tween the N-CoR C terminus and AR was ligand independent. Moreover, in the mammalian two-hybrid assay for AR–N-CoR interaction analysis, the authors used AR-ligand binding domain, not the full-length of the receptor. In fact, in a recent study by Hodgson et al., DHT-dependent interaction between the C terminus of N-CoR and the full-length AR was not detected (20), which corresponds with our present results (Fig. 1 and 6). Therefore, it is supposed that the middle region may be important for the agonist-dependent interaction between N-CoR and AR, whereas the C terminus of N-CoR may be important for the antagonist-induced interaction. In the present study, the middle region of the N-CoR, N-CoR(1134–1798), but not the amino- or carboxyl-terminal region of the N-CoR, was recruited by agonist-bound AR, GRα, and ERα in living cells (Fig. 5). Furthermore, coimmunoprecipitation experiments revealed that N-CoR(1134–1798) containing RD3 was able to interact with the AR. Therefore, N-CoR(1134–1798) containing RD3 is strongly suggested to be the major region for the repression of transactivation function of agonist-bound steroid hormone receptors. The N-CoR(1–1133) also contributes to the interaction with agonist-bound receptors but does not seem to be responsible for the repression of their transactivation function (Fig. 6 and 7).

Many coactivators have been shown to regulate AR transactivation function through an interaction with the N-terminal domain of the AR. The p160 coactivators such as SRC-1 and TIF2 directly interacted with the N-terminal domain of the AR (2). It has previously been reported that SRC-1 and TIF2 were recruited into the AR–AF-1 foci (41). The N-CoR protein was also shown to interact with the N-terminal domain of steroid hormone receptors (20, 44, 60). In the present study, the N-CoR was also recruited to the AR–AF-1 foci, and the interaction between N-CoR and AR–AF-1 was confirmed by mammalian one-hybrid assay. The N-CoR is thus suggested to repress AR-mediated transactivation through an interaction with the N-terminal domain of the AR.

We demonstrated that the N-CoR inhibited the AR N-C interaction in the mammalian two-hybrid assay and also in the AR-mediated transactivation. A specific HDAC inhibitor, TSA, could not recover the N-CoR-mediated suppression of AR-dependent transactivation (Fig. 12A). It has been recently reported that SMRT-mediated inhibition of the steroid hormone receptor function is not recovered by TSA (1, 23) and is largely due to the blockage of the AR N-C interaction (1, 34). In the present study, we confirmed that N-CoR also had such inhibitory effects on the AR N-C interaction. In a recent paper (32), it has been reported that the intramolecular N-C interaction controls AR chromatin binding and is required for the recruitment of SWI/SNF, which remodels chromatin. Inhibition of intramolecular interactions by N-CoR might affect chromatin remodeling, resulting in repression of the transactivation function.

Recently, it was proposed that coactivators and corepressors exert equilibrium interactions with nuclear receptors (61) and that the properties of nuclear receptors are determined by the intracellular ratio of coactivators and corepressors (45, 51, 61). It was also proposed that the transcriptional regulation by nuclear receptors requires the corepressor-coactivator exchange via the TBL1/TBLR1-containing exchange complex (39). Competition between N-CoR and coactivators to regulate N-C interaction and the transactivation activity of AR was revealed in the present study, which seems consistent with the above hypothesis.

The appearance of intranuclear focus formation has been shown to be closely related to the transcriptionally active conformation of steroid hormone receptors with coactivators (13, 30, 41, 56). Upon treatment with DHT, AR-GFP alone formed complete foci with recruitment of coactivators such as SRC-1, TIF2, and CBP (41). Such focus formation was clearly demonstrated for endogenous AR in the present study (Fig. 8A). Treatment with some antagonists, which inhibit the transcriptional activation, translocates the receptors from the cytoplasm into the nucleus; however, the translocated receptors are not able to form intranuclear foci and are diffusely distributed (13, 56). The N-C interaction is also required for complete focus formation of ARs (41). However, the present study clearly demonstrated that the quality of AR foci or ERα foci containing the N-CoR was different from that of AR foci or ERα foci without N-CoRs. The focus pattern of AR-GFP and ERα-GFP without N-CoRs (Fig. 2Ab and Gb and 9A and K) seemed much more distinct than the speckle pattern of AR-GFP and ERα-GFP coexpressed with the N-CoR (Fig. 2Ci and Ii and 9C and M), but such differences in the appearance of the intranuclear compartments were not confirmed only by simple two-dimensional images. However, a quantitative analysis of fluorescence intensity fluctuation in two-dimensional images and a three-dimensional imaging method we previously developed (41, 56) clearly demonstrated impaired formation of AR or ERα foci (incomplete focus formation) in the presence of the N-CoR (Fig. 9 and 10). The transactivation activities of AR and ERα were parallel at the level of their distinct (complete) focus formation (Fig. 6 and 10). It was thus suggested that only complete focus formation is related to the full transactivation function of ARs. An antagonist, tamoxifen, was shown to induce ERα focus formation, which was similar to foci induced by estradiol (Fig. 8Bd and Ca). However, the present study demonstrated that the tamoxifen-induced foci of ERα are not complete foci (Fig. 8Cb and Cc). The biochemical analyses using the two-hybrid and reporter gene assays revealed the functional competition between the N-CoR and CBP/SRC-1 on transactivation activity of the AR. This finding would further conceive that the N-CoR and coactivators mutually and exclusively bind to ARs, namely, binding of CBP and SRC-1 leads to a change in molecular conformation of the AR that hinders further binding of N-CoRs and vice versa. The present three-dimensional imaging analysis provided evidence for the above concept. Additional expression of SRC-1 or CBP in DHT-bound ARs coexpressed with N-CoRs led to release of N-CoRs from incomplete foci containing DHT-bound ARs and N-CoRs and to formation of distinct/complete foci with the coactivators (Fig. 13). Essentially the same phenomena were observed for endogenous N-CoR (Fig. 14). We speculate that there is a conformational equilibrium between transcriptionally active and inactive steroid hormone receptors in the presence of agonists, determined by the relative ratio of coactivators and corepressors.

The present observations that transcription inhibitors, actinomycin D and α-amanitin, disrupt intranuclear foci of steroid hormone receptors (Fig. 8B) revealed that the focus formation is considered not to precede the transcriptional activation. The

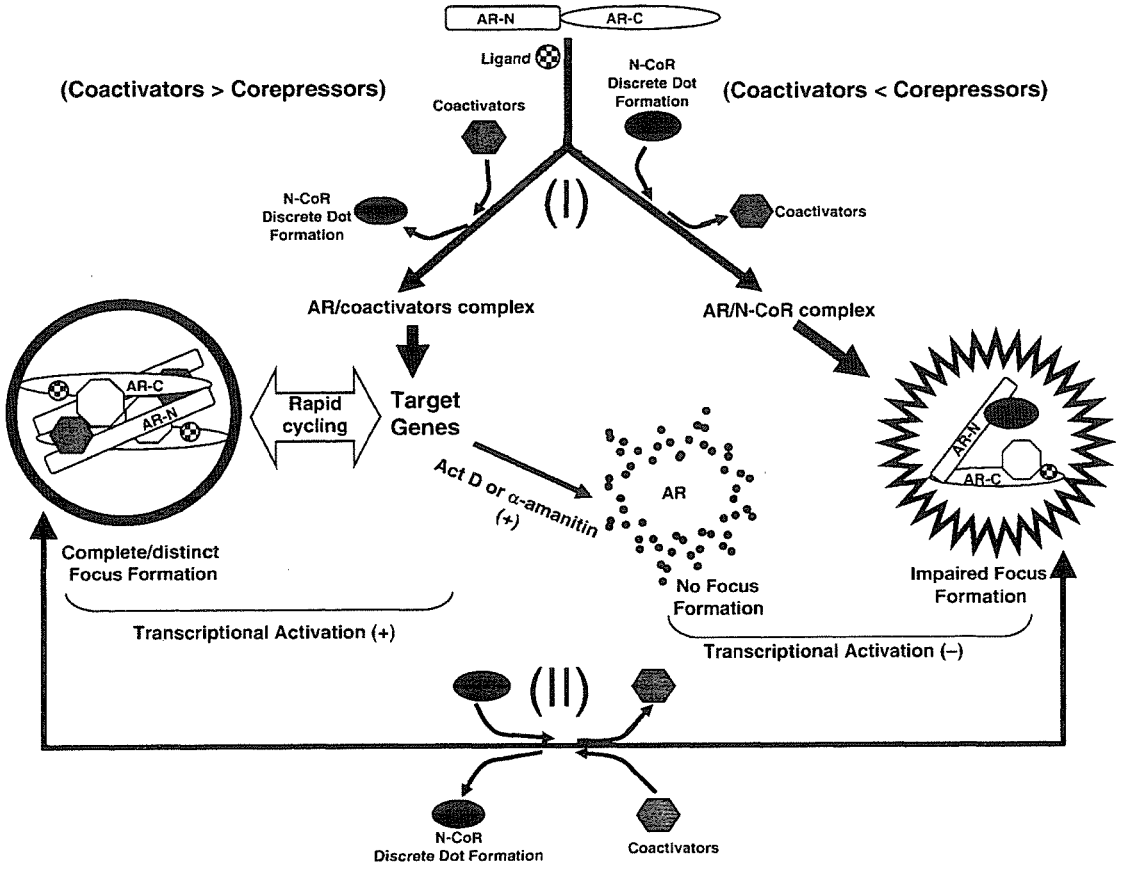

FIG. 15. Speculation of N-CoR action on intranuclear focus formation and transactivation function of the AR. DHT induces a conformational change of the AR and binding domains for coactivators, and the corepressors, N-CoRs, in the amino termini of the AR are unmasked. Under the intranuclear environmental conditions in which the content of the N-CoR is considerably low compared with that of coactivators, DHT-bound AR takes proper N-C interaction with bound coactivators and forms a transcriptionally active conformation, and the AR-coactivator complexes transactivate on the target genes. The transcriptionally active AR-coactivator complexes undergo a rapid cyclic exchange between the subnuclear compartment (complete/distinct foci) and transcriptionally active sites of the target genes. When transcription is prevented by a transcription inhibitor, actinomycin D or α-amanitin, a rapid exchange may be inhibited and then the focus formation is destroyed. The appearance of complete/distinct foci would be an indicator for the transcription active status. Under the environmental conditions in which the N-CoR content is relatively high, the N-CoR binds to liganded AR (apparent overlapping) and interferes with the N-C interaction, resulting in a transcriptionally inactive conformation, which shows the intranuclear impaired focus formation. There are several possible phases (I and II) for the conformational equilibrium between transcriptionally active and inactive DHT-bound AR dependent on the relative ratio of coactivators and N-CoR.

steroid hormone receptors and coactivators show multiphasic on and off switching for binding to promoter elements of the target genes. The receptor-coactivator complexes also undergo a rapid exchange between the target genes and their subnuclear compartments (34, 35, 48). Based on these reported observations and the present findings that complete focus formation is related to the transcriptional activation, it is suggested that complete focus formation of steroid hormone receptors reflects the accumulation of steroid hormone receptor-coactivator complexes released from their target genes. Therefore, it is further suggested that focus formation is a mirror of transcriptionally active complex formation of steroid hormone receptors and coactivators at transcription sites. Thus, investigation of focus formation would be useful for the elucidation of the mechanism of steroid hormone receptor-dependent transactivation in living cells. A physiological role for focus formation still remains to be clarified. One speculated possibility is as follows. Numerous important molecules are functioning in the nucleus. Some regulatory mechanisms for these molecules should be present. Focus formation might be one of these mechanisms, and foci function as temporary storing and buffering sites that are related to steroid hormone receptor cycling. Figure 15 summarizes the speculation of the intranuclear compartmentalization and transactivation function of the AR based on the present findings.

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Pivotal role of Notch signaling in regulation of erythroid maturation and proliferation

Tachikawa Y, Matsushima T, Abe Y, Sakano S, Yamamoto M, Nishimura J, Nawata H, Takayanagi R, Muta K. Pivotal role of Notch signaling in regulation of erythroid maturation and proliferation.

Abstract: Notch signaling plays an important role in cell fate decisions in developmental systems. To clarify its role in committed hematopoietic progenitor cells, we investigated the effects of Notch signaling in erythroid colony forming cells (ECFCs) generated from peripheral blood: ECFCs express Notch receptors, Notch1 and Notch2, and Notch ligands Delta1, Delta4, and Jagged1. When we assayed the effects of Notch ligands on erythroid maturation by flow cytometry, we found that immobilized Delta1 and immobilized Delta4 in particular inhibited maturation, whereas Jagged1 had no effect. In addition, Delta4 inhibited proliferation without reducing cell viability. Increases in expression levels of the Notch target gene hairy enhancer of split (HES) -1 were evident by real-time PCR after stimulation with immobilized Delta4. The effect of soluble Delta4 on expression of HES-1 was less pronounced than that seen with the immobilized form, indicating that all surface-bound ligands are important for effective signal transduction. When ECFCs were cultured in the presence of soluble Delta4 at a low cell concentration, erythroid maturation was slightly inhibited, but at a high concentration, maturation was promoted via competition of soluble Delta4 with endogenous ligands. These results indicate a pivotal role of Notch signaling in regulating erythroid maturation and proliferation, and further suggest that cell–cell interactions modulate growth of erythroid progenitor cells via Notch system.

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Key words: notch signalling; ECFCs; notch ligand; delta4; erythroid progenitor cells; normal hematopoietic cells

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Notch receptors play an important role in cell fate decisions by binding to their ligands on adjacent cells in various developmental systems (1–3). At present, four Notch receptors, Notch-1, -2, -3, -4 (4–6), and their ligands, Delta-1, -3, and -4, and Jagged-1 and -2 (7–10), have been identified in mammals. Notch ligands bind to Notch receptors through their extracellular domains, trigger proteolytic processing, and release the Notch intracellular domain (NIC) from the cell membrane. Cleaved NIC interacts with the DNA-binding transcription factor CBF1/RBP-Jκ, and the complex translocates into the nucleus (11). This complex binds to the Hairy enhancer of split (HES) -1 promoter and stimulates the transcription of the HES-1 gene (1, 12). Moreover, over-expressed NIC acts as a constitutively active form of Notch receptor (13, 14).

Recently it has been demonstrated that cell–cell signaling via Notch regulates lineage commitment at various points during hematopoiesis. For example, NIC transgenic mice have defective B-cell development, and Notch signaling influences the decision to differentiate into αβ or γδ T cells (15), as well as that of differentiation into CD4 or CD8 T cells (16). These observations indicate that Notch signaling plays a central role in regulation of cell fate decisions of multi- or bi-potent precursors (17, 18). Notch receptors expressed by bi-potential progenitors are activated by Notch ligands expressed on neighboring progenitors, leading to inhibition of differentiation of the Notch-expressing cells along a fate-specific pathway. These cells remain undifferentiated or differentiate along an alternate pathway in the presence of appropriate stimuli (namely, lateral inhibition) (1). The Notch system is known to play crucial roles in the regulation of blood cell production, modulation of proliferation, and lineage commitment. Even so,

the role of Notch signal in growth of committed progenitors is not fully understood.

Human erythroid progenitor cells purified from peripheral blood (erythroid colony forming cells; ECFCs) are committed erythroid progenitor cells and it has been known that in erythroid maturation and proliferation, several growth factors, such as interleukin-3 (IL-3), erythropoietin (EPO), stem cell factor (SCF), and insulin-like growth factor 1 (IGF 1), play essential roles at each maturation stage. Highly purified peripheral erythroid burst-forming units (BFU-E) are dependent on IL-3 for the first 2–3 d of *in vitro* culture. SCF is required in the earliest BFU-E through day 8 ECFCs. ECFCs proliferate and mature from erythroid progenitor cells into late erythroblasts in serum-free cultures with EPO and IGF-1 (19). However, the role of Notch signaling in these committed human erythroid progenitor cells has not yet been investigated.

In this study, we investigate whether components of the Notch pathway are expressed in human ECFCs and whether Notch signaling influences erythroid maturation. We found that components of the Notch pathway were expressed in human erythroid progenitor cells and that maturation and proliferation of these cells can be altered by activation of the Notch signaling pathway. The results suggest that cell–cell interactions modulate growth of erythroid progenitor cells via the Notch system.

Materials and methods

Reagents

Recombinant human erythropoietin (rhEPO) was kindly provided by Chugai Pharmaceutical Co Ltd (Tokyo, Japan); recombinant human interleukin 3 (rhIL-3), and recombinant human stem cell factor (rhSCF) were kindly provided by Kirin-Brewery Co Ltd (Tokyo, Japan). Mouse anti-IgG1 antibody was purchased from Life Technologies (Rockville, MD, USA), and human IgG1 kappa from The Binding Site (Birmingham, UK).

Purification and expansion of ECFCs

The ECFCs were prepared as previously described (20–22). Briefly, light-density mononuclear cells were obtained from 40 mL of heparinized peripheral blood buffy coat from healthy Japanese volunteers after informed consent. Cells were isolated by density centrifugation using lymphocyte separation medium (LSM, density 1.0770–1.0800 g/mL; ICN Biomedicals, Aurora, OH, USA). Red blood cells were lysed by suspending the mononuclear cell pellet in red cell lysis buffer (0.16 mol/L ammonium chloride, 10 mmol/L potassium bicarbonate, 5 mmol/L EDTA). Platelets were removed by cell centrifugation through phosphate-buffered saline (PBS) containing 10% human serum albumin (HSA, kindly provided by the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). Adherent cells were depleted by a 1-h incubation in a polystyrene tissue culture flask at 4 °C. Non-adherent cells were then collected and two cycles of negative selection were performed using anti-CD3, -CD11b, -CD15, and -CD45RA antibodies and immunomagnetic beads with VarioMACS columns (Miltenyi Biotech, Auburn, CA, USA). The remaining cells were cultured in Iscoves modified Dulbecco medium (IMDM; GIBCO BRL, Grand Island, NY, USA) containing 15% heat-inactivated fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Vic., Australia), 15% pooled human AB serum, 2 U/mL EPO, 20 ng/mL SCF, 10 ng/mL IL-3, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO) at 37 °C in a high humidity, 5% CO_2, 95% air incubator (day 0). On day 3, the cells, referred to as day 3 ECFCs, were centrifuged over LSM, then collected and incubated under the same conditions, but without IL-3. The purity of day 3 ECFCs, with proerythroblastic features, was 79.8% ± 11.6%, as determined by cytospin preparation. Cell purity was assessed in each experiment.

Serum-free liquid culture of day 3 ECFCs

Day 3 ECFCs (1.0×10^4 cells/mL) were incubated in serum-free liquid medium containing 50% IMDM/50% F-12 medium (Sigma Chemical, St Louis, MO, USA) with 0.1% detoxified bovine serum albumin (BSA; Stem Cell Technologies, Vancouver, BC, USA), 300 μg/mL iron-saturated transferrin (652202, Boehringer Mannheim, Mannheim, Germany), lipid suspension prepared, as described (21), 10 U/mL rhEPO, and 50 ng/mL rh SCF at 37 °C in a high humidity, 5% CO_2, 95% air incubator. Cultured cells were collected on days 5, 7, and 9 (referred to as day 5 ECFCs, day 7 ECFCs, and day 9 ECFCs).

Cloning and expression of human Notch receptors and ligands

Purified human Notch ligand-immunogloblin (Ig) G1 chimeric proteins and a human Delta4-FLAG octapeptide chimeric protein were prepared as described previously (23). Briefly, isolation of a complementary DNA (cDNA) clone of human Notch ligands was performed by plaque hybridization. cDNA encoding the extracellular domain of Notch ligands was fused in frame to a sequence of human IgG1 or of FLAG octapeptide. The Notch

ligand-IgG or -FLAG fusion proteins were produced by transfection of the fused genes in the expression vector pcDNA3 or pM-KITneo into Chinese hamster ovary cells via electroporation, followed by G418 selection. The Notch ligand fusions were detected using anti-human IgG1 or -FLAG antibody. The chimeric proteins were purified from culture supernatants by affinity chromatography and stored in PBS. The purity was more than 90%, as determined by SDS-PAGE.

Determination of cell viability

Viability of the cells was determined by trypan blue exclusion using a hemocytometer.

MTT colorimetric assay

Cell growth was examined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasodium bromide (MTT) colorimetric assay (Chemicon International Inc., Osaka, Japan). After incubation, cells were washed with IMDM and plated into serum-free liquid medium in 96-well tissue culture plates. Then MTT solution (1 mg/mL) was added to each well, and the cells were further incubated for 4 h at 37 °C. The absorbance at the wavelength of 570 nm was measured by a micro-ELISA reader. The negative control well contained medium only. The effect of additives on cell survival was estimated as a percentage of the value of untreated control.

Assessment of erythroid maturation by flow cytometry

To determine the effect of Notch ligands on erythroid maturation, expression of the transferrin receptor (TfR) and glycophorin A (GPA) on ECFCs were analyzed by flow cytometry as described (24). The cells (1.0×10^5) were washed twice with PBS and resuspended in PBS with 0.05% BSA. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-GPA MoAb (11E4B-7-6, IM2212; Immunotech, Marseilles, France) and phycoerythrin (PE)-conjugated anti-CD71 MoAb (YDJ1.2.2, IM2001; Immunotech) for 30 min on ice. Samples were analyzed using the Epics Elite ESP flow cytometer (Coulter, Miami, FL, USA).

Plasma clot assay

The erythroid colony-forming capacity of ECFCs was determined by the plasma clot method (22). Day 3 ECFCs were incubated for 6 d in serum-free liquid medium containing 50% IMDM/50% F-12 medium with 0.1% BSA, 300 μg/mL iron-saturated transferrin, lipid suspension, 10 U/mL rhEPO, and 50 ng/mL rhSCF at 37 °C in a high humidity, 5% CO_2, 95% air incubator. The cells (day 9 ECFCs) were then collected, and 600 cells with the media consisting of IMDM, 20% FCS, 1% BSA, 10 ng/ml rhSCF, 2 U/ml rhEPO, and 10% citrated human AB plasma were plated in triplicate 35-mm culture dishes and incubated for an additional 7 d. The clots were then fixed and stained with 3, 3′ dimethoxybenzidine. The number of hemoglobinized cells in randomly selected colonies was counted for each plate.

Reverse transcriptase PCR (RT-PCR) and real-time quantitative RT-PCR

Total RNA was isolated from 10^6 cells with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using 1 μg total RNA by the RT-PCR kit (Takara, Shiga, Japan) according to the protocol of the manufacturer. To determine the expression of Notch signaling components, RT-PCR reactions for Notch receptors and ligands were performed in a Perkin-Elmer 9700 cycler (Foster City, CA, USA) for a total of 40 cycles. Reactions were optimized for each set of primers as shown in Table 1.

To serve as an internal control, β-actin was amplified from all cDNA templates generated. PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed.

Quantitative real-time PCR assay of transcripts were carried out using gene-specific double fluorescently labeled probes in LightCycler Software Version 3.5 (Roche, ID, USA). Target quantities were normalized to 18S rRNA and calibrated using day 6 values and defined as a value of 1.0. All quantities were expressed as n-fold relative to the calibrator (day 6).

Statistical analysis

The Student's *t*-test was used to determine significant differences between the groups and results are expressed as the mean ± SD.

Results

Notch receptors and ligands are expressed on ECFCs

To determine the expression pattern of Notch receptors and ligands in erythroid progenitor cells, we examined the expression of all of four Notch receptors and three of five ligands, Delta1, Delta4, and Jagged1, during liquid culture of ECFCs via RT-PCR. As shown in Fig. 1A, Notch1 and

Table 1. Specific primer pairs used for RNA analysis

Gene	Size (bp)	Annealing temperature (°C)	5′ primer	3′ primer
Notch1	547	58	CAGGTCAGTACTGTACCGAG	TGGCACTCTGGAAGCACTGC
Notch2	398	58	ACATCATCACAGACTTGGTC	CATTATTGACAGCAGCTGCC
Notch3	659	58	TGGATGAGTGTCAGCTGCAG	AGGTGCAGCTGAAGCCATTG
Notch4	622	61	ACATACCCCCAGATCCAGTG	AGAGGCAGGAGAAAGAGCCC
Delta1	469	58	TGCAGGAGTTCGTCAACAAG	TCCGTAGTAGTGTTCGTCAC
Delta4 first	656	58	GTTCATCAACGAGCGCGGCG	GGGATGCATTCGTTACACAG
Delta4 second	642	62	TCAACGAGCGCGGCGTAGTG	TCGTTACACAGCCGGCCCTG
Jagged1	436	58	GATCCTGTCCATGCAGAACG	GGATCTGATACTCAAAGTGG
HES-1	374	59	GACAGCATCTGAGCACAGAAATG	GTCATGGCATTGATCTGGGTCAT
β–actin	266	57	TACCTCATGAAGATCCTCA	TTCGTGGATGCCACAGGAC
18S rRNA	269	66	ACTCAACACGGGAAACCTCA	GGACATCTAAGGGCATCACA

18S rRNA Probe: LightCycler – Red 640 – 5′ – TCCGATAACGAACGAGACTCTGGCAT – 3′
5′ –GTTGGTGGAGCGATTTGTCTGGTTAA – 3′ – FITC
HES, hairy enhancer of split.

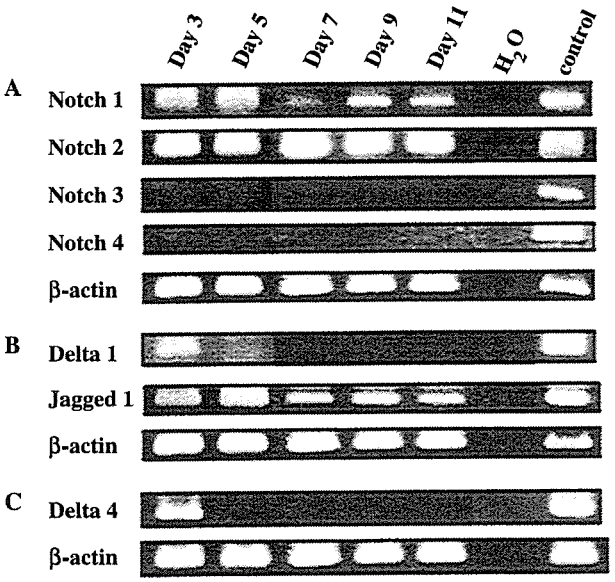

Fig. 1. Expression of Notch receptors and ligands on erythroid colony forming cells (ECFCs). Day 3 ECFCs (1.0×10^4 cells/well) were transferred to serum-free medium containing recombinant human erythropoietin (10 U/mL) and recombinant human stem cell factor (50 ng/mL). At the indicated times, RNA was extracted and subjected to RT-PCR followed by electrophoresis on an agarose gel and staining with ethidium bromide. (A) Expression of Notch-1, -2, -3, -4. Notch1 and Notch2 are expressed during erythroid maturation. Neither Notch3 nor Notch4 can be detected. (B) Expression of Delta1 and Jagged1. Delta1 is expressed only on day 3. Jagged1 is detected throughout erythroid maturation. (C) Expression of Delta4 as detected by nested PCR. The purity of ECFCs collected at day 3 ECFCs was 79.8% ± 11.6%; day 5, 91.8% ± 0.3%; day 7, 93.6% ± 5.1%; day 9, 98.5% ± 0.8%; and day 11, 99.8% ± 0.2% (mean ± SD).

Notch2 were expressed at all stages investigated. In contrast, Notch3 and Notch4 were not detected at any stage of maturation we tested. In addition, Delta1 and Delta4 were expressed only on day 3 ECFCs, and Jagged1 was expressed at all stages investigated (Fig. 1B and C). Although Delta4 was not detectable in first-round RT-PCR, Delta4 transcripts could be detected by nested PCR in day 3 ECFCs. The purity of day 3 ECFCs prepared for RT-PCR was 79.8% ± 11.6% after the second negative selection; the purity of day 5 ECFCs was 91.8% ± 0.3%; day 7 ECFCs, 93.6% ± 5.1%; day 9 ECFCs, 98.5% ± 0.8%; and day 11 ECFCs, 99.8% ± 0.2%. Regarding the stage of erythroid differentiation, days 7 and 9 ECFCs were in basophilic erythroblast stages and about 75% of day 11 ECFCs were in polychromatic stages and the others, orthochromatic.

Immobilized Delta1 and Delta4 inhibit maturation of ECFCs

The fact that ECFCs expressed both Notch receptors and their ligands suggested the possibility that Notch signaling induced by cell–cell interaction might affect the maturation of erythroid progenitors. Thus, we next examined the effects of Notch ligands on maturation of ECFCs. When day 3 ECFCs were incubated in serum-free media, the cells first began to express TfR and then these TfR+ cells expressed GPA; i.e., the percentage of mature cells (TfR+/GPA+) gradually increased during culturing (Fig. 2A, upper panel). When day 3 ECFCs were cultured with immobilized Delta4-IgG1 fusion proteins (30 mg/mL) that had been plated on 24-well plastic plates coated with BSA, the percentage of mature erythroid cells (TfR+/GPA+) was lower than the percentage detected in controls at any time point (Fig. 2A, lower panel).

Next, we looked at the effect of other Notch ligands, Delta1 and Jagged1, on maturation of ECFCs. On day 9, the percentage of TfR+/GPA+ cells was significantly lower after incubation with immobilized Delta1 and Delta4 (vs. control; Delta1, $P < 0.05$, Delta4, $P < 0.001$), whereas immobilized Jagged1 had no effect on erythroid maturation even at day 9 (Fig. 2B).

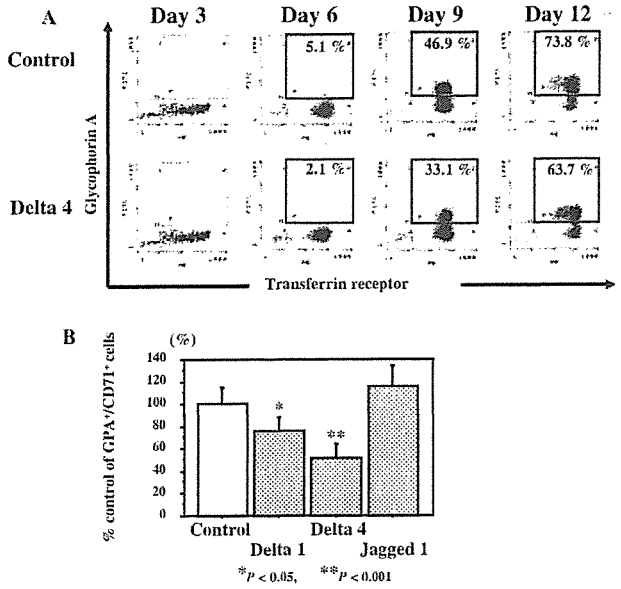

Fig. 2. Effects of immobilized Notch ligands on maturation of erythroid colony forming cells (ECFCs). Day 3 ECFCs (1.0×10^4/mL) were cultured with human IgG1-kappa or immobilized Notch ligands (30 mg/mL) in serum-free medium and expression of transferrin receptor (TfR) and glycophorin A (GPA) was determined by flow cytometry at the indicated days. Data are representative of three experiments. Numbers in corners show the percentage of gated events. (A) Serial change of expression of GPA and TfR during erythroid maturation in control (upper panel) and with Delta4 (lower panel). (B) Each bar represents the ratio of mature cells (TfR+/GPA+) on day 9 after culture with Notch ligand to the number of mature cells in the control. Each point shows the mean ± SD of triplicate experiments.

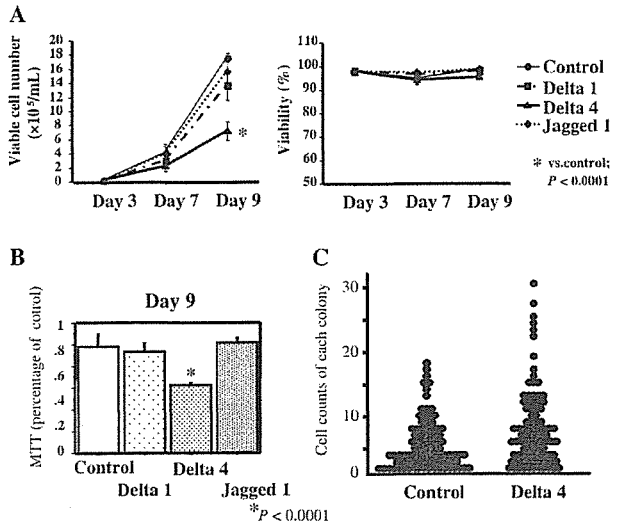

Fig. 3. Effects of immobilized Notch ligands on proliferation of erythroid colony forming cells (ECFCs). Day 3 ECFCs (1.0×10^4/mL) were incubated with or without immobilized Notch ligands (30 mg/mL), and (A) viable cell number (left panel) and cell viability (right panel) were measured by trypan blue exclusion at the indicated days. (B) Measurement of cell growth by MTT assay. (C) Effect of immobilized Delta4 on function of erythroid colony formation. Each point shows the number of hemoglobinized cells in randomly selected colonies. Data are representative of two experiments.

Immobilized Delta4 inhibits proliferation of ECFCs without reducing cell viability

Next we examined the effect of Notch ligands on cellular proliferation of ECFCs. Immobilized Delta4 significantly reduced the number of ECFCs without having an effect on viability, as measured by the trypan blue exclusion test on day 9 (control vs. Delta4; $17.56 \pm 0.60 \times 10^5$/mL vs. $7.13 \pm 1.32 \times 10^5$/mL, $P < 0.0001$; Fig. 3A). This effect on cellular proliferation was also confirmed by performing an MTT assay on day 9 ECFCs (Fig. 3B). The other two ligands assayed did not have a detectable effect on cell proliferation. Furthermore, we were able to confirm that none of the four ligands examined induced apoptosis of ECFCs using flow cytometry with Annexin V-FITC and phosphatidylserine staining (data not shown).

We examined the effect of immobilized Delta4 on colony-forming capacity of ECFCs (Fig. 3C). Immobilized Delta4 did not reduce colony-forming capacity of ECFCs, despite the fact that Delta4 has been shown to act as an inhibitor of proliferation in other contexts. Moreover, ECFCs incubated with immobilized Delta4 tended to form erythroid colonies that are larger than control colonies.

Expression of HES-1 mRNA increases after stimulation with immobilized Delta4

To investigate if Delta4 actively induces Notch related intracellular signaling in erythroid progenitor cells, we determined the ratio between the number of copies of mRNA of HES-1 (a Notch target gene) and the number of copies of 18S rRNA (an internal control) on days 6 and 9 using real-time PCR. The relative expression of HES-1/number of copies of 18S in the culture with ligand was then compared with the control. Increased expression of HES-1 mRNA became evident after stimulation with immobilized Delta4 on day 6 ($P = 0.0003$; Fig. 4A). In contrast, no significant change in expression of HES-1 could be detected when cells were stimulated by non-immobilized, soluble Delta4-FLAG fusion protein (10 mg/mL; Fig. 4B).

Soluble Delta4 slightly inhibits maturation of ECFCs

We next investigated the effects of soluble Delta4 on maturation of ECFCs by using flow cytometry to determine the level of expression of TfR and GPA. When day 3 ECFCs were cultured with soluble Delta4 (10 mg/mL) at a low cell concentra-

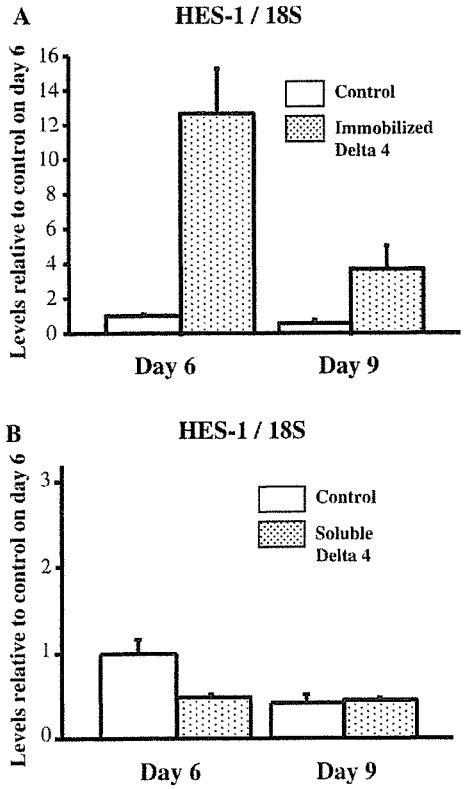

Fig. 4. Induction of the Notch target gene, hairy enhancer of split (HES) -1, by Delta4. Relative expression of mRNA of the Notch target gene, HES-1, and the number of copies of 18S rRNA (a ubiquitously expressed gene) were determined by real-time PCR on days 6 and 9 for cells cultured with IgG1-kappa (white bar), immobilized Delta4 (gray bar) or soluble Delta4 (gray bar). Relative expression of hairy enhancer of split (HES) -1/number of copies of 18S in the culture with ligand was compared with the control. (A) Expression of HES-1 mRNA increases after stimulation with immobilized Delta4. (B) Expression of HES-1 after stimulation by soluble Delta4.

tion, cell–cell interaction among ECFCs is likely to be at negligible levels. After 6 d of culture, the percentage of GPA+ cells was lower than that in the control (63.7% vs. 52.1%, Fig. 5A, left panel). The percentage of TfR+/GPA+ cells also decreased in cultures with soluble Delta4 (64.3% vs. 53.1%, Fig. 5A, right panel). However, this effect of soluble Delta4 was less significant than that seen in cells cultured with immobilized Delta4 (Fig. 2B). This finding is consistent with the expression data for HES-1 mRNA (Fig. 4).

In order to ask if soluble Delta4 inhibited maturation of ECFCs in cultures at a high density of day 3 ECFCs, in which endogenous cell–cell interaction among ECFCs might exist, we cultured day 3 ECFCs with soluble Delta4 (10 mg/mL) at a high density (1.0×10^5/mL). Unexpectedly, the percentage of GPA+ cells was higher than that seen in the control (46.6% vs. 65.6%, Fig. 5B, left panel). In addition, the percentage of TfR+/

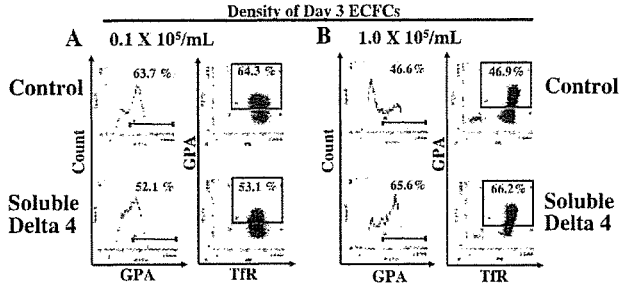

Fig. 5. Effects of non-immobilized Delta4 on erythroid maturation. Day 3 erythroid colony forming cells (ECFCs) were incubated at low density (A; 0.1×10^5/mL) or at high density (B; 1.0×10^5/mL) with or without soluble Delta4 (10 mg/mL), and expression of transferrin receptor and glycophorin A (GPA) on day 9 ECFCs were determined by flow cytometry. In the left panel of each figure, numbers in corners represent the percentage of GPA+ cells. In the right panel of each figure, numbers in corners represent the percentage of gated events within that quadrant. Data are representative of three experiments.

GPA+ cells significantly increased with Delta4 treatment (46.9% vs. 66.2%, Fig. 5B, right panel). These results suggested that maturation of ECFCs could be promoted by the addition of soluble Delta4 to the culture medium when the cells are at high density. In contrast, immobilized Delta4 inhibited maturation of ECFCs plated at high cell density (1.0×10^5/mL; data not shown).

Effects of soluble Delta4 differ with changes in cell density

To further examine the effects of cell–cell interaction in modulation of erythroid maturation, we examined the effect of soluble Delta4 in cells at different densities at day 3 (Fig. 6A). In each of four independent experiments, at low cell-density, the percentage of TfR+/GPA+ cells decreased as compared with controls with addition of soluble Delta4. When cell density of the culture was increased, the effect of soluble Delta4 was lost, and finally, at high cell density, the percentage of TfR+/GPA+ cells was higher with soluble Delta4 than that seen in the control. Cumulative data are shown in Fig. 6B. The percentage of TfR+/GPA+ cells was significantly lower than the control at low cell density (0.25×10^5/mL, $P = 0.02$) and higher at high cell density (1.0×10^5/mL, $P = 0.03$).

Discussion

Notch signaling maintains an immature, precursor state of cells and affects the lineage outcome in a number of developmental and differentiation systems (15–18, 25–29). When progenitors have both Notch receptors and ligands, Notch receptors on one cell are activated by ligands on neighboring cells. This results in inhibition of differentiation of

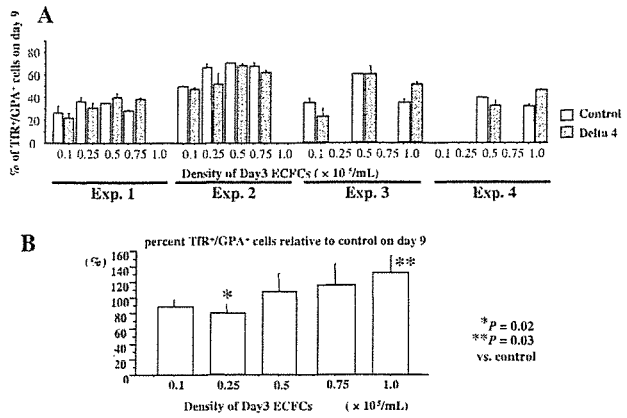

Fig. 6. Effect of soluble Delta4 on maturation at different cell densities. Day 3 erythroid colony forming cells were incubated at various cellular densities (0.1, 0.25, 0.5, 0.75, 1.0×10^5/mL) in serum-free liquid medium with or without soluble Delta4 for 6 d. (A) The percentage of transferrin receptor (TfR)+/glycophorin A (GPA)+ cells on day 9 was determined in four independent experiments. (B) Each bar represents the ratio of TfR+/GPA+ cell number in soluble Delta4-treated cultures to the number of double-positive cells in the control cultures at each cellular density.

receptor-expressing cells at the same time that ligand-expressing cells differentiate along a fate-specific pathway. The result is that the two cell populations exist in different stages of differentiation as a result of the effects of differential Notch signaling.

In studies of hematopoietic cells, Notch signaling has mainly been investigated in multipotent hematopoietic stem cells. With regard to the effect of Notch signaling on commitment to an erythroid lineage fate, activation of Notch signaling inhibits differentiation along erythroid lineage in mouse stem cells (18, 30). Moreover, in human and mouse erythroleukemic cells, Notch signaling inhibits erythroid differentiation (31, 32). Despite these advances in our understanding, the role of Notch signaling in committed hematopoietic progenitor cells has not been fully explored.

Here, we have shown that Notch receptors and their ligands are expressed in human erythroid progenitor cells purified from peripheral blood (ECFCs). In addition, we found that Notch signaling through immobilized Delta4 inhibits erythroid maturation and proliferation without affecting their function or inducing apoptosis.

The level of the expression patterns of Notch receptors and ligands on ECFCs differs between cells at different stages of maturation, as seen in the B-cell lineage (33). These observations suggest the possibility that the pattern of functional Notch receptors and ligands differs at different stage of maturation of ECFCs.

We detected a maturation inhibition effect of Notch signaling by comparing the percentage of TfR+ and GPA+ cells. The effect of Notch signaling on ECFCs was rather faint and thus difficult to detect by less sensitive methods, such as benzidine staining (data not shown). Kumano *et al.* reported that Notch1 signaling inhibited erythroid differentiation in mouse erythroleukemic cell lines F5-5 and that the expression of GATA-2, one of the transcription factors, which expression level decreased during erythroid differentiation, was sustained after Notch1 activation (34). However, it has not been known if any transcription factors relevant to erythroid differentiation, such as GATA-2, were affected by activation of Notch signaling in primary human hematopoietic progenitor cells. Although we analyzed the expression level of GATA-2 mRNA by real-time PCR, we found no significant difference between control cells and cells stimulated by immobilized Delta4. GATA-2 is known to be important in decision of lineage commitment of multipotent progenitor cells toward erythroid lineage and in primary progenitor cells Notch may play a role at more differentiated stage (i.e., at a stage where GATA-2 mRNA level has already decreased).

We show that immobilized Delta4 induced elevation of HES-1 mRNA expression in ECFCs, a cell type in which Delta4 also delayed maturation. Ishiko *et al.* reported that forced expression of HES-1 inhibited erythroid differentiation in an erythroid/megakaryocytic cell line (K562) and in murine normal hematopoietic stem/progenitor cells. Thus, in human erythroid progenitor cells, similar HES-1-directed mechanisms may also take part in Notch signaling.

The observation that ECFCs expressed Notch and Delta4, together with the observation that immobilized Delta4 delayed erythroid maturation, indicate that erythroid progenitors may modulate maturation of one another via cell–cell interaction. Moreover, the data also suggest that interaction occurs between ECFCs and endothelial cells. In vertebrates, some of Notch receptors and ligands are expressed in the vascular endothelium (35–37). Delta4 is predominantly expressed in arterial endothelium (10). Recently, it was shown that Delta4 can function as a ligand for Notch4 in a human primary endothelial cell line derived from neonatal dermal microvasculature (38). It is possible that ECFCs stimulate Notch signaling in primary endothelial cells through Delta4-Notch binding. It was also reported that VEGF induces expression of Notch1 and of its ligand, Delta-like 4, in human arterial endothelial cells (39). Given that ECFCs produce VEGF (data not shown), and given that VEGF stimulates the proliferation of endothelial cells, negative feedback via Delta-like 4-Notch binding may occur between ECFCs and endothelial cells.

Our study shows that the elevation of HES-1 mRNA expression and inhibition of maturation of ECFCs induced by soluble Delta4 are less prominent than what is seen with immobilized Delta4. Similar results were obtained using immobilized or soluble Delta1 in C2 myoblasts and U20S cells. In these cell lines, Notch is not activated by soluble Delta1. Furthermore, soluble Delta1 blocks the effect of immobilized Delta1 (40). These results imply that ligation of Notch receptor by a stabilized ligand may be essential for full activation of Notch signaling.

Surprisingly, effects of soluble Delta4 differ with changes in cell density. When the density of day 3 ECFCs is high, there may exist many cell–cell interactions among ECFCs and thus, signaling between the Notch receptor on one ECFC and the endogenous Notch ligand on neighboring ECFCs may occur. When soluble Delta4 is added to day 3 ECFCs, some of the soluble Delta4 (but not the endogenous Delta4) binds to Notch receptors. Consistent with the idea that the effect of soluble Delta4 is smaller than that of endogenous Delta4, inhibitory effect of Notch signaling on maturation is less prominent than that seen in the culture without soluble Delta4. On the other hand, when the density of day 3 ECFCs is low, the frequency of cell–cell interaction among ECFCs is rare and thus, we expect that signaling between endogenous Notch receptors and ligands will only rarely be seen. But when soluble Delta4 is added to this culture, soluble Delta4 can bind to Notch receptors on ECFCs and the ligand weakly inhibits the maturation of receptor-expressing cells. The result is that in the presence of soluble Delta4, maturation is inhibited slightly in comparison with what is seen in cells without soluble Delta4. Based on these observations, we conclude that there exists a cell–cell interaction among ECFCs that acts via the Notch signal transduction pathway.

In conclusion, we demonstrate that Notch signaling plays a pivotal role in the regulation of erythroid maturation and proliferation. Additionally, the data suggest that cell–cell interactions modulate growth of erythroid progenitor cells via the Notch pathway. However, the precise molecular mechanism(s) by which Notch signaling modulates erythroid maturation is not fully understood. Further investigation of the mechanisms of inhibition of erythroid progenitor cell maturation by Notch signaling components may be revealing.

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An increase in the serum amylase level in patients after peroral double-balloon enteroscopy: an association with the development of pancreatitis

Background and study aims: Double-balloon enteroscopy (DBE) is a novel technique that allows the enteroscope to be inserted deep into the small intestine. The procedure has been thought to be safe, but cases of acute pancreatitis after peroral DBE have recently been observed. The aim of this study was to confirm the occurrence of hyperamylasemia after peroral DBE.

Patients and methods: Peroral DBE was carried out in 13 patients from July 2005 to February 2006. Blood samples were taken before and 3 h after the procedure, and serum pancreatic amylase levels were measured. The patients were also evaluated for pancreatic-type abdominal pain after the procedure. Hyperamylasemia after peroral DBE was defined as an elevation of the serum pancreatic amylase level to more than the upper normal limit and twice the level before the procedure. Pancreatitis was diagnosed on the basis of both pancreatic-type abdominal pain and hyperamylasemia.

Results: Hyperamylasemia after peroral DBE occurred in six patients (46.2 %). One of the six patients with hyperamylasemia had pancreatic-type abdominal pain after the procedure and developed acute pancreatitis. The average procedure time was 105 min (range 65 – 155 min) in the patients with hyperamylasemia, and did not significantly differ from that in the group without hyperamylasemia (99 min).

Conclusions: Hyperamylasemia after peroral DBE occurs frequently and may be associated with development of pancreatitis.

Introduction

Double-balloon enteroscopy (DBE), which is a novel technique, allows the insertion of the instrument deep into the small intestine via either the peroral or peranal approaches, allowing visualization of the entire small intestine [1 – 3]. The system includes a 200-cm enteroscope and a 145-cm overtube, with two balloons attached to the tips. The balloons can be inflated or deflated, allowing for shortening of the proximal intestine and thus enabling the enteroscope to be inserted deep into the small intestine. The enteroscopes are currently available with two different diameters: the EN-450P5/20 (Fujinon-Toshiba ES System Co., Tokyo, Japan) has an outer diameter of 8.5 mm, with an overtube that has an outer diameter of 12.2 mm, and the EN-450T5 (Fujinon-Toshiba ES System Co.) has an outer diameter of 9.4 mm, with an the overtube that has an outer diameter of 13.2 mm. With a larger accessory channel, the EN-450T5 allows a variety of endoscopic treatments to be carried out. The technique of DBE is considered to be safe, as few complications have been reported [4, 5].

We recently encountered a patient who developed acute pancreatitis after peroral DBE using the EN-450T5 instrument [6]. In this case, it was considered that the thicker diameter of the EN-450T5 instrument might possibly have been associated with the development of pancreatitis. However, there have recently been several cases of acute pancreatitis after peroral DBE using not only the EN-450T5 but also the EN-450P5/20 in Japan. In ad-

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Table 1 Clinical details and serum amylase levels before and after peroral double-balloon enteroscopy (DBE)

Patient	Age (y)/ sex	Total procedure time (min)	Reason for DBE	Pancreatic-type abdominal pain after procedure	Pancreatic amylase before/3 h after (normal: 10–65 U/l)	Lipase before/3 h after (normal: 16–51 U/l)
1	15/M	100	Severe IDA	Absent	38/35	Not measured
2	47/F	120	Follicular lymphoma	Absent	42/58	Not measured
3	38/F	145	Obscure GI bleeding	Absent	20/189	Not measured
4	78/M	85	Obscure GI bleeding	Absent	23/23	Not measured
5	31/F	90	Chronic diarrhea	Absent	22/35	Not measured
6	67/F	155	Small intestinal tumor	Absent	28/823	27/2093
7	67/M	75	Obscure GI bleeding	Absent	45/228	57/634
8	40/M	85	Carcinoid of the duodenum	Absent	45/66	33/39
9	28/F	115	Peutz–Jeghers syndrome	Absent	37/50	33/33
10	53/M	75	Follicular lymphoma	Absent	53/48	34/25
11	50/M	85	Follicular lymphoma	Absent	42/96	43/125
12	39/M	90	Obscure GI bleeding	Present	36/632	20/1124
13	58/M	80	Follicular lymphoma	Absent	54/168	50/330

GI, gastrointestinal; IDA, iron-deficiency anemia.

dition, three cases of acute pancreatitis after peroral DBE using the EN-450P5/20 in the Netherlands have been reported [7,8]. The peroral DBE procedure itself might thus be regarded as a cause of acute pancreatitis. However, the mechanism underlying the development of pancreatitis after peroral DBE has not yet been clarified, and the evidence for an association between peroral DBE and pancreatitis has yet to be confirmed.

It is well known that endoscopic retrograde cholangiopancreatography (ERCP) is a cause of acute pancreatitis. Elevated serum amylase levels frequently occur after ERCP, and there have been some reports showing that post-ERCP hyperamylasemia after 2 or 4 h is predictive of the subsequent development of pancreatitis [9–11]. We hypothesized that hyperamylasemia may occur in patients undergoing peroral DBE as well as ERCP if peroral DBE is associated with acute pancreatitis. The aim of the present study was therefore to determine whether or not hyperamylasemia occurs in patients who undergo peroral DBE.

Patients and methods

From July 2005 to February 2006, peroral DBE was carried out in 13 patients (five women and eight men; age 15–78, median 47) (Table 1). Written informed consent to the enteroscopic procedure was obtained from all of the patients. The reasons for DBE included the following; obscure gastrointestinal bleeding (four patients), severe iron-deficiency anemia (one patient), suspected small-intestinal lesions with duodenal follicular lymphoma (four patients), suspected small-intestinal lesions with duodenal multiple carcinoids (one patient), suspected small-intestinal lesions because of chronic diarrhea and malabsorption (one patient), a small intestinal tumor detected by a barium meal study (one patient), and suspected small-intestinal polyps in Peutz–Jeghers syndrome (one patient). None of the patients had a history of pancreatitis, gallstones, or alcohol abuse. Serum pancreatic amylase levels (normal range 10–65 U/l) were measured both before and 3 h after the peroral DBE procedure. Isoamylase was used, as it is well known that hyperamylasemia even after upper gastrointestinal endoscopy sometimes occurs due to an increase in salivary amylase, probably associated with hypersalivation [12]. Serum lipase levels were also measured in eight patients (normal range 16–51 U/l).

Pancreatic-type abdominal pain (persistent epigastric pain radiating to the back) was also evaluated after the procedure. Elevation of the serum pancreatic amylase level was defined as an increase greater than the upper normal limit (65 U/l) and twice the level before the procedure. Pancreatitis was defined as a combination of pancreatic-type abdominal pain and elevation of the serum pancreatic amylase level.

Peroral double-balloon enteroscopy procedure

Twelve patients underwent peroral DBE using the EN-450P5/20 instrument, and one patient with Peutz–Jeghers syndrome was examined with the EN-450T5. Peroral DBE was carried out using the same procedures described by Yamamoto et al. [4]. When necessary, biopsy specimens were obtained using biopsy forceps.

Statistics

The average procedure time in the groups with elevated pancreatic amylase and without elevated pancreatic amylase was compared using Student's t-test and the difference was considered to be statistically significant at $P < 0.05$.

Results

The total procedure time, including insertion of the enteroscope and observation, ranged between 75 and 155 min (mean 102 min). No small-intestinal lesions were detected in patients 1, 3, 4, 7, and 12. In patients 2 and 8, no other lesions apart from the known duodenal lesions were found. In patients 10, 11, and 13, with duodenal follicular lymphoma, other lesions were detected in the small intestine and biopsies were taken. In patient 5, marked villous atrophy from the upper jejunum to the deep

In brief

An interesting series based on the recent observation of pancreatitis cases after double-balloon enteroscopy (DBE). In this prospective study of 13 patients who underwent DBE, one developed clinical pancreatitis and almost half had hyperamylasemia. This requires systematic assessment with larger numbers of patients.

small intestine was found, and a biopsy was therefore performed. In patient 6, a tumor with ulceration in the jejunum was found and a biopsy was performed. The serum pancreatic amylase levels measured before and 3 h after peroral DBE are shown in Table **1**. In seven patients (patients 1, 2, 4, 5, 8, 9, and 10; 53.8%), the serum pancreatic amylase level did not exceed the upper normal limit. Six patients (patients 3, 6, 7, 11, 12, and 13, 46.2%) had elevated serum pancreatic amylase levels above the upper normal limit and twice as high as their levels before the procedure. Patients 3, 7, 11, and 13 had no pancreatic-type abdominal pain after the procedure and their serum pancreatic amylase level normalized 24 h after the procedure; computed tomography (CT) was therefore not carried out. Patient 6 had marked hyperamylasemia and hyperlipemia, but no pancreatic-type abdominal pain after the procedure. Abdominal CT performed later revealed no inflammation of the pancreas. Two days after peroral DBE, both her serum P-amylase and lipase levels had normalized. One patient (patient 12) had pancreatic-type abdominal pain after the procedure, and his serum pancreatic amylase and lipase levels were increased to 632 IU/l and 1124 IU/l, respectively. He was therefore diagnosed as having acute pancreatitis. A laboratory test showed an elevated C-reactive protein level, at 14.52 mg/dl (normal range < 0.06 mg/dl). Abdominal CT revealed acute pancreatitis with edema of the pancreas and infiltration of the peripancreatic fat. The pancreatitis resolved with conservative therapy after 28 days in hospital.

There were no complications apart from acute pancreatitis associated with the peroral DBE procedure.

The average procedure time in patients with an elevated pancreatic amylase level was not significantly longer than that in patients who had no elevation of the pancreatic amylase level (105 min vs. 99 min).

Discussion

The present study clearly demonstrates that latent hyperamylasemia without the development of pancreatitis occurs after peroral DBE more frequently than was previously thought. The high incidence of postperoral DBE hyperamylasemia strongly indicates that there is indeed a link between peroral DBE and pancreatitis. In peroral DBE, the duodenum and proximal small intestine were markedly shortened, and it the duodenum was sometimes found to be nearly straight from the pyloric ring to the ligament of Treitz on fluoroscopic guidance. In these conditions, the papilla of Vater may be subject to severe strain, and intraluminal pressure in the duodenum may increase in such a way as to disturb the secretion of pancreatic juice. This mechanism might be associated with the occurrence of hyperamylasemia and pancreatitis after peroral DBE. Groenen et al. [8] hypothesized that duodenal hypertension caused by inflation of the two balloons, inducing reflux of the duodenal contents into the pancreatic duct and leading the activation of pancreatic enzymes, could lead to the development of acute pancreatitis. The animal model of acute pancreatitis due to duodenal hypertension involves a closed duodenal loop [13]. In this model, a closed loop is created by ligating the duodenum at two points on the oral and anal sides of the major duodenal papilla, and severe pancreatitis is induced due to reflux of duodenal fluid containing activated pancreatic fluid, increased pressure within the pancreatic duct, and impaired pancreatic blood flow [14–16]. Blackstone [15] noted the importance of impairment of the pancreatic microcirculation, with increased pressure in the closed loop and in the pancreatic duct, in association with edema-induced pancreatic tissue ischemia and vascular injury induced by refluxed bile.

Pancreatitis may therefore be a major complication not only of ERCP but also of peroral DBE. On the basis of the present results showing frequent hyperamylasemia, the factors affecting the outcome of the latent hyperamylasemia and the onset of pancreatitis need to be determined. As the number of patients examined in this study is small, further research is necessary to clarify which factors may induce hyperamylasemia and potential pancreatitis.

In conclusion, the results of the present study show that hyperamylasemia occurs frequently after peroral DBE and that it appears to be associated with pancreatitis following the procedure.

Competing interests: None

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Genetic polymorphism of cholesterol 7α-hydroxylase (CYP7A1) and colorectal adenomas: Self Defense Forces Health Study

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Bile acids have long been implicated in colorectal carcinogenesis, but epidemiological evidence is limited. Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme producing bile acids from cholesterol. A recent case-control study showed a decreased risk of proximal colon cancer associated with the *CC* genotype of the *CYP7A1 A-203C* polymorphism. The present study examined the relationship between the *CYP7A1 A-203C* polymorphism and colorectal adenoma, which is a well-established precursor lesion of colorectal cancer. The study subjects comprised 446 cases of colorectal adenomas and 914 controls of normal total colonoscopy among men receiving a preretirement health examination at two hospitals of the Self Defense Forces (SDF). The *CYP7A1* genotype was determined by the polymerase chain reaction–restriction fragment length polymorphism method. Statistical adjustment was made for age, hospital, rank in the SDF, smoking, alcohol use, body mass index, physical activity and parental history of colorectal cancer. The *CYP7A1* polymorphism was not measurably related to the overall risk of colorectal adenomas. However, the *CC* genotype was associated with a decreased risk of proximal colon adenomas, but not of distal colon and rectal adenomas. Adjusted odds ratios of proximal colon adenomas (95% confidence intervals) for the *AC* and *CC* genotype versus *AA* genotype were 0.82 (0.54–1.24) and 0.56 (0.34–0.95), respectively. The findings add to evidence for the role of bile acids in colorectal carcinogenesis. The *CC* genotype of the *CYP7A1 A-203C* polymorphism probably renders lower activity of the enzyme synthesizing bile acids. (*Cancer Sci* 2006; 97: 406–410)

Bile acids have long been implicated in colorectal carcinogenesis.[1] Primary bile acids, such as cholic and chenodeoxycholic acids, are produced from cholesterol in the liver, and more than 95% of those passing through the ileum are reabsorbed and return to the liver. Secondary bile acids, mainly deoxycholic and lithocholic acids, are formed by the anaerobic bacterial flora in the large bowel from primary bile acids that escape absorption in the ileum.[1] Secondary bile acids are known to promote colorectal carcinogenesis in animals,[2,3] and molecular mechanisms have been found regarding the effect of bile acids promoting colorectal carcinogenesis.[4,5] However, epidemiological evidence remains elusive regarding the relationship between bile acids and colorectal cancer. Fecal concentrations of secondary bile acids are higher in populations at high risk of colorectal cancer.[6,7] Several case-control studies have shown that fecal or serum levels of secondary bile acids are higher in patients with colorectal cancer or adenoma than in those without these lesions.[8–11] A high ratio of serum deoxycholic acid to cholic acid tended to be associated with an increased risk of colorectal cancer in a prospective study.[12]

Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme that converts cholesterol into cholesterol 7α-hydroxycholesterol in the first step of the classical pathway of bile acid synthesis.[13] Overexpression of cholesterol 7α-hydroxylase activity in hamsters results in a dose-dependent decrease in plasma cholesterol concentrations.[14] In mice deficient in cholesterol 7α-hydroxylase, fecal excretion of bile acids as well as the bile acid pool is decreased.[15] In humans, a polymorphism in the promoter region of the *CYP7A1* gene (*CYP7A1 A-203C*) is associated with plasma concentrations of total or low density lipoprotein (LDL) cholesterol, suggesting lower enzyme activities in those with the *-203CC* genotype,[16,17] although this finding was not replicated in another study.[18] The *CYP7A1 A-203C* polymorphism may modulate transcription of the *CYP7A1* gene and consequently the rate of bile acid synthesis.

In a case-control study of colorectal cancer,[19] individuals with the *CYP7A1-203CC* genotype were associated with a lower risk of proximal colon cancer, but not of distal colon and rectal cancer. In the present study, we examined the relationship between the *CYP7A1 A-203C* polymorphism and colorectal adenomas, which is a well-established precursor lesion of colorectal cancer.[20,21]

Materials and Methods

Subjects

Study subjects were male self-defense officials who received a preretirement health examination at the Self Defense

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Forces (SDF) Fukuoka or Kumamoto Hospital from January 1997 to March 2001. The preretirement health examination is a program offering comprehensive medical examination for those retiring from the SDF. Details of the health examination and a lifestyle survey have been described elsewhere.[22,23] In addition to blood samples for routine use in the health examination, a sample of 7 mL fasting venous blood was obtained for the purpose of medical research with written informed consent. The study was approved by the ethical committee of Kyushu University.

The study subjects comprised 446 cases of colorectal adenomas and 914 controls of normal total colonoscopy. In the consecutive series of 2454 men, except for eight who refused to participate in the survey, 2377 (97%) underwent total or partial colonoscopy. We excluded 242 men with a prior history of colectomy ($n = 17$), colorectal polypectomy ($n = 212$), malignant neoplasm ($n = 27$) or inflammatory bowel disease ($n = 1$). Numbers of men according to the macroscopic findings of colonoscopy were: normal 1073, colorectal polyps 938, carcinoma one and other non-polyp benign lesions 123. Of the 938 with colorectal polyps, 461 were found to have at least one histologically confirmed adenoma without *in situ* or invasive carcinoma. Of the 1073 men with a normal result, 949 underwent total colonoscopy and were used as controls subjects. Of these cases and controls, DNA was not available for six cases and 13 controls, and genotyping was unsuccessful with nine cases and 22 controls, thus the remaining 446 cases and 914 controls were used in the analysis.

Numbers of adenoma cases by subsite were: proximal colon only 146, distal colon only 180, rectum only 42, and multiple sites 78. Proximal colon included cecum, ascending colon and transverse colon. Cases of adenoma with sizes of <5 mm, 5–10 mm and ≥10 mm (the largest size for multiple adenomas) numbered 260, 149 and 37, respectively.

Genotyping

DNA was extracted from buffy coat stored at −80°C by using a commercial kit (QIAGEN, Hilden, German). The *CYP7A1* genotype was determined by the polymerase chain reaction (PCR)–restriction fragment length polymorphism method, as described previously by Han *et al.*,[18] using primers 5′-AATGT TTTTC CCAGT TCTCT TTC-3′ (sense) and 5′-AATTA GCCAT TTGTT CATTC TATTA G-3′ (antisense). The PCR was carried out in a reaction mixture of 10 μL containing 0.5 IU of *Taq* and 1 μL of template DNA with a concentration of approximately 50–150 ng/μL. After the initial denaturation at 94°C for 4 min, 30 cycles of PCR were carried out for 30 s at 94°C, 30 s at 53°C and 30 s at 72°C, with a final extension at 72°C for 7 min. The PCR product of 393 bp was digested with 10 IU of *Bsa*I in a reaction mixture of 20 μL for 3 h at 50°C. The digestion resulted in fragments of 300 and 93 bp for the *A* allele, and fragments of 261, 93 and 39 bp for the *C* allele. The digested fragments were electrophoresed on a 3% agarose gel (NuSieve GTG), and visualized using ethidium bromide. The polymorphism was described as A-204C by Couture *et al.*,[17] but we confirmed, by sequencing, the actual site of the polymorphism at 203 bp upstream of the transcription start site. Genotyping was carried out with case and control status unknown.

Lifestyle factors

Height and bodyweight were recorded, and body mass index (kg/m^2) was calculated. Body mass index was categorized by using the 30th, 60th and 90th percentiles in the distribution of the controls. A self-administered questionnaire ascertained smoking habit, alcohol use, leisure-time physical activity and other lifestyle factors. Lifetime exposure to cigarette smoking was expressed as cigarette-years (the average number of cigarettes smoked per day multiplied by years of smoking), and classified into four levels of 0, 1–399, 400–799 and ≥800 cigarette-years. Alcohol drinking was defined as having drunk alcoholic beverages at least once per week for 1 year or longer, and former alcohol use was separated from lifetime non-use of alcohol. The amount of alcohol consumed per day was calculated for current alcohol drinkers on the basis of consumption frequencies and amounts per occasion of five types of alcoholic beverages on average in the past year. Alcohol use was categorized into never, former and current use with consumption of <30, 30–59, or ≥60 mL of ethanol per day.

Questions on leisure-time physical activity were slightly changed in April 1999. In the earlier version, subjects were first asked about the frequency of regular participation in exercise and sport during leisure time on average in the past year using a closed-ended question (none, 1–2, 3–4, 5–6 times per week and daily). If the subjects participated in recreational physical activity at least once per week, they reported type of activity and time spent per occasion regarding at most three types of regular exercise. In the revised questionnaire, the subjects were first asked whether they participated in recreational activity regularly (one or more times per week) in the past year. Those with a regular participation reported at most three types of physical activities together with frequency per week and time spent per occasion for each activity. Type of physical activity was classified into light, moderate, heavy or very heavy activity in terms of metabolic equivalent (MET).[24] The time spent in recreational exercise was multiplied by the corresponding MET value (light 2, moderate 4, heavy 6 and very heavy 8) to yield a MET-hour score per week. Individuals were classified into four groups with the quartiles in the control group as cut-off points. Parental history of colorectal cancer was also elicited.

Statistical analysis

The association between the *CYP7A1* polymorphism and colorectal adenomas was assessed by means of adjusted odds ratio (OR) and 95% confidence interval (CI), which were derived from multiple logistic regression analysis. Statistical adjustment was made for age (continuous variable), hospital, rank of the SDF (low, intermediate and high), cigarette smoking, alcohol use, body mass index, physical activity and parental colorectal cancer. Interactions of the *CYP7A1* polymorphism with selected lifestyle factors were evaluated by the likelihood ratio test. Statistical significance was declared if a two-sided *P*-value was less than 0.05 or if the 95% CI did not include unity. All statistical analyses were carried out using SAS version 8.2 (SAS Institute, Cary, NC, USA).

Results

The characteristics of colorectal adenoma cases and controls are summarized in Table 1. The age ranges were 50–57 years in the cases and 47–59 years in the controls, but the mean ages were identical in the two groups. Cigarette and alcohol consumption were greater in the cases than in the controls, and body mass index was also greater in the former group. Men with low physical activity and those with parental history of colorectal cancer were slightly more frequent in the cases.

In the cases, proportions of the *AA*, *AC* and *CC* genotypes were 26, 50 and 25%, respectively (Table 2). The corresponding proportions in the control group were 24, 49, and 27%, respectively. These frequencies were in agreement with the Hardy–Weinberg equilibrium ($P = 0.98$ for cases and $P = 0.95$ for controls). Overall, the *CYP7A1* polymorphism was not measurably associated with colorectal adenomas, although the OR for the *CC* genotype versus *AA* genotype was slightly lower than unity.

When the association with the *CYP7A1* polymorphism was examined for adenomas of the proximal colon, distal colon and rectum separately, the OR of proximal colon adenomas showed a statistically significant decrease among individuals with the *CC* genotype compared with those with the *AA* genotype. The *CYP7A1* polymorphism was unrelated to adenomas at the distal colon and rectum (Table 3).

Adjusted OR of small colorectal adenomas (<5 mm) for the *AC* and *CC* genotypes versus *AA* genotype were 1.11 (95% CI 0.78–1.58) and 0.87 (95% CI 0.58–1.32), respectively. The corresponding OR of large colorectal adenomas (≥5 mm) were 0.74 (95% CI 0.50–1.09) and 0.86 (95% CI 0.55–1.33), respectively. Adjusted OR of small proximal colon adenomas ($n = 86$) were 1.09 (95% CI 0.64–1.86) for the *AC* genotype and 0.41 (95% CI 0.20–0.87) for the *CC* genotype compared with the *AA* genotype, and adjusted OR of large proximal colon adenomas ($n = 60$) for the *AC* and *CC* genotypes were 0.52 (95% CI 0.28–0.98) and 0.71 (95% CI 0.36–1.41), respectively.

Finally, we explored interactions between the *CYP7A1* polymorphism (three genotypes) and lifestyle factors for the risk of proximal colon adenomas with two categories used for smoking (<400 cigarette-years vs ≥400 cigarette-years), alcohol use (<30 mL/day including past alcohol use versus ≥30 mL/day), body mass index (<25.0 kg/m^2 vs ≥25.0 kg/m^2), and physical activity (<15 MET-hours/week vs ≥15 MET-hours/week). There was no measurable interaction for any of the lifestyle factors under study: smoking ($P = 0.38$), alcohol use ($P = 0.93$), body mass index ($P = 0.62$) and physical activity ($P = 0.39$).

Table 1. Characteristics of colorectal adenoma cases and controls

Variable	Cases (n = 446)	Controls (n = 914)	P-value†
Age (years), mean (SD)	52.4 (0.83)	52.4 (0.92)	0.82
Hospital (%)			
Fukuoka	71.1	68.4	0.31
Kumamoto	28.9	31.6	
Rank (%)			
Low	60.3	62.3	0.68
Intermediate	25.3	23.2	
High	14.3	14.6	
Cigarette-years (%)			
0	21.3	34.1	<0.0001
1–399	14.3	19.1	
400–799	44.8	33.8	
≥800	19.5	12.9	
Alcohol use (%)			
None	11.2	14.6	0.0002
Past	2.9	3.1	
<30 (mL/day)	21.5	31.0	
30–59	34.1	28.8	
≥60	30.3	22.6	
BMI (kg/m^2), mean (SD)	24.1 (2.79)	23.7 (2.46)	0.007
MET-hours/week (%)			
<5	28.5	23.9	0.32
5–14	23.8	25.9	
15–24	24.2	24.9	
≥25	23.5	25.3	
Parental CRC (%)			
Negative‡	95.3	96.6	0.23
Positive	4.7	3.4	

†Based on t-test or χ^2- test. ‡Including two cases and two controls with parental colorectal cancer (CRC) unknown. BMI, body mass index; MET, metabolic equivalent; SD, standard deviation.

Table 2. *CYP7A1 A-203C* polymorphism and risk of colorectal adenoma

Genotype	Cases		Controls		Crude OR	Adjusted OR (95% CI)†
	n	%	n	%		
AA	115	25.8	219	24.0	1.00	1.00 (referent)
AC	221	49.6	452	49.5	0.93	0.93 (0.70–1.24)
CC	110	24.7	243	26.6	0.86	0.87 (0.62–1.20)

†Adjusted for age, hospital, rank, body mass index, cigarette smoking, alcohol use, physical activity and parental history of colorectal cancer. CI, confidence interval; OR, odds ratio.

Table 3. *CYP7A1 A-203C* polymorphism and risk of colorectal adenoma by location

Genotype	Proximal colon		Distal colon		Rectum	
	n†	OR (95% CI)‡	n†	OR (95% CI)‡	n†	OR (95% CI)‡
AA	44/219	1.00 (referent)	42/219	1.00 (referent)	10/219	1.00 (referent)
AC	73/452	0.82 (0.54–1.24)	86/452	1.01 (0.67–1.52)	23/452	1.07 (0.49–2.35)
CC	29/243	0.56 (0.34–0.95)	52/243	1.14 (0.72–1.80)	9/243	0.89 (0.35–2.31)

†Numbers of cases/controls. ‡Adjusted for age, hospital, rank, body mass index, cigarette smoking, alcohol use, physical activity and parental history of colorectal cancer. CI, confidence interval; OR, odds ratio.