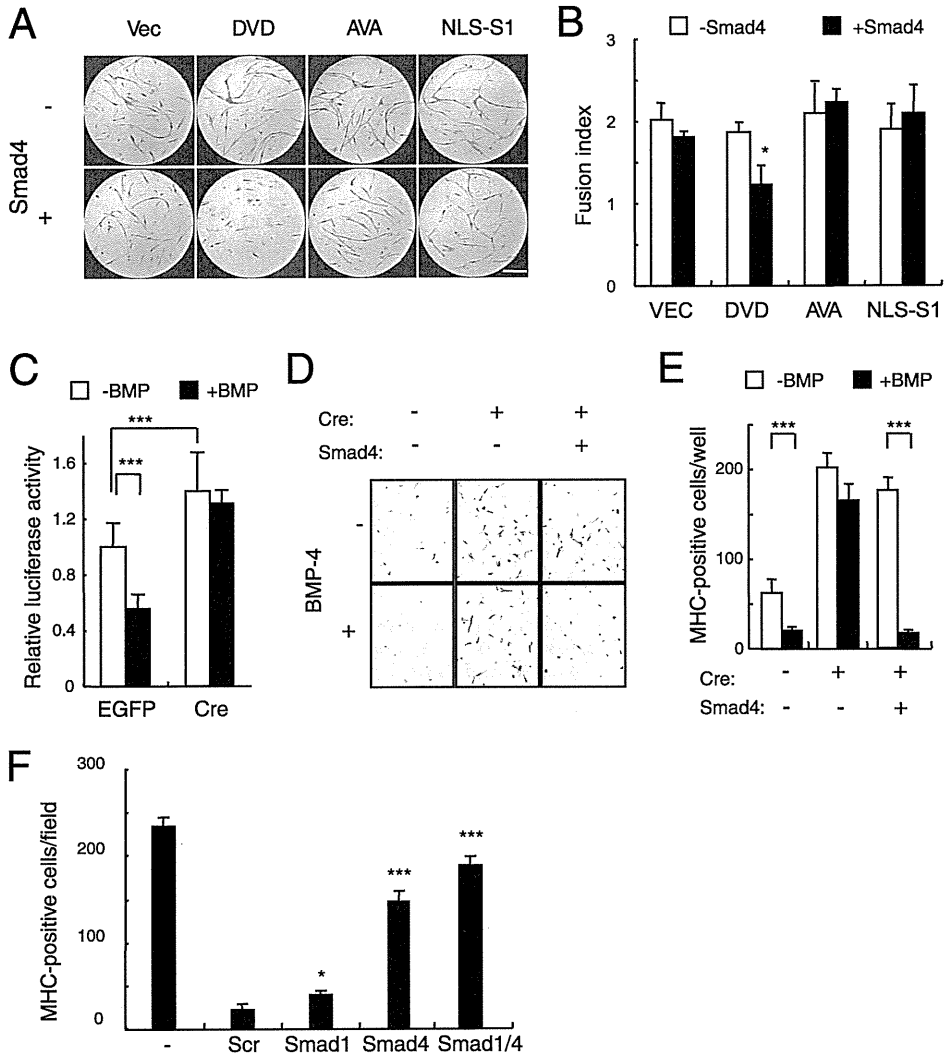

FIGURE 6. **Smad4 is involved in the inhibition of myogenic differentiation.** *A* and *B*, Smad1(DVD) inhibits myogenic differentiation only in the presence of Smad4. C3H10T1/2 fibroblasts were co-transfected with *MyoD* and empty vector (*Vec*), *Smad1(DVD)*, *Smad1(AVA)*, or *NLS-Smad1* (*NLS-S1*) in the absence (*upper panels*) or presence (*lower panels*) of *Smad4* (*A*). Myogenic cells were immunostained for MHC (*red*) on day 5. *Scale bar*, 400 μm. *B*, fusion index was determined in the presence (*closed bars*) or absence (*open bars*) of Smad4. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$. *C*, Smad4 is required for suppression of transcriptional activity of MyoD by BMP-4. MEFs prepared from *Smad4*$^{floxed/floxed}$ were infected with adenovirus expressing EGFP or Cre recombinase, and then the transcriptional activity of MyoD was determined in the presence or absence of 100 ng/ml of BMP-4. Values are mean ± S.D. ($n = 10$). ***, $p < 0.001$. *D* and *E*, Smad4 is essential for inhibition of myogenic differentiation by BMPs. Clonal cell line 16 infected with Cre-expressing or EGFP-expressing control adenovirus was transfected with *MyoD* and stained for MHC on day 5 (*D*). *E*, the numbers of MHC-positive cells were counted in cultures prepared as in *D*. Values are mean ± S.D. ($n = 6$). ***, $p < 0.001$. *F*, 20 nmol of *Smad1* or *Smad4* siRNA or a scrambled oligonucleotide was transfected into C2C12 cells. These cells were treated with 100 ng/ml of BMP-4, and MHC-positive cells were counted on day 5. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$ compared with a scrambled siRNA transfection group.

(Fig. 6*C*). This hypothesis was further confirmed using the floxed *Smad4* MEF cell line, clone 16. Transient transfection of MyoD in clone 16 induced a small number of MHC-positive cells, which were reduced in number by BMP-4 (Fig. 6, *D* and *E*). Infection by Cre-expressing adenovirus not only increased the number of MHC-positive cells, but also maintained this increase in the presence of BMP-4 (Fig. 6, *D* and *E*). Transient transfection of Smad4 in Cre-adenovirus-infected cultures restored the suppression of myogenic differentiation in response to BMP-4 (Fig. 6, *D* and *E*). Moreover, RNAi knockdown of Smad4 increased the number of MHC-positive cells in C2C12 cell cultures treated with BMP-4 (Fig. 6*F*). In contrast, knockdown of Smad1 had minimal effects on these cultures (Fig. 6*F*).

Inhibition of Myogenic Differentiation by Nuclear Smad4—Because Smad4 may translocate into the nucleus as a complex with Smad1/5/8 in response to BMP stimulation, we generated NLS-Smad4 and deletion mutants lacking amino-terminal MH1 (NLS-Smad4(ΔMH1)) and carboxyl-terminal MH2 (NLS-Smad4(ΔMH2)) domains, respectively (Fig. 7*A*). Nuclear localization of these NLS-Smad4 mutants was confirmed, although wild-type Smad4 was mainly detected in the cytoplasm (Fig. 7*B*). Overexpression of full-length NLS-Smad4 suppressed the myogenic differentiation of C3H10T1/2 cells induced by MyoD in a dose-dependent manner (Fig. 7, *C* and *D*, and data not shown). Unexpectedly, NLS-Smad4(ΔMH1), but not NLS-Smad4(ΔMH2), stimulated myogenic differentiation, suggesting that NLS-Smad4(ΔMH1) behaved in a dominant-negative fashion (Fig. 7, *C* and *D*). The Smad4 MH2 domain may thus interact with other molecules essential for inhibition of myogenic differentiation.

Involvement of E4F1 in the Inhibition of Myogenic Differentiation by BMP Signaling—We searched a protein-protein interaction data base that was constructed based on the mammalian two-hybrid method established by the RIKEN group (33) and found several proteins that formed complexes with Smad4. Among these proteins, we focused on E4F1, which contains six zinc fingers and a ubiquitin E3 ligase domain (Fig. 9*A*), because it appeared to be one of the transcription factors principally responsible for inhibition of myogenic differentiation by Smad4.

FLAG-tagged E4F1 was expressed in nuclei, co-localized with NLS-Smad4, and bound to larger amounts of NLS-Smad4 than wild-type Smad4 (Fig. 8, *A* and *B*). Interaction between endogenous Smad4 and FLAG-E4F1 was also detected in C2C12 cells (Fig. 8*C*). NLS-Smad4(ΔMH1), but not NLS-Smad4(ΔMH2), bound to E4F1, confirming that the complex is formed via the MH2 domain of Smad4 (Fig. 8*D*).

Both wild-type E4F1 and E4F1(ΔE3), but not zinc finger mutants, suppressed myogenic differentiation, suggesting that

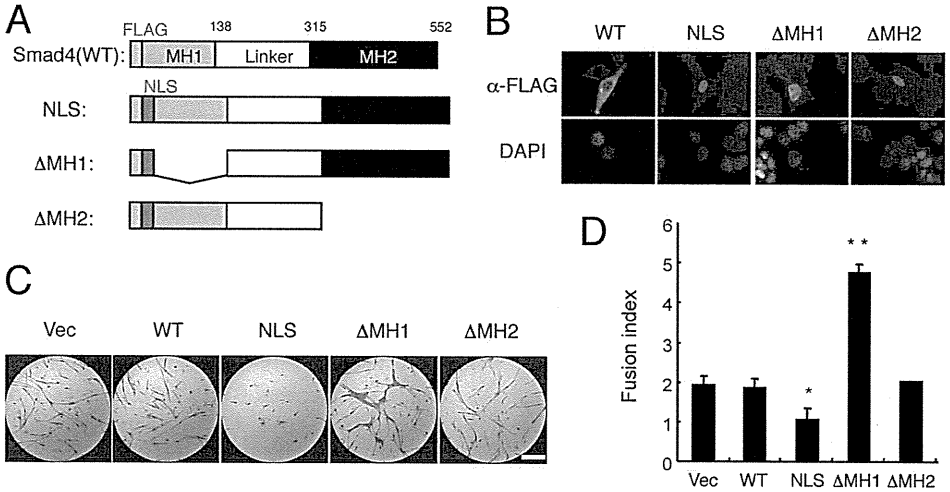

FIGURE 7. **Nuclear Smad4 inhibits myogenic differentiation.** *A*, scheme of construction of FLAG-tagged Smad4 mutants. *B*, cellular localization of FLAG-tagged Smad4 mutants. The cells were immunostained with α-FLAG antibody. *Scale bar*, 25 μm. *C*, effects of Smad4 mutants on myogenic differentiation. C3H10T1/2 cells were co-transfected with *MyoD* and one of the *Smad4* constructs and immunostained for MHC on day 5. *Scale bar*, 400 μm. *D*, fusion index was determined from cultures prepared as in *C*. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$. *DAPI*, 4′,6-diamidino-2-phenylindole.

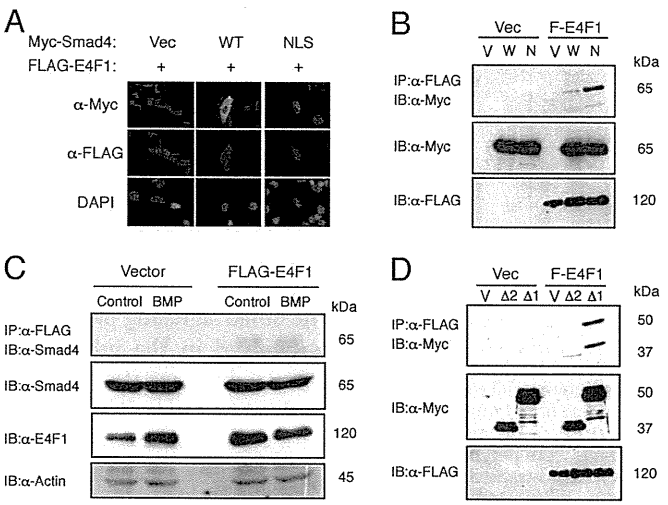

FIGURE 8. **E4F1 inhibits myogenic differentiation in cooperation with nuclear Smad4.** *A*, E4F1 and Smad4 overlapped in nuclei in C2C12 cells. FLAG-tagged *E4F1* and empty vector (*Vec*), *Myc-Smad4* (*WT*), or *Myc-NLS-Smad4* (*NLS*) were cotransfected and stained with α-Myc and α-FLAG antibodies. *B* and *C*, E4F1 interacts with Smad4 *in vivo*. *B*, COS-7 cells were co-transfected with *FLAG-E4F1* and empty vector (*Vec*), *Myc-Smad4* (*WT*), or *Myc-NLS-Smad4* (*NLS*). Whole cell lysates were immunoprecipitated (*IP*) with α-FLAG antibody followed by immunoblotting (*IB*) using α-Myc antibody. *C*, C2C12 cells transfected with *Flag-E4F1* were treated for 1 h without or with 100 ng/ml of BMP-4. Whole cell lysates were immunoprecipitated with M2-agarose beads followed by immunoblotting with antibodies for E4F1, Smad4, and actin. *D*, the MH2 domain of Smad4 is required for interaction with E4F1. The interaction between E4F1 and Smad4 was determined by cotransfection of *Flag-E4F1* and *Myc-NLS-Smad4*(*ΔMH2*) or *Myc-NLS-Smad4*(*ΔMH1*) in COS-7 cells. *DAPI*, 4′,6-diamidino-2-phenylindole.

E4F1 may inhibit myogenesis as a transcription factor rather than as a ubiquitin ligase (Fig. 9*B*). Although *Myogenin* is one of the targets of MyoD and is markedly suppressed by BMP signaling, we could not detect Smad4 or E4F1 binding to the *Myogenin* promoter in response to BMP-4 (supplemental Fig. S2). RNAi knockdown of E4F1 increased the number of MHC-positive C2C12 cells in the presence of BMP signaling (Fig. 9, *D* and *E*). Similar results were obtained using a plasmid-based microRNA expression vector for E4F1 (supplemental Fig. S3). We further examined the role of E4F1 in myogenesis in cell line clone 16 established from *Smad4*$^{floxed/floxed}$ MEFs. Again, deletion of *Smad4* by Cre-adenovirus infection increased the number of MyoD-induced MHC- and myogenin-positive myogenic cells (Fig. 9, *F* and *G*). Co-transfection of *Smad4* and *E4F1* markedly reduced the number of myogenic cells, suggesting that E4F1 acts cooperatively with Smad4 (Fig. 9*F*).

Id1–3 suppress myogenesis and are targets of BMP signaling. Transfection of *E4f1* increased Id1-, Id2-, and Id3-luc activities in C2C12 cells treated with and without BMP-4 (Fig. 9*G*, and data not shown). This stimulation by E4F1 seemed to be Smad4 dependent because the activity was lost by Smad4 ablation and restored by Smad4 overexpression in MEF clone 16 (Fig. 9*G*).

DISCUSSION

In the present study, we examined the molecular mechanisms underlying the conversion of myoblasts by BMPs, allowing their differentiation into osteoblastic cells. It has been suggested that a unique type of intracellular BMP signaling is involved in this conversion, because other inhibitors of myogenic differentiation, such as TGF-β and fibroblast growth factors, do not induce ectopic bone formation *in vivo* or osteoblastic differentiation *in vitro* (12). We found that the inhibition of myogenic differentiation by BMP-4 required treatment for less than 1 h, although induction of osteoblastic differentiation required treatment for more than 9 h. Both activities of BMPs were dependent on the Smad pathway, suggesting that related but distinct mechanisms regulate the conversion of myoblasts into osteoblastic cells. Because we failed to detect cells positive for both MHC and ALP in C2C12 cell cultures treated with BMPs (9, 12), it appeared that osteoblastic differentiation is activated only in immature myoblasts that have not yet initiated myogenic differentiation (34). This hypothesis was confirmed by our preliminary observation that BMPs did not induce ALP activity in mature multinucleated myotubes.[3]

BMP treatment can convert the differentiation pathway of myoblasts into osteoblastic cells and overexpression of constitutively activated BMP type I receptors such as BMPR-IA, BMPR-IB, and ALK2 can have the same effect without requiring the addition of BMPs (35, 36). However, we found that levels of endogenous Smad1 and Smad5 were low in C2C12 cells and that overexpression of wild-type Smad1 was required for induction of osteoblastic differentiation by BMPR-IA(Q233D). These findings suggested that downstream signaling of BMP type I receptors, rather than BMP type II and co-receptors,

[3] J. Nojima, T. Takada, and T. Katagiri, unpublished data.

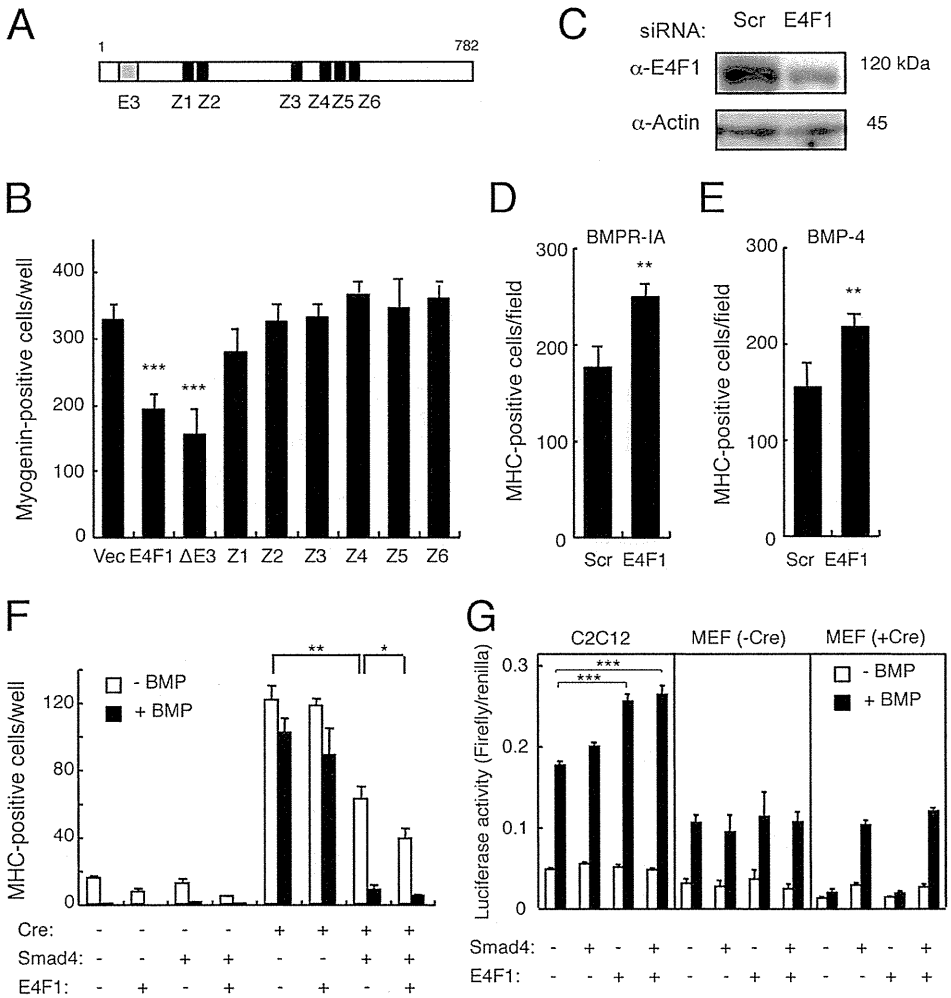

FIGURE 9. **E4F1 is involved in the BMP-induced inhibition of myogenesis.** *A*, schematic structure of E4F1. *E3*, an E3 ubiquitin ligase domain; *Z*, zinc fingers. *B*, overexpression of E4F1 inhibits myogenic differentiation. C3H10T1/2 cells were co-transfected with *MyoD* and wild-type *E4F1*, *E4F1*(*ΔE3*), or a zinc finger mutant *E4F1* and immunostained for myogenin on day 3. Values are mean ± S.D. ($n = 3$). ***, $p < 0.001$. *C*, 20 nmol of *E4F1* siRNA or scrambled oligonucleotide was transfected in C2C12 cells, and protein levels were determined by Western blots at 24 h post-transfection. *D* and *E*, the siRNA-transfected C2C12 cells were co-transfected with *Bmpr-Ia*(*Q233D*) (*D*) or treated with 100 ng/ml of BMP-4 (*E*), and MHC-positive cells were counted on day 5. Values are mean ± S.D. ($n = 3$). **, $p < 0.01$ compared with each scrambled siRNA transfection group. *F*, E4F1 suppresses myogenesis cooperatively with Smad4. *Smad4*$^{floxed/floxed}$-derived clonal cell line 16 was infected with or without a Cre-expressing adenovirus. The cells were transfected with or without *Smad4* in the presence or absence of Smad4, cultured with or without 100 ng/ml of BMP-4, and stained for MHC on day 3. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$. *G*, E4F1 stimulates *Id1* expression. C2C12 cells and MEF clone 16 infected with Cre adenovirus were transfected with *Id985-luc*, *E4F1*, and *Smad4* and were then cultured in the presence or absence of 50 ng/ml of BMP-4. Luciferase activity was determined on day 1. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

plays an important role in the conversion of myoblast differentiation. We established a constitutively activated Smad1, Smad1(DVD), to directly examine the role of the Smad pathway without activation of other signaling pathways induced by BMP receptors, such as the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. Serine residues were substituted with aspartic residues in Smad1(DVD) to introduce negative charges in the SVS motif at the carboxyl terminus, the site of phosphorylation by type I BMP receptors. These substitutions may induce conformational changes and stimulate interaction with coactivators such as p300, OAZ, and Runx2 (11). Indeed, Smad1(DVD) was directly recognized by the α-phospho-Smad1/5/8 antibody, suggesting that its three-dimensional structure is similar to that of native Smad1 phosphorylated by the receptors. We found that co-transfection of *Smad1*(*DVD*) with *Smad4* was capable of inducing osteoblastic differentiation of C2C12 myoblasts, and this induction was not inhibited by co-expression with Smad7, suggesting that Smad1(DVD) induces activation downstream of BMP type I receptors without activating endogenous BMPs or receptors. However, the ALP activity induced by Smad1(DVD) and Smad4 was lower than that induced by cotransfection of a constitutively activated BMPR-IA receptor and wild-type Smad1, although it was higher than that induced by BMPR-IA(Q233D) alone or Smad1(EVE), in which serine residues had been substituted with glutamic acids instead of aspartic acids.[3] These findings suggested that native phosphorylated Smad1 may have higher affinity for the coactivators required for osteoblastic differentiation than Smad1(DVD) or Smad1(EVE). This hypothesis will require further testing.

The Smad signaling pathway was also involved in the inhibition of myogenic differentiation. In contrast to osteoblast differentiation, however, this inhibitory activity of the Smad pathway appeared to be mainly dependent on Smad4 rather than R-Smads. In particular, the nuclear-targeted Smad4 markedly suppressed myogenic differentiation, although overexpression of NLS-Smad4 did not induce osteoblastic differentiation,[3] suggesting that Smad4 in complex with R-Smads inhibits myogenic differentiation after translocation from the cytoplasm to the nucleus in response to BMP stimulation. Because Smad4 is a common Smad among the TGF-β superfamily members, it may also be involved in mediating the effects of other myogenic inhibitors, such as TGF-βs, myostatin, and activin (37, 38).

The MH1 and MH2 domains of Smads have been shown to be involved in DNA binding and interaction with other proteins, respectively (39). Our deletion analysis suggested that nuclear Smad4 may interact with other transcriptional factor(s) and recruit them to the target DNA sequences via the MH2 and MH1 domains, respectively, to suppress myogenesis. This hypothesis was further supported by the finding of

stimulation of myogenic differentiation by NLS-Smad4(ΔMH1); this mutant Smad4 lacking DNA-binding activity may quench the transcriptional activity of the complex via the MH2 domain. It also appeared that a component of the Smad4 complex, interacting through the MH2 domain, is critical for inhibition of myogenic differentiation in response to BMPs. In the present study, we identified E4F1 as one of the components of the Smad4 complex in the nucleus, interacting through the MH2 domain. E4F1 is a zinc finger DNA-binding protein, identified as a cellular target of viral oncoproteins and shown to regulate the cell cycle (40–42). Our findings indicated that overexpression of E4F1 inhibited myogenic differentiation cooperatively with Smad4. Moreover, RNAi knockdown of E4F1 prevented the inhibition of myogenic differentiation by BMP signaling. Although E4F1 was recently shown to act as a ubiquitin E3 ligase of p53 (43), our findings indicated that deletion of the ubiquitin E3 ligase domain from E4F1 still allowed inhibition of myogenic differentiation. However, all of the zinc finger structures of E4F1 seemed to be important for this inhibitory activity. Taken together, these findings suggest that Smad4, which undergoes nuclear translocation in response to BMP stimulation, may interact with E4F1 in the nucleus to suppress myogenic differentiation as a transcription factor, independent of its ubiquitin E3 ligase activity. Recently, it was reported that Smad4 regulates the processing of pri-microRNA into mature microRNA in response to BMP-2 treatment (44). The direct target gene(s) of the complex still needs to be identified. It is interesting to note that loss-of-function mutations of p53 and Smad4 were identified in some tumors, suggesting that mutations in the Smad4-E4F1-p53 axis might play a role in tumorigenesis (45, 46).

We found that E4F1 stimulated the expression of *Id1–3* in the presence of Smad4. Id proteins inhibit myogenesis and are targets of BMP signaling. Recently, insufficient skeletal muscle repair was reported in $Id1^{+/-}Id3^{-/-}$ mice after muscle injury (47). BMP signaling may also up-regulate *Id* expression in healing muscle tissue (47). Because expression of *Ids* leads to cell cycle progression, the E4F1-induced Ids may suppress myogenic differentiation and maintain myoblast proliferation. Further studies are needed to elucidate the physiological roles of Smads and E4F1 in muscle development and regeneration *in vivo*.

In the present study, we obtained an unexpected finding related to Smads. BMP-induced osteoblastic differentiation was not completely blocked in the *Smad4*-deleted MEFs. There are some possible explanations for this finding: 1) undetectable levels of Smad4 still remained in the MEFs expressing Cre recombinase, 2) an alternative pathway, including a novel Co-Smad, transduced BMP signaling, or 3) Smad4 is not essential for the osteoblastic differentiation induced by BMPs. Recently, evidence has been presented that bone and cartilage tissues were formed during development in the absence of functional Smad4 in mice, although such mice exhibited abnormalities (48). Deletion of *Smad4* in mouse mature osteoblasts using a Cre-loxP system significantly reduced bone volume and osteoblast function *in vivo*, but they still had bone tissues and osteoblasts (48). Further study will be required to elucidate the roles of Smad4 in bone metabolism.

In conclusion, we found that the Smad-dependent pathway regulates both the inhibition of myogenic differentiation and the induction of osteoblastic differentiation induced by BMPs. The introduction of negative charges at the carboxyl terminus of Smad1 may play an important role in the induction of osteoblast differentiation in response to BMPs. In contrast, nuclear Smad4, rather than R-Smad, and E4F1, a novel partner of nuclear Smad4, are responsible for the inhibition of myogenic differentiation by BMPs.

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Surgical treatment of gastric cancer: 15-year follow-up results of the randomised nationwide Dutch D1D2 trial

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Background Historical data and recent studies show that standardised extended (D2) lymphadenectomy leads to better results than standardised limited (D1) lymphadenectomy. Based on these findings, the Dutch D1D2 trial, a nationwide prospectively randomised clinical trial, was undertaken to compare D2 with D1 lymphadenectomy in patients with resectable primary adenocarcinoma of the stomach. The aim of the study was to assess the effect of D2 compared with D1 surgery on disease recurrence and survival in patients treated with curative intent.

Methods Between August, 1989, and July, 1993, patients were entered and randomised at 80 participating hospitals by means of a telephone call to the central data centre of the trial. The sequence of randomisation was in blocks of six with stratification for the participating centre. Eligibility criteria were a histologically proven adenocarcinoma of the stomach without evidence of distance metastasis, age younger than 85 years, and adequate physical condition for D1 or D2 lymphadenectomy. Patients were excluded if they had previous or coexisting cancer or had undergone gastrectomy for benign tumours. Strict quality control measures for pathological assessment were implemented and monitored. Analyses were by intention to treat. This study is registered with the NCI trial register, as DUT-KWF-CKVO-8905, EU-90003.

Findings A total of 1078 patients were entered in the study, of whom 996 were eligible. 711 patients underwent the randomly assigned treatment with curative intent (380 in the D1 group and 331 in the D2 group) and 285 had palliative treatment. Data were collected prospectively and all patients were followed up for a median time of 15·2 years (range 6·9–17·9 years). Analyses were done for the 711 patients treated with curative intent and were according to the allocated treatment group. Of the 711 patients, 174 (25%) were alive, all but one without recurrence. Overall 15-year survival was 21% (82 patients) for the D1 group and 29% (92 patients) for the D2 group (p=0·34). Gastric-cancer-related death rate was significantly higher in the D1 group (48%, 182 patients) compared with the D2 group (37%, 123 patients), whereas death due to other diseases was similar in both groups. Local recurrence was 22% (82 patients) in the D1 group versus 12% (40 patients) in D2, and regional recurrence was 19% (73 patients) in D1 versus 13% (43 patients) in D2. Patients who had the D2 procedure had a significantly higher operative mortality rate than those who had D1 (n=32 [10%] *vs* n=15 [4%]; 95% CI for the difference 2–9; p=0·004), higher complication rate (n=142 [43%] *vs* n=94 [25%]; 11–25; p<0·0001), and higher reoperation rate (n=59 [18%] *vs* n=30 [8%]; 5–15; p=0·00016).

Interpretation After a median follow-up of 15 years, D2 lymphadenectomy is associated with lower locoregional recurrence and gastric-cancer-related death rates than D1 surgery. The D2 procedure was also associated with significantly higher postoperative mortality, morbidity, and reoperation rates. Because a safer, spleen-preserving D2 resection technique is currently available in high-volume centres, D2 lymphadenectomy is the recommended surgical approach for patients with resectable (curable) gastric cancer.

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Introduction

After several decades of debate on what the optimum surgical treatment of gastric cancer should be, it is now possible to treat patients using evidence-based principles established by well designed and conducted studies. Adequate surgery, the only treatment known to offer cure, is still the cornerstone of gastric-cancer treatment; however, local regional control remains an issue. In western Europe and the USA, optimum local control and survival seemed to be reached with surgery as a single-modality treatment, based mainly on two large European trials, the Dutch Gastric Cancer Trial (DGCT)[1] and the UK Medical Research Council (MRC) randomised trial.[2] In both trials, standardised extended (D2) lymphadenectomy did not improve survival, and was associated with significantly higher morbidity and mortality compared with standardised limited (D1) lymphadenectomy. The unfavourable outcomes were mostly associated with pancreatico-splenectomy, which was an integral part of the D2 resection in both trials. In 2004, results from a study by Degiuli and colleagues[3] suggested a survival benefit after pancreas-preserving D2 resections, and in 2006, a Taiwanese single-institution trial[4] found that extended lymph-node dissection (D2) led to better results (no postoperative mortality) than D1 lymphadenectomy. More extended resection (D2 plus para-aortic nodal dissection) was not found to be better than D2 resections in Japanese patients.[5]

As a result of the INT0116 trial,[6] a combination of surgery and postoperative chemoradiotherapy became the standard treatment for curable gastric cancer in the USA. In this trial, fluorouracil plus leucovorin given concomitantly with 45 Gy radiation after potentially curative surgery improved 3-year survival from 41% to 50%, compared with surgery alone. In Europe, following the results of the UK MAGIC trial,[7] perioperative chemotherapy with the ECF (epirubicin, cisplatin, and fluorouracil) became the new treatment standard for gastric cancer. The MAGIC trial found that perioperative systemic chemotherapy improved 5-year survival from 23% to 36%, compared with surgery alone. Randomised controlled trials in Japanese patients have shown significant improvement in overall survival with postoperative adjuvant chemotherapy with S-1 (an orally active combination of tegafur, gimeracil, and oteracil) after D2 dissection.[8] Therefore, S-1 after D2 surgery is becoming the standard treatment for patients with gastric cancer in Japan.

Early results from the DGCT showed significantly higher postoperative morbidity and mortality in the D2 group compared with the D1 group.[9] With the 5-year follow-up results showing no significant survival benefit in the D2 group, a conclusion was drawn that D2 resection could not be advised in patients with curable gastric cancer.[10] However, 11-year follow-up data showed better survival results in exploratory analyses in patients with stage II and IIIa disease who had D2 compared with D1 resections.[1] The current report is the 15-year follow-up data of the DGCT.

Methods

Patients

The DGCT was approved by the medical ethics committees of the Leiden University Medical Center and all participating hospitals. Written or oral informed consent was obtained according to the principles of the institution. 80 hospitals participated in the trial. Eligible patients had histologically proven adenocarcinoma of the stomach without evidence of distance metastasis, were younger than 85 years, and were in adequate physical condition for D1 or D2 lymphadenectomy. Patients were excluded if they had previous or coexisting cancer or had undergone gastrectomy for benign tumours. Randomisation was done before surgery to allow scheduling for the presence of specially trained supervising surgeons. If a supervising surgeon could not attend a planned surgery, the patient was considered ineligible. No patients received adjuvant chemotherapy, because at the time of the trial chemotherapy was not standard therapy.

All patients were assessed every 3 months during the first year and every 6 months thereafter. In accordance with common practice at that time, a clinical diagnosis was considered sufficient evidence of recurrence; however, radiological or endoscopic confirmation was sought for most patients. Evidence of recurrent disease was accepted only if one of the following criteria were present: cytological puncture under CT scan or ultrasonography guidance; local recurrence found by endoscopy or with relaparotomy; peritoneal dissemination on ultrasonography or CT scan; liver metastasis on ultrasonography or CT scan; distant metastasis, including supraclavicular lymph-node involvement or metastases in the Douglas pouch; lung metastasis on chest radiography; or bone metastasis on radiography or bone scan. After recurrence, no data are available on additional treatments, and only the date of death was registered. Post-mortem examination was desired to confirm death due to disease, although not all patients had a post-mortem examination.

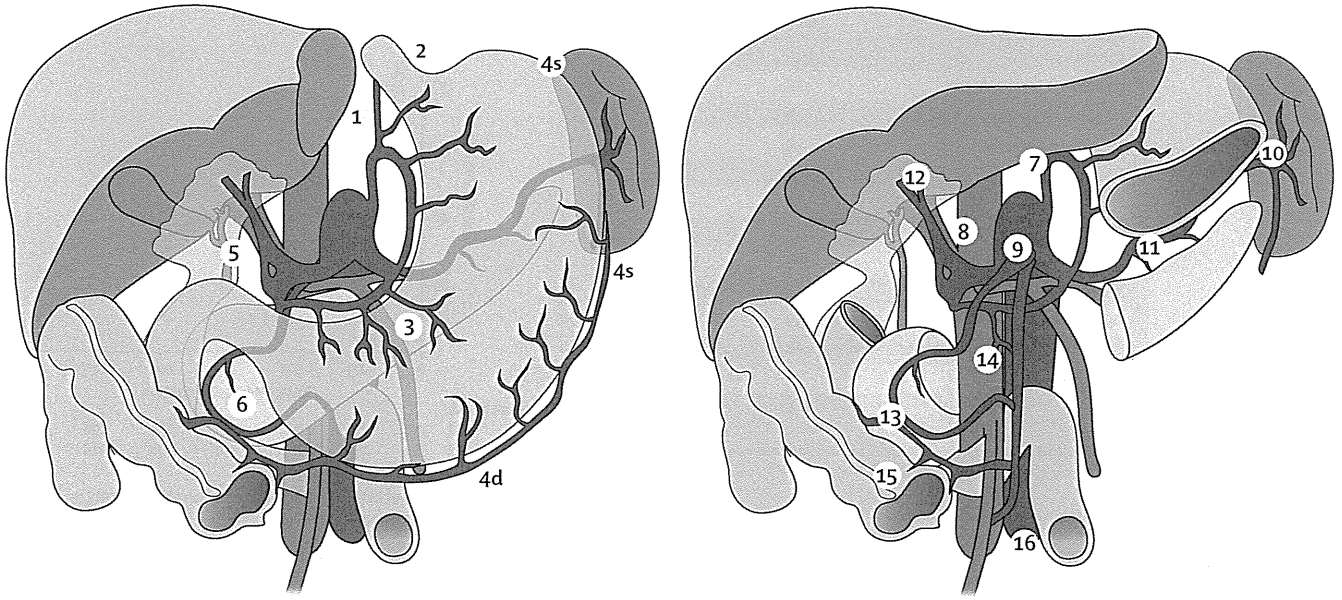
Randomisation and masking

After establishing the diagnosis and curative resectability, participating centres registered patients by means of a telephone call to the central data centre of the trial at the Leiden University Medical Center, Germany. The following information was requested: the institution's name, patient's name and date of birth, surgeon's name, and a check of inclusion and exclusion criteria. The sequence of randomisation was in blocks of six with stratification according to the participating centre. The surgical procedure (D1 or D2) was then assigned and a date for the surgery was agreed on. After registration, reference to the case was by a reference number, thus ensuring the privacy of the patient. Because of the nature of the treatment, concealment from the surgeons was not possible; the surgeons assessing outcomes or analysing data were not masked to group assignment.

Procedures

A D1 dissection entailed removal of the involved part of the stomach or the entire stomach (distal or total resection), including the perigastric lymph nodes (N1 level, station numbers 1–6) and the greater and lesser omenta. For a D2 dissection, both N1 and N2 lymph nodes (station numbers 7–11) were removed, along with omental bursa and the front leaf of the transverse mesocolon (figure 1). At the time of the trial, resection of the spleen and pancreatic tail were regarded as necessary for adequate removal of D2 lymph-node stations 10 and 11 in proximal tumours, and in D1 in the case of tumour invasion. Station numbers 12–14 were grouped as N3 and station numbers 15 and 16 as N4, but were outside the scope of our trial because involvement of N3 and N4 were considered distant metastases.

Assessment of curability was done by the supervising surgeon at laparotomy. Patients were regarded as able to undergo resection with curative intent and underwent the randomly assigned treatment (D1 or D2) if, at laparotomy, they had a macroscopically completely



N1 Lymph nodes (perigastric)

1 Right cardiac nodes
2 Left cardiac nodes
3 Nodes along the lesser curvature
4d Lymph nodes along the short gastric and the left gastroepiploic vessels
4s Lymph nodes along the right gastroepiploic vessels
5 Suprapyloric nodes
6 Infrapyloric nodes

N2 Lymph nodes (branches coeliac axis)

7 Nodes along root left gastric artery
8 Nodes along common hepatic artery
9 Nodes around coeliac axis
10 Nodes at splenic hilum
11 Nodes along splenic artery

N3 Lymph nodes

12 Nodes at the hepatoduodenal ligament
13 Retropancreatic (periduodenal) nodes
14 Nodes at the root of the mesentery

N4 Lymph nodes

15 Nodes along the middle colic vein
16 Para-aortic nodes

Figure 1: **Location and grouping of the lymph nodes**
D1 resection: removal of the N1 lymph nodes. D2 resection: removal of the N1 and N2 lymph nodes. Courtesy of Cornelis van de Velde.

removable tumour without peritoneal spread, liver metastases, or distant lymph-node metastases. For the latter criterion, a frozen-section examination of one or two para-aortic lymph nodes was required. Patients who met these criteria constituted the group treated with curative intent. To detect free abdominal tumour cells, abdominal fluid obtained by irrigation of the abdominal cavity immediately after laparotomy was recommended. The results were not used for immediate assessment of curability. The type of gastrectomy performed (distal or total) was independent of randomisation. Distal gastrectomy was allowed if there was a tumour-free margin of 5 cm beyond the proximal resection line. All other patients had total gastrectomy. Reconstruction of the alimentary tract was done mainly by the local surgeon, who used their preferred method. Histological examination of the resected specimens was done by the local pathologist, and the results were reviewed by a panel of supervising pathologists. After the final pathological examination, resections were considered R0 (radical resection, as opposed to R1, microscopic remnant tumour, and R2, macroscopic remnant tumour) when the cytology of the abdominal washing fluid was tumour negative, the resection lines were microscopically tumour negative, no distant lymph-node stations (beyond N2) were involved, and there were no distant metastases. Patients who did not meet these criteria constituted the group treated non-curatively. These patients underwent a palliative surgical procedure or exploratory laparotomy at the discretion of the surgeon and irrespective of the assigned treatment. None of the patients treated curatively underwent adjuvant radiotherapy or chemotherapy.

The primary endpoint of the trial was overall survival, calculated from the day of randomisation until the day of death (event) or the day of last follow-up (censored) and recurrence, defined as the time from randomisation to disease recurrence; the data of a patient were censored when at last follow-up the patient was alive with no evidence of disease, or had died of diseases other than gastric cancer without evidence of a recurrence. Disease-free survival was defined as the time from randomisation to recurrence or death due to any cause. All analyses were based on patients treated with curative intent and were according to the group to which patients were randomised.

	D1 group	D2 group
Randomised	539	539
Ineligible	26	56
No supervising surgeon available	5	30
Metastases or second tumour	9	11
No adenocarcinoma	8	10
Physical condition	4	6
Eligible	513	483
Non-curative*	133	152
Remnant tumour	90	102
Peritoneal metastases	59	83
Distant lymph-node metastases	36	37
Liver metastases	27	22
Curative	380	331

D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. *Patients may be included in more than one category.

Table 1: **Numbers of patients in the Dutch Gastric Cancer Trial**

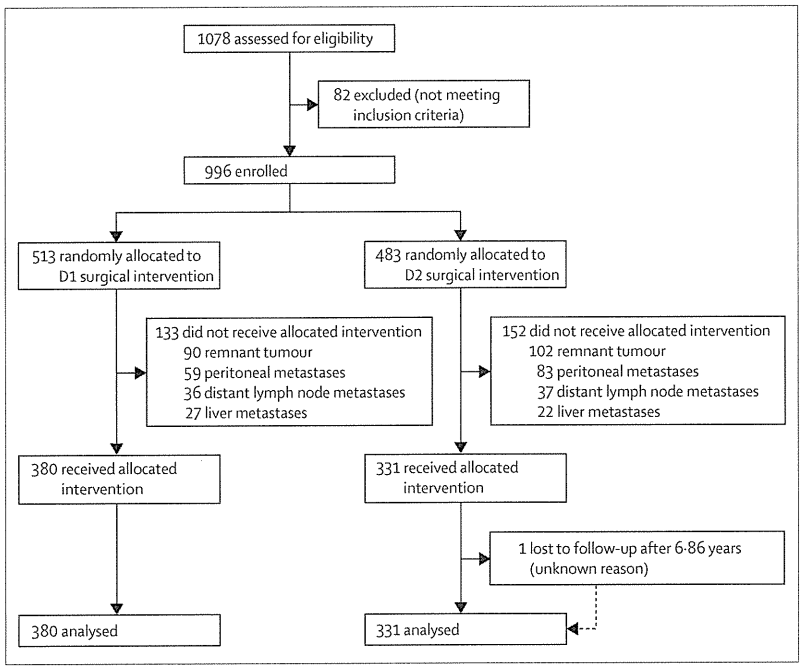


Figure 2: **Trial profile**

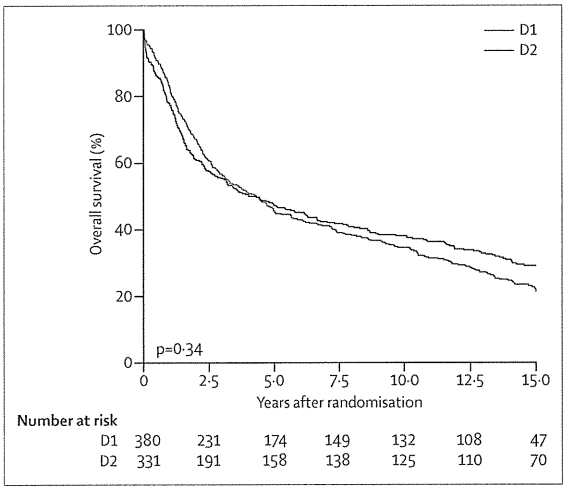


Figure 3: **Overall survival in patients treated with curative intent (n=711)**
D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy.

Strict quality control measures for surgery and pathology were implemented and monitored. Participating surgeons received a videotape and an instruction booklet about the technique and were instructed in the operating theatre by an expert gastric-cancer surgeon. The expert was present during the first 4 months of intake, which served as an instruction period, and regularly thereafter. All surgeries involving D2 dissection were attended by one of 11 surgeons who had been trained in D2 dissection. The study coordinator attended nearly all D1 procedures. The supervising surgeons monitored the technique and the extent of lymph-node dissection. After the surgery, the perigastric tissue was divided into lymph-node stations and fresh specimens were sent to the pathologist. All locations dissected not en-bloc were prepared and labelled by the surgeon. Regular meetings about the technique were held with the supervising surgeons, the study coordinator, and the instructing surgeon. Quality control was done by relating the number and location of the lymph nodes detected at pathological examination to the guidelines of the study protocol.[1,9,10]

Statistical analysis

The study size was based on an expected 5-year survival of 20% for patients undergoing D1 dissection with curative intent and 32% for those undergoing D2 dissection with curative intent. Using a two-sided significance level of 5%, a power of 90%, and an expected curability rate of 40%, 1062 patients had to be randomised.

The SPSS program (SPSS Inc, Chicago, IL, USA) and R version 2.9.1 were used for statistical analysis. A two-sided p value of 0·05 was considered statistically significant. Logistic regression was used to assess the influence of prognostic factors on postoperative mortality. The χ^2 test was applied to assess differences in proportions, and the log-rank test was used to assess the difference in survival and recurrence rates between groups, although the assumption of proportional hazards was not always satisfied.[11] The Kaplan-Meier method was used to estimate survival curves for overall and disease-free survival. For the analysis of death due to gastric cancer and due to other causes, a competing-risks analysis was done. Cumulative incidences were calculated and Gray's test was done to test between treatment groups.[12] Hazard ratios reported for these competing-risks analyses are based on the Fine and Gray model.[13]

	D1 group (n=380)	D2 group (n=331)
R0 resection	339	297
Cytology tumour-positive	15	10
Resection-line involvement		
Proximal	10	13
Distal	15	9
Distant metastases	8	3
Distant lymph-node involvement		
Station # 12	1	3
Station # 13	2	4
Station # 14	2	0
Station # 16	8	0
Total number of patients excluded	41	34

Patients may be included in more than one category. D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. R0=radical resection. R1=microscopic remnant tumour.

Table 2: **Reasons for R1 rather than R0 resections**

	D1 group		D2 group		HR (95% CI)	Log-rank p value	Interaction p value
	N	15-year overall survival (95% CI)	N	15-year survival overall (95% CI)			
Total	380	21% (17–26)	331	29% (24–34)	0·92 (0·78–1·09)	0·34	..
Sex							
Male	215	21% (15–27)	186	24% (18–30)	1·08 (0·86–1·35)	0·50	0·04
Female	165	21% (15–28)	145	35% (27–43)	0·75 (0·58–0·98)	0·03	
Age, years							
≤70	252	30% (25–36)	229	36% (30–42)	0·93 (0·75–1·16)	0·54	0·89
>70	128	3% (0–6)	102	13% (6–20)	0·88 (0·67–1·16)	0·36	
Pathological stage*							
T1	98	39% (29–49)	85	53% (42–63)	0·79 (0·53–1·18)	0·25	0·79
T2	181	21% (14–27)	152	25% (18–32)	0·95 (0·75–1·22)	0·70	
T3	94	5% (1–10)	82	15% (7–22)	0·94 (0·68–1·28)	0·68	
Lymph nodes							
Negative	171	35% (27–42)	144	39% (31–47)	0·98 (0·74–1·30)	0·88	0·33
Positive	209	10% (6–14)	187	22% (16–27)	0·87 (0·70–1·08)	0·20	
N stage							
N0	171	35% (27–42)	144	39% (31–47)	0·98 (0·74–1·30)	0·88	0·15
N1	138	15% (9–21)	113	28% (19–36)	0·87 (0·66–1·15)	0·33	
N2	50	0% (0)	47	19% (8–30)	0·68 (0·44–1·04)	0·07	
N3	21	0% (0)	27	0% (0)	0·73 (0·40–1·25)	0·28	
TNM stage (UICC, 1997)†							
IA	75	41% (29–52)	69	53% (42–65)	0·79 (0·50–1·25)	0·32	0·14
IB	97	36% (26–45)	72	27% (17–38)	1·29 (0·89–1·85)	0·18	
II	93	15% (7–22)	77	33% (23–44)	0·68 (0·48–0·97)	0·03	
IIIA	60	3% (0–8)	54	19% (8–29)	0·84 (0·57–1·25)	0·39	
IIIB	24	0% (0)	20	10% (0–23)	0·81 (0·44–1·51)	0·51	
IV	28	0% (0)	36	3% (0–8)	0·71 (0·42–1·18)	0·18	
Gastrectomy							
Total	115	15 (9–22)	126	19 (12–26)	1·00 (0·76–1·32)	0·99	0·35
Partial	265	24 (18–29)	205	35 (29–42)	0·82 (0·66–1·02)	0·08	

D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. HR=hazard ratio. TNM=tumour, node, metastasis. UICC=International Union Against Cancer. *Does not include two patients with missing data, two patients with T0 stage tumours, and three patients with T4 stage tumours in the D1 group, and three patients with T0 stage tumours and nine patients with T4 stage tumours in the D2 group. †Does not include one patient with missing data and two patients with TNM0 in the D1 group, and three patients with TNM0 in the D2 group.

Table 3: **Univariate analysis of overall survival at 15 years (median) follow-up**

Risk of recurrence is also reported as cumulative incidence, accounting for death due to other causes as a competing risk.

For the subgroup analysis, no adjustment for multiple testing was applied. Interpretation of the results of subset analyses have to be judged carefully and any significant results should be viewed as hypotheses that require validation in subsequent studies. A p value of 0·05 might not be strict enough for these subgroups. Cox proportional-hazards model was used to test for interaction between prognostic factors and lymph-node dissection.

This study is registered with the NCI trial register, identifier DUT-KWF-CKVO-8905, EU-90003.

Role of the funding source

The funding source had no role in the study design, collection, analysis, or interpretation of data, or writing of the report. The corresponding author had full access to all data and the final responsibility to submit for publication.

Results

Between August, 1989, and July, 1993, 1078 patients with gastric adenocarcinoma were entered and randomised (539 to each group) in the DGCT. 996 patients met the eligibility criteria and were randomly assigned to have a D1 or D2 lymph-node dissection. Because of peritoneal, hepatic, or distant lymph-node metastasis or locally irresectable disease, 285 patients (29%) underwent palliative surgery without a formal lymph-node dissection, according to the discretion of the surgeon. Of these 285 patients, 156 (55%) had a palliative resection, 51 (18%) had a bypass, and 78 (27%) had exploration only. Characteristics of the patients judged ineligible and non-curative are summarised in table 1 and patient flowchart in figure 2. The remaining 711 patients underwent a D1 or D2 resection with curative intent, according to random assignment. Data were collected prospectively and all patients were followed up: median follow-up for all eligible patients was 15·2 years (range 6·9–17·9). The lower limit is 6·9 years because one patient was lost to follow-up at that point. The trial is now closed. This analysis focuses on the 711 patients (71%) who had a curative resection with D1 (n=380) or D2 (n=331) lymphadenectomy. The characteristics of the 711 curative patients are well balanced between the two treatment groups, except for pancreatico-splenectomy, which was expected because of the protocol and type of gastrectomy (webappendix pp 1[9,10]).

See Online for webappendix

At last follow-up, 217 patients (31%) died without recurrence (110 in the D1 group and 107 in the D2 group)

	D1 group (n=380)	D2 group (n=331)	p value
Alive	82 (22%)	92 (28%)	0·34*
Deaths from gastric cancer	182 (48%)	123 (37%)	0·01†
Deaths from other causes	116 (31%)	116 (35%)	0·12†
Other diseases	94 (25%)	77 (23%)	..
Toxicity treatment	15 (4%)	32 (10%)	..
Unknown	7 (2%)	7 (2%)	..

Data are number of patients (%) or p value. D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. *Log-rank p value. †Gray's test p value.

Table 4: Causes of death

and 320 patients (45%) died with recurrence (188 in D1 and 131 in D2). 173 patients (24%) were alive without recurrence (82 in D1 and 91 in D2), and one patient was alive with recurrence in the D2 group. 15-year overall survival for patients who had curative resections was 21% (85 of 380, 95% CI 17–26) for D1 and 29% (98 of 331, 24–34) for D2 (log-rank p=0·34; figure 3).

D2 patients had significantly higher operative mortality than D1 patients (32 of 331 [10%] *vs* 15 of 380 [4%], 95% CI for the difference 2–9%, p=0·004), higher complication rate (142 of 331 [43%] *vs* 94 of 380 [25%], 11–25%, p<0·0001, and higher reoperation rate (59 of 331 [18%] *vs* 30 of 380 [8%] 5–15%, p<0·00016; previously published[9,10]). Significant risk factors for postoperative mortality in logistic regression analysis were: age >70 versus ≤70 years (overall risk [OR] 3·55, 95% CI 1·91–6·61, p<0·0001), D2 versus D1 lymphadenectomy (OR 2·64, 1·38–5·04, p=0·003), male versus female (OR 2·50, 1·25–4·99, p=0·01) and total versus partial gastrectomy (OR 2·04, 1·01–3·79, p=0·02; data not shown). Other factors included in the analysis but that had no significant association with postoperative mortality were T stage, lymph-node involvement (N-negative *vs* N-positive and N0–N3), and TNM stage. Overall survival for patients who had an R0 resection was 25% (85 of 324, 95% CI 20–30) for D1 and 35% (97 of 268, 30–41) for D2, when postoperative deaths (15 of 380 [4%] in D1 and 32 of 331 [10%] in D2) are excluded (log-rank p=0·08). The reasons for no R0 resection are reported in table 2.

The following analyses are based on the 711 patients who had curative resections. Table 3 shows the results of univariate analyses of 15-year overall survival and hazard ratios (HR), according to subgroups based on several prognostic variables. Overall survival was significantly higher for female patients who had D2 versus D1 dissection (35% *vs* 21%, p=0·03), and for patients with TNM stage II disease (33% in D2 *vs* 15% in D1, p=0·03). Patients with N2 disease had better survival after a D2 dissection (19% in D2 *vs* 0% in D1, p=0·07), as did those who had partial gastrectomy (35% in D2 *vs* 24% in D1, p=0·08), however this was not statistically significant.

Cause of death was analysed for all patients and the distribution for the D1 and D2 groups is summarised in table 4. Gastric-cancer-related death was significantly higher in the D1 group compared with the D2 group (HR 0·74 for D2 *vs* D1, 95% CI 0·59–0·93, p=0·01), whereas death due to other causes was not different between groups (HR 1·22 for D2 *vs* D1, 0·95–1·58, p=0·12; figure 4). Five patients in the D1 group had recurrence at the time of death (two patients with locoregional recurrence, two patients with local and distant recurrence,

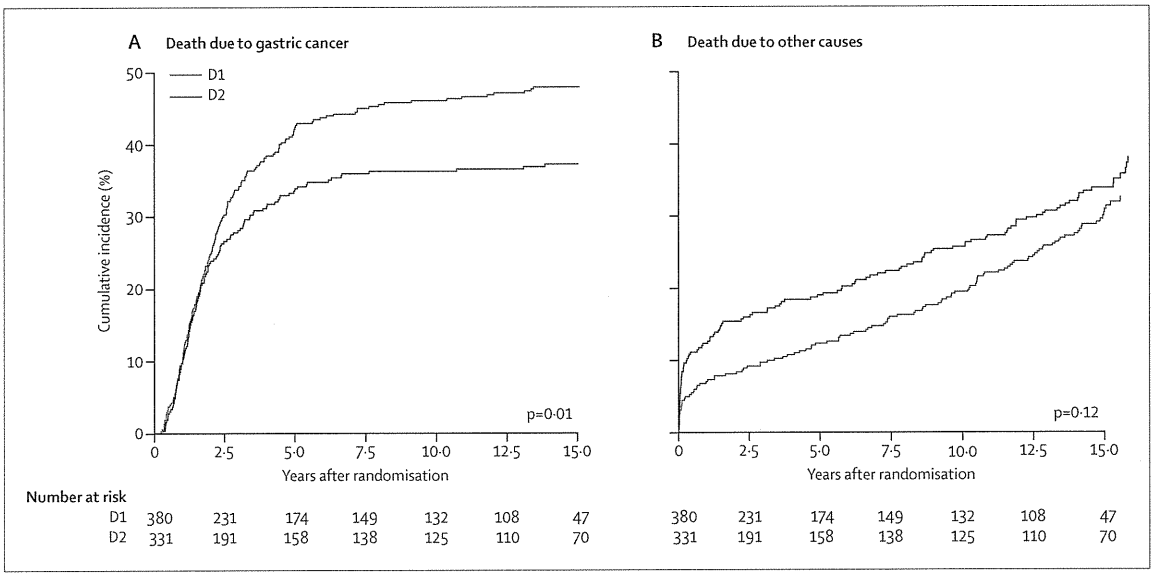


Figure 4: Cumulative risk of death due to gastric cancer and due to other causes in patients treated with curative intent (n=711)
D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy.

	D1 group (n=380)	D2 group (n=330)*
Alive, no recurrence†	82 (22%)	91 (28%)
Dead, no recurrence†	110 (29%)	107 (32%)
Dead, with recurrence‡	188 (49%)	131 (40%)
Local	82	40
Regional	73	43
Adjacent organs	37	26
Liver metastasis	65	37
Lung metastasis	12	6
Other organs	48	38
Dead, local+regional†	58 (15%)	42 (13%)
Dead, local+distant†	100 (26%)	56 (17%)
Dead, distant†	30 (8%)	34 (10%)

Data are number of patients (%). D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. *One patient is alive with recurrence. †Overall p value for recurrence pattern is 0·015. ‡Patients may be included in more than one category.

Table 5: **Recurrence sites and patterns at time of death**

and one patient with distant recurrence) and seven patients in the D2 group (three patients with locoregional recurrence, three patients with local and distant recurrence, and one patient with distant recurrence), although they died of other causes. In the group of patients who died from gastric cancer, postmortem examinations were done for ten patients in the D1 group and in 18 patients in the D2 group. Except for 31 patients in the D1 group and 25 patients in the D2 group, all recurrences were established by means of additional investigations (CT scan or ultrasonography, or both, biopsy or cytology, chest radiography or bonescan) depending on the recurrence.

Local recurrence was significantly higher in the D1 versus D2 group (82 of 380 [22%] *vs* 40 of 330 [12%]). Regional recurrence (73 of 380 [19%] in D1 *vs* 43 of 330 [13%] in D2) and liver metastases (65 of 380 [17%] in D1 *vs* 37 of 330 [11%] in D2) were also more common in the D1 group (table 5). Locoregional, distant, and distant-only recurrence are reported in table 5. Results for disease-free survival (p=0·31) and risk of recurrence (p=0·10) are shown in figure 5. Most patients had recurrence within 2–2·5 years, and after 5 years recurrence occurs less frequently. The difference in recurrence rates between the D1 and D2 group seems to present between 2·5 and 5 years of follow-up.

Patients older than 70 years had significantly lower overall survival in both the D1 and the D2 treatment groups, male patients had significantly lower survival compared with female patients in the D2 group (mean 7·07 years *vs* 8·73 years, p<0·0001), and patients undergoing splenectomy and pancreatectomy had significantly lower overall survival in both D1 and D2 (table 6). Subgroup analysis of patients without pancreatico-splenectomy (339 patients in D1 and 206 in D2, data not shown) shows a significantly higher overall survival in those who had D2 lymphadenectomy, with 15-year survival of 22% (75 of 339, 95% CI 17–26) in D1 and 35% (71 of 206, 29–42) in D2 (HR 1·34, 95% CI 1·09–1·65; log-rank p=0·006).

Discussion

Our findings based on 15-year follow-up data of the DGCT show that D2 lymphadenectomy is associated with lower locoregional recurrence and fewer gastric-cancer-related deaths than D1. The drawback of a D2 resection is its association with significantly higher

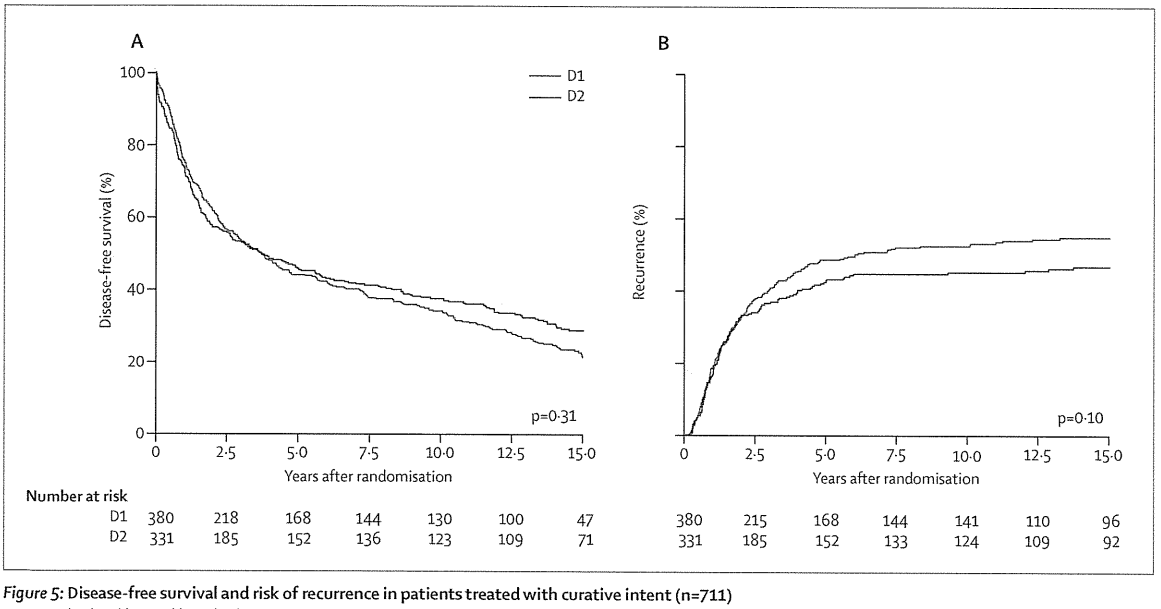


Figure 5: **Disease-free survival and risk of recurrence in patients treated with curative intent (n=711)**
D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy.

	N	D1 group mean OS (95% CI)	D2 group mean OS (95% CI)
Age			
≤70 years	481	8·23 (7·38–9·09)	8·69 (7·71–9·66)
>70 years	230	4·97 (4·10–5·82)	5·35 (4·21–6·49)
p value*	..	<0·0001	<0·0001
Sex			
Male	401	7·19 (6·33–8·05)	6·75 (5·75–7·74)
Female	310	7·07 (6·06–8·08)	8·73 (7·53–9·93)
p value*	..	0·83	0·02
Splenectomy			
Yes	165	5·14 (3·16–7·12)	5·19 (4·07–6·31)
No	546	7·37 (6·68–8·06)	9·09 (8·09–10·08)
p value*	..	0·02	<0·0001
Pancreatectomy			
Yes	108	2·34 (0·00–5·23)	4·85 (3·64–6·07)
No	603	7·27 (6·61–7·93)	8·81 (7·87–9·73)
p value*	..	0·0007	<0·0001

D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. OS=overall survival. *Log-rank p value.

Table 6: **Effect of age, sex, splenectomy, and pancreatectomy on mean overall survival**

postoperative mortality and morbidity. However, at the time of the trial, resection of the spleen and pancreatic tail were regarded as necessary for adequate removal of D2 lymph-node stations 10 and 11 in proximal tumours, and in D1 in case of tumour invasion. Considering that a safer, spleen-preserving D2 resection is currently available in high-volume centres, and our findings of better recurrence and gastric-cancer-related survival rates, D2 resection now seems likely to be the recommended surgical approach for patients with resectable (curable) gastric cancer, despite the earlier follow-up results.

The extent of lymphadenectomy for curative resections in patients with gastric cancer has been debated for several decades. Better survival results reported for extended lymphadenectomy in Asian countries and better results with D2 in historical controls in western Europe and the USA were the reasons for the DGCT investigation. The MRC trial[2] in the UK addressed the same issue—eg, whether standardised D2 leads to better survival than D1 in patients with resectable gastric cancer. In both the MRC trial and the DGCT, no adjuvant chemotherapy was given. Both trials showed similar results: significantly increased morbidity and postoperative mortality after D2 surgery, without improvement in 5-year overall survival.[9,10] Long-term follow-up analysis of the DGCT after 11 years also showed no overall survival benefit with D2 lymphadenectomy.[1] In subgroup analysis, patients with N2 disease in the D2 group had higher survival than those in the D1 group (19% *vs* 0%, p=0·07). Since N2 disease is difficult to identify preoperatively, the conclusion was that extended lymph-node dissections might be beneficial if morbidity and mortality can be avoided. Meanwhile, it was reported that D2 lymphadenectomy can also be done safely in western Europe and the USA, without increased morbidity and mortality and after an adequate learning period, when routine pancreatico-splenectomy is avoided.[3,14]

After a median follow-up of 15 years after randomisation, these data show a more favourable recurrence pattern and a cancer-related survival benefit for patients who had a D2 rather than D1 lymphadenectomy. Patients older than 65 years, male sex and type of gastrectomy, splenectomy, and pancreatectomy were identified as important risk factors for the increased morbidity seen in the D2 group.[15] The increased mortality in this group of the pancreatico-splenectomy probably offset the survival benefit of D2 compared with D1 lymphadenectomy in the earlier analysis of the DGCT.

Current practice for treatment of patients with gastric cancer in Europe has become surgery with perioperative chemotherapy after the promising results of the MAGIC trial.[7] However, in the MAGIC trial, both D1 and D2 resections were done according to the discretion of the physician and 68% of the gastrectomies were D2 resections. A French trial[16] provided additional evidence for improved disease-free survival (DFS) and overall survival in resectable adenocarcinoma of the stomach and lower oesophagus with preoperative chemotherapy. Continuous infusion of fluorouracil and cisplatin resulted in a significantly higher R0 resection rate

	Time period	Surgery only			Multimodality treatment		
		N	RFS	OS	N	RFS	OS
MacDonald et al (2001)[6]	1991–1998	275	31% (3-year)	41% (3-year)	281 CRT	48% (3-year)	50% (3-year)
Cunningham et al (2006)[7]	1994–2002	253	NA	23% (5-year)	250 ECF	NA	36% (5-year)
Sakuramoto et al (2007)[8]	2001–2004	530	60% (3-year)	70% (3-year)	529 S-1	72% (3-year)	80% (3-year)
Boige et al (2007)[16]	1995–2003	111	21% (5-year)	24% (5-year)	113 FP	34% (5-year)	38% (5-year)

RFS=relapse-free survival. OS=overall survival. NA=not available. CRT=postoperative chemoradiotherapy (fluorouracil plus leucovorin followed by 45 Gy radiotherapy). ECF=Three preoperative and three postoperative cycles of epirubicin, cisplatin, and fluorouracil. S-1=cycles of S-1 (orally active combination of tegafur, gimeracil, and oteracil) for 1 year postoperatively. FP=2–3 cycles of preoperative fluorouracil and cisplatin; postoperative FP was recommended for patients with a response or stable disease with pN+.

Table 7: **Randomised trials of surgery only versus surgery combined with chemotherapy or chemoradiotherapy**

	Time period	Group 1				Group 2			
		N	Morbidity	Mortality	5-year overall survival	N	Morbidity	Mortality	5-year overall survival
Cuschieri et al (1999)[2]	1987–1994	200 (D1)	28%	6·5%	35%	200 (D2)	46%	13%	33%
Bonenkamp et al (1999)[9] and Hartgrink et al (2004)[3]	1989–1993	380 (D1)	25%	4%	45% and 30% (11-year)	331 (D2)	43%	10%	47% and 35% (11-year)
Degiuli et al (2004)[14]	1999–2002	76 (D1)	10·5%	1·3%	NA	86 (D2)	16·3%	0%	NA
Wu et al (2006)[4]	1993–1999	110 (D1)	7·3%	0%	53·6%	111 (D3)	17·1%	0%	59·5
Sasako et al (2008)[5]	1995–2001	263 (D2)	20·9%	0·8%	69·2%	260 (D2+PAND)	28·1%	0·8%	70·3

D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. NA=not available. D3=referred to as D3 in this trial, but the same as D2. PAND=para-aortic nodal dissection.

Table 8: **Randomised trials comparing the extent of lymphadenectomy**

(73% *vs* 84%, p=0·04), a 13% improvement in 5-year DFS (21% *vs* 34%, p=0·003), and a 14% improvement in overall survival (24% *vs* 38%, p=0·02).[16] The INT0116 trial[6] found a significant improvement in 3-year overall survival, from 41% to 50%, with postoperative chemoradiotherapy in patients with adenocarcinoma of the stomach or gastro-oesophageal junction; only 10% of the patients had D2 resections, most had gastrectomy with D0 or D1 lymph-node dissection. The significant improvement in survival seemed to be merely a compensation of inadequate surgery. Nevertheless, as a result of this trial, a combination of surgery and postoperative chemoradiotherapy became the standard treatment for curable gastric cancer in the USA. A randomised controlled trial in Japanese patients with gastric cancer showed a significant improvement in 3-year overall survival with postoperative adjuvant chemotherapy with S-1 after D2, from 70·1% to 80·1%, compared with D2 dissection alone[8] (table 7). In non-Asian patients, however, meta-analyses of adjuvant chemotherapy showed only a small advantage in survival, insufficient to be considered standard of care.[17–21]

The results of these studies suggest that D2 surgery alone results in much better survival than limited surgery plus adjuvant CRT, as shown by the INT0116 trial, and that (neo)adjuvant therapy improves results even after D2 resection. Promising results from the Maruyama Index[22–24] and nomograms[25–27] that predict disease-specific survival might also help distinguish between patients with a high risk of relapse, and select those who will most likely benefit from tailored multimodality treatment. Because there are no other studies with such long-term follow-up data, including the MRC trial with similar design, the results of the DGCT are unique and of paramount clinical relevance. The main outcomes of the surgical trials discussed in this paper are summarised in table 8.

In selecting patients with gastric cancer for surgery, we do not think that elderly patients should be denied surgery. However, we cannot advocate extensive surgery, especially in elderly male compared with female patients. In this study, patients treated without curative intent were excluded from the analyses, according to the study protocol, and analyses were by intention to treat. Postoperative deaths were excluded from the analyses in one example as an illustration of whether patients would benefit from a D2 resection if excessive postoperative mortality could be prevented. Nowadays, surgery for gastric cancer can be done with a spleen-preserving and pancreas-preserving D2 resection technique (unless removal is indicated because of tumour invasion into these organs).[15,28–30] This technique is safer and is applicable in experienced, high-volume hospitals, where the outcome of patients with gastric cancer has been shown to be better.[31–33] Several countries (eg, Scandinavian countries) have already implemented a high-volume hospital setting and centralisation for gastric-cancer treatment, because it is recognised as high-risk surgery. Bilimoria and colleagues[34] studied the effect of differences in hospital surgical volume on perioperative mortality and long-term survival using the National Cancer Data Base. When comparing low-volume hospitals with high-volume hospitals, hazard ratios for perioperative mortality were substantially larger than for long-term survival. The effect of hospital volume in the DGCT has also been analysed and published,[9] and no association was noted between the number of patients randomised at centres (five *vs* five–14 *vs* ≥15 patients per centre) and complication, hospital death, or reoperation rates (webappendix pp 2). However, the importance of standardisation and surgical training is shown in a population-based study from the Netherlands.[35] The 5-year overall and relative survival of patients with curatively resected non-cardia gastric cancer was evaluated over time on a regional basis. The evaluation periods were before, during, and after the DGCT. Survival improved over time, most likely because of the standardisation and surgical training in D1 and D2 resection during the national DGCT, with extensive quality control measures for surgery and pathology implemented and monitored. With 5-year survival of 45% and 47% and 11-year survival of 30% and 35% for D1 and D2, respectively,[1] the results are the best recorded survival rates in western Europe and the USA.

Our results suggest that a D2 resection provides better locoregional control and significantly better cancer-specific survival compared with limited D1 surgery. Still, the significantly better gastric-cancer-specific survival

after D2 has to be interpreted carefully; a p value of <0·01 could be considered appropriate, since not all patients had post-mortem examinations (this was not mandatory according to the trial protocol) to confirm death due to gastric cancer.

Since a D2 resection can now be done safely with the spleen-preserving method, and more extended resections (D2 plus para-aortic nodal dissection) do not further improve survival outcome,[5] we believe D2 resection should be recommended as the standard surgical approach to resectable gastric cancer. With the surgical standard determined, a logical next step for investigation is whether patients with low Maruyama Index derive a survival benefit and improved locoregional control from chemoradiotherapy combined with gastrectomy and optimum standardised lymphadenectomy—ie, D2 resection without splenectomy—since treatment of gastric cancer requires a multidisciplinary approach. These issues are currently being addressed in the CRITICS trial (participating countries: the Netherlands, Sweden, and Denmark), a multicentre randomised phase 3 trial investigating whether chemoradiotherapy (45 Gy in 5 weeks with daily cisplatin and capecitabine) after preoperative chemotherapy (3×ECC [epirubicin, cisplatin, capecitabine]) and standardised D2 surgery without splenectomy leads to improved survival compared with postoperative chemotherapy (3×ECC).

For more on the **CRITICS trial** see http://www.critics.nl

Contributors

CJHV conceived and designed the trial. MS instructed surgeons during the first 6 months of the trial. Data collection was done by EM-KK. HP and IS did the statistical analyses. IS wrote the report with revisions from all other authors.

Conflicts of interest

The authors declared no conflicts of interest.

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ORIGINAL ARTICLE

Anastomotic Leakage Contributes to the Risk for Systemic Recurrence in Stage II Colorectal Cancer

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Abstract
Purpose In stage II colorectal cancer (CRC), high-risk patient selection is required, but no candidate markers have been elucidated. Our concern was whether anastomotic leakage (Lk) is a potential available clinicopathological factor for selecting high-risk stage II.
Methods Two hundred seven patients with stage II CRC who underwent curative resection were analyzed. Clinical variables were tested for their relationship to survival.
Results The 5-year disease-free survival rate (DFS) was 87.0%. The univariable prognostic analyses indicated that Lk (P=0.003) was the only significant factor. The multivariable prognostic analysis revealed that Lk remained to be potently independent [hazard ratio (HR), 4.21, P=0.021), and the DFS was 58.3% in cases with Lk, while 88.7% in the counterpart. The multivariable logistic regression analysis revealed perioperative blood transfusion (P=0.001) was independently associated with Lk. Intriguingly, Lk was closely associated with hematogenic recurrence (P=0.003) rather than peritoneal or local recurrence. Although sustained increase of the serum C-reactive protein at 2 weeks after operation predicted poor prognosis, the mutitivariable analysis including the C-reactive protein level revealed that Lk still indicated the prognostic potential (HR, 3.70, P=0.075).
Conclusions The findings concluded that Lk may be a high risk for systemic recurrence in stage II CRC.

Keywords Colorectal cancer · Stage II · Prognosis · Anastomotic leakage

Introduction

Colorectal cancer (CRC) is the second most prevalent cancer,[1], and chemotherapy has dramatically improved prognostic outcome of CRC patients over the past decades.[2,3] Nevertheless, CRC remains the fourth leading cause of cancer death worldwide with about 530,000 deaths every year.[1] Recently, as the prognostic outcome of stage III patients has been dramatically improved due to prevalent use of adjuvant chemotherapy and improvement of chemotherapy regimens,[2,4] adjuvant chemotherapy is consented as standard therapy in stage III CRC. Similarly, application of adjuvant chemotherapy is under discussion for patients with high-risk stage II disease[5] although no selecting marker has been clinically identified at present. In stage II patients, approximately 20% of the patients have yet suffered from recurrence in spite of potentially curative resection.[6] Therefore, pre- or postoperative prognostic markers have been anticipated for selecting high-risk patients who may benefit from adjuvant

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