Illumina Genome Analyser (Illumina, San Diego, CA, USA) (Ley *et al.*, 2008). All of our 11 somatic changes were confirmed by analysis of both genomic DNA and cDNA of the corresponding specimens with a capillary sequencer (data not shown). These data thus support the necessity of examining paired noncancerous specimens to pinpoint somatic changes in the cancer genome.

One of the gene mutations found only in the $CD34^+$ fractions results in a Met-to-Ile change at amino-acid position 511 of JAK3. A heterozygous *JAK3* mutation responsible for the amino-acid change was confirmed in both genomic DNA and cDNA from the $CD34^+$ fraction, but not in those from the corresponding $CD4^+$ fraction of patient ID JM07 (Supplementary Figure S3), who had *de novo* AML (M1 subtype) and a normal karyotype (Supplementary Table S1). In contrast to JAK2, activating mutations in which are preferentially associated with myeloproliferative disorder, several gain-of-function mutations (such as I87T, P132T, Q501H, A572V, R657Q and V722I) of JAK3 have recently been associated with acute megakaryoblastic leukemia of children (Walters *et al.*, 2006; Sato *et al.*, 2008). Other JAK3 mutations (such as A573V and A593T) were also identified in the same disorder, and an M576L substitution was detected in an adult with acute megakaryocytic leukemia (AML, M7 subtype) (Kiyoi *et al.*, 2007), although the transforming potential of these changes remains unknown.

Given that the M511I mutant of JAK3 has not previously been described and that the relevance of JAK3 to the pathogenesis of adult AML has not been extensively investigated, we first focused on the function of JAK3(M511I). The M511 residue is located in the linker region between the Src homology 2 (SH2) domain and the pseudokinase domain of JAK3 (Figure 2a). The transforming mutation Q501H that is associated with juvenile acute megakaryoblastic leukemia (Sato *et al.*, 2008) is also located in this region. Given that JAK3 is abundant in and has an essential role in the development of lymphocytes (Russell *et al.*, 1995), we examined the expression level of *JAK3* in AML blasts. The gene was expressed at a high level in most AML specimens ($n = 52$), with its expression level being greater than that of *JAK2* in all but three cases (Supplementary Figure S3).

To examine the transforming potential of JAK3(M511I), we introduced the mutant or wild-type protein into the interleukin-3 (IL-3)-dependent mouse cell line 32D (Greenberger *et al.*, 1983). Although 32D cells forced to express wild-type JAK3 underwent rapid apoptosis after withdrawal of IL-3, those expressing JAK3(M511I) continued to grow even in the absence of IL-3, although at a reduced rate compared with that of cells expressing the artificially generated, highly transforming mutant JAK3(V674A) (Choi *et al.*, 2007) (Supplementary Figure S3). 32D cells differentiate into terminal granulocytes in the presence of granulocyte colony-stimulating factor. However, cells expressing the M511I or V674A mutant of JAK3 maintained an exponential rate of growth, without any sign of differentiation, in the presence of granulocyte colony-stimulating factor (Figure 2b, Supplementary Figure S3), supporting the notion that the M511I mutant has transforming potential.

To directly examine the leukemogenic activity of JAK3(M511I), we generated a recombinant retrovirus encoding this mutant and used it to infect murine hematopoietic stem cells. Reconstitution of the bone marrow of lethally irradiated mice with such infected cells resulted in marked lymphocytosis in peripheral blood and enlargement of the spleen in the recipient animals (Figure 2c). The cells in the peripheral blood, spleen and bone marrow of the recipients responsible for these phenotypes manifested a medium-sized, blastic morphology, and flow cytometric analysis revealed them to be $CD8^+$ T cells (Figure 2c). The clonal nature of these proliferating T cells was further confirmed by Southern blot analysis (Supplementary Figure S4), indicative of the development of T-cell acute lymphoblastic leukemia in the recipient mice.

To assess the prevalence of *JAK3* mutations in adult leukemia, we further examined the nucleotide sequence of the entire coding region of *JAK3* cDNA in an additional 266 specimens of leukemic blasts. The coding region of *JAK3* cDNA was successfully amplified by PCR from 83 specimens. We could further identify 4 distinct *JAK3* sequence changes in 8 of these 83 samples: 1 case with G62S, 4 cases with Q501H, 2 cases with R657Q and 1 case with R918C (Figure 2a). Taking into account the 20 cases evaluated in the phase I analysis, we thus identified a total of 9 cases with a mutant form of JAK3 (3.1%) among 286 cases of leukemia (Supplementary Table S3). Our identification of known transforming JAK3 mutants (Q501H and R657Q) originally associated with acute megakaryoblastic leukemia prompted us to determine the prevalence of these two changes in another cohort of AML ($n = 148$), revealing two cases with JAK3(Q501H) and one case with JAK3(R657Q). In addition, analysis of a hematopoietic cell line (KCL22) (Kubonishi and Miyoshi, 1983) established from a patient with chronic myeloid leukemia in BC revealed yet another mutation (L1017M) of JAK3 (Figure 2a).

To directly compare the transforming potential of these various JAK3 mutants, we introduced each protein into the IL-3-dependent mouse B-cell line BA/F3 and examined the growth properties of the resulting transfectants. Whereas all cells expressing the JAK3 mutants proliferated in a similar manner in the presence of IL-3 (data not shown), culture without IL-3 revealed marked differences in the transforming potential among the mutants. JAK3(M511I) was the most efficient oncokinase, with a transforming activity similar to that of JAK3(V674A). The frequent mutants JAK3(Q501H) and JAK3(R657Q) exhibited weaker but still pronounced transforming potential, whereas the remaining mutants (G62S, R918C and L1017M) showed an even lower potential (Figure 2d).

Somatic mutations of DNMT3A

Another somatic mutation identified in the phase II data set was a heterozygous change in *DNMT3A* that results

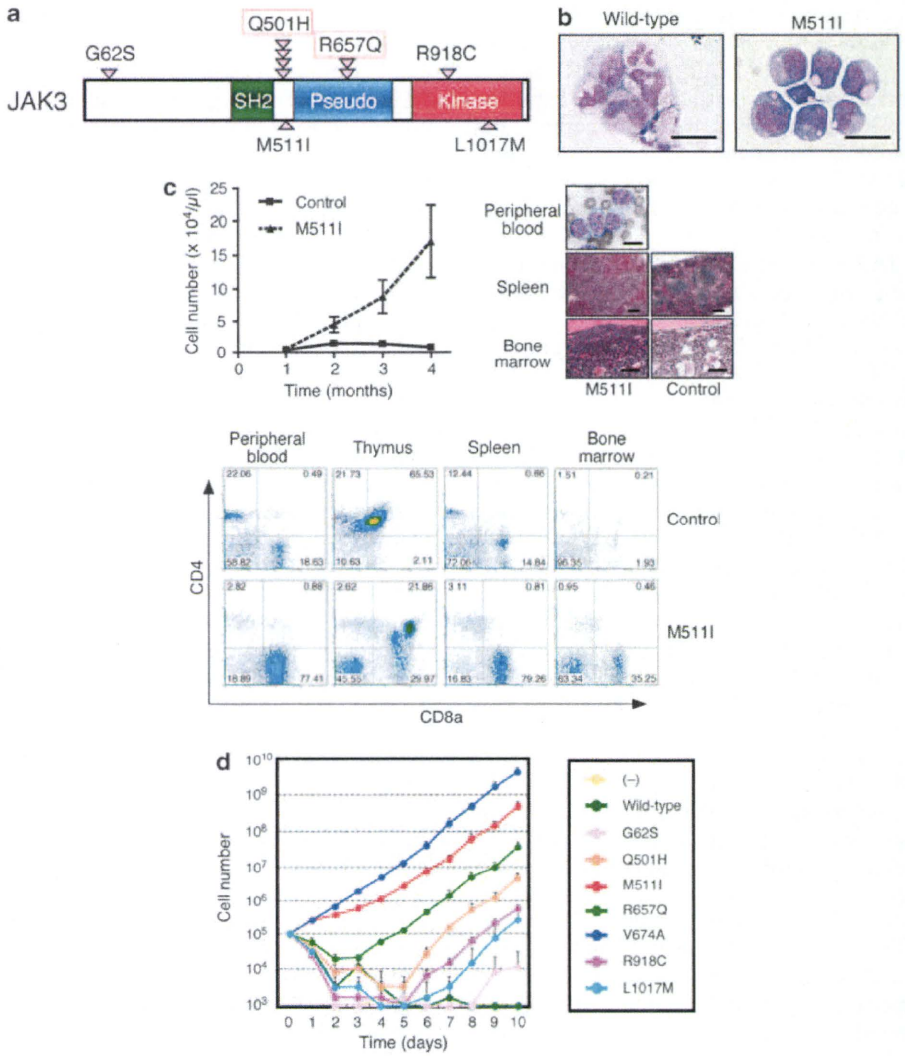

Figure 2 Identification of JAK3 mutants in leukemia. (**a**) Amino-acid substitutions detected in this study are shown relative to the domain organization of JAK3. The mutations M511I (one case) and Q501H (four cases) are located in the linker region between the SH2 and pseudokinase domains of JAK3, whereas G62S (one case), R657Q (two cases) and R918C (one case) are located in the amino-terminal region, the pseudokinase domain and the kinase domain, respectively. The KCL22 cell line also harbors an L1017M mutation within the kinase domain of JAK3. Previously known activating mutations of JAK3 (Q501H and R657Q) are indicated by red rectangles. (**b**) Mouse 32D cells expressing wild-type human JAK3 or the JAK3(M511I) mutant were incubated with G-CSF (0.5 ng/ml) for 14 days, stained with Wright–Giemsa solution and examined by light microscopy. Scale bars, 20 μm. (**c**) C57BL/6 mice were irradiated and then injected intravenously with syngeneic CD34$^-$KSL hematopoietic stem cells infected with a retrovirus encoding JAK3(M511I) or the corresponding empty virus (control). The number of white blood cells in peripheral blood was counted at the indicated times thereafter; data are means ± s.d. for 10 mice in each group (upper left panel). Peripheral blood, spleen and bone marrow isolated from recipient mice 3 months after cell injection were stained with the Wright–Giemsa solution (peripheral blood) or hematoxylin–eosin (spleen and bone marrow) and were then examined by light microscopy (upper right panel); scale bars represent 10, 200 and 50 μm, respectively. Mononuclear cells isolated from peripheral blood, thymus, spleen and bone marrow of recipient mice 3 months after cell injection were subjected to flow cytometric analysis of surface expression of CD4 and CD8a (lower panel). (**d**) Control BA/F3 cells (–) or those expressing the indicated JAK3 mutants were cultured without IL-3 for the indicated times, after which the cell number was determined. Data are means + s.d. of triplicates from a representative experiment.

in an R882H substitution in the encoded protein (Figure 3a, Supplementary Figure S5). DNMT3A, together with DNMT3B, has an essential role in *de novo* methylation of the human genome (Okano *et al*., 1999), and an aberrant methylation profile (hypermethylation of CpG islands and hypomethylation of other regions) is a hallmark of cancer cells (El-Osta, 2004). Despite a direct linkage between such methylation changes and silencing of tumor-suppressor genes in cancer, the molecular mechanism responsible for such abnormal methylation remains unknown. Our data thus provide the first evidence of somatic mutation of a DNA methyltransferase gene in cancer cells. Mutations in the catalytic domain of DNMT3B have been shown to be

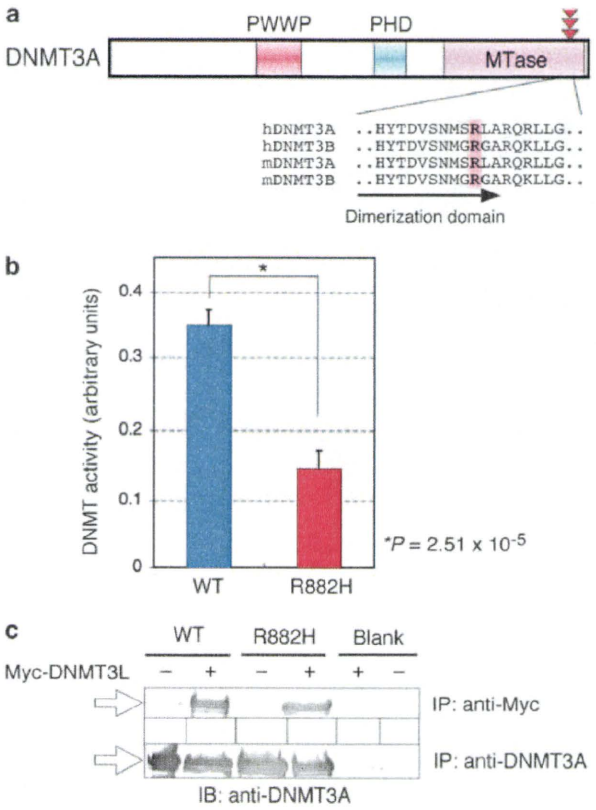

Figure 3 Identification of a DNMT3A mutant in leukemia. (**a**) Domain organization of human DNMT3A showing that the R882 residue found to be mutated in leukemia is conserved among human (h) and mouse (m) members of the DNMT3 family. DNMT3A contains a tetrapeptide PWWP domain, polybromo homology domain (PHD) and methyltransferase (MTase) domain. The R882 residue is located in the homodimerization region present within the MTase domain. (**b**) Wild-type (WT) and R882H forms of DNMT3A were expressed in and purified from insect cells and then subjected to an *in vitro* assay of methyltransferase activity. Data are means + s.d. of triplicates from a representative experiment. The *P*-value was determined by Student's *t*-test. (**c**) Lysates of HEK293 cells expressing Myc epitope-tagged DNMT3L and wild-type or R882H forms of DNMT3A, as indicated, were subjected to immunoprecipitation (IP) with antibodies to Myc or to DNMT3A, and the resulting precipitates were subjected to immunoblot analysis (IB) with antibodies to DNMT3A. The position of DNMT3A (wild-type or mutant) is indicated by an open arrow.

responsible for a hereditary syndrome characterized by ICF (immunodeficiency, instability of the centromeric region of chromosomes and facial anomalies) in humans (Ehrlich, 2003). One of the mutation sites of DNMT3B (R823) associated with the ICF syndrome corresponds to the residue of DNMT3A (R882) shown to be mutated in this study.

The R882 residue of DNMT3A is considered to participate in the homodimerization and activation of the protein (Jia *et al.*, 2007) (Figure 3a). To determine whether the R882H mutation affects the catalytic activity of DNMT3A, we expressed mutant and wild-type proteins separately in insect cells, purified them to near homogeneity and subjected them to an *in vitro* assay of methyltransferase activity with a synthetic substrate (Suetake *et al.*, 2003). The catalytic activity of DNMT3A(R882H) was $<50\%$ of that of the wild-type protein (Figure 3b). DNMT3L acts as a coactivator for the methyltransferase activity of DNMT3A or DNMT3B through its association with the latter proteins (Jia *et al.*, 2007). The R882H mutation did not affect the interaction of DNMT3A with DNMT3L in transfected mammalian cells (Figure 3c) or its sensitivity to DNMT3L as examined by the *in vitro* assay of methyltransferase activity (data not shown). These data thus suggested that the R882H mutation directly inhibits the enzymatic activity of DNMT3A.

Screening of another cohort of leukemia cases ($n=54$) for mutant forms of *DNMT3A* revealed another two patients with a mutation of the same amino acid (R882H in one patient and R882C in the other) (Supplementary Table S4). Therefore, we identified a total of 3 cases with an R882 mutation (4.1%) among 74 cases of leukemia. Screening for mutations of *DNMT3B* failed to detect any somatic changes in the same individuals (data not shown), suggesting that *DNMT3A* is a preferential target in leukemia.

Multistep transformation in leukemia

Although $>99\%$ of nucleotide changes in the phase I data were also observed in the paired CD4$^+$ cells, it is unlikely that all of these changes are actually germline polymorphisms because they include established oncogenic mutations. They thus include 190 nucleotide changes previously described in cancer cells (Supplementary Table S5), such as those giving rise to NRAS(Q61H) in patient ID JM17 and to FLT3(D835Y) in patient ID JM08 (Figure 4a). Given that both NRAS(Q61H) and FLT3(D835Y) are well-characterized oncoproteins (Yamamoto *et al.*, 2001), it is unlikely that these individuals harbored such nucleotide changes in the germ line. There are at least two possible explanations for these findings. First, it is possible that purification of the CD4$^+$ fraction was not efficient, with the result that this fraction was contaminated by CD34$^+$ cells. However, the *CD4* expression ratio for the CD4$^+$ and CD34$^+$ fractions of each individual was $\geqslant 17.1$ (median $=40.1$) (Figure 4b), and contamination of the CD4$^+$ fraction with CD34$^+$ cells at such a level would not likely produce detectable changes in Sanger sequencing outputs (compare, for instance, the signal intensities of the normal and mutant alleles in Figure 4a).

Furthermore, although CD4 expression has been occasionally observed in AML blasts (Schwonzen *et al.*, 2007), quantitation of *CD4* and *CD34* mRNA within our purified CD34$^+$ fractions failed to detect a significant level of the former message in the blasts (Supplementary Figure S6). Therefore, it is unlikely that contamination of CD4$^+$ leukemic blasts within the purified, control CD4$^+$ fraction substantially affected the sequencing results in our phase II experiment.

Rather, it is more likely that leukemia may develop in a stepwise manner with a substantial time interval

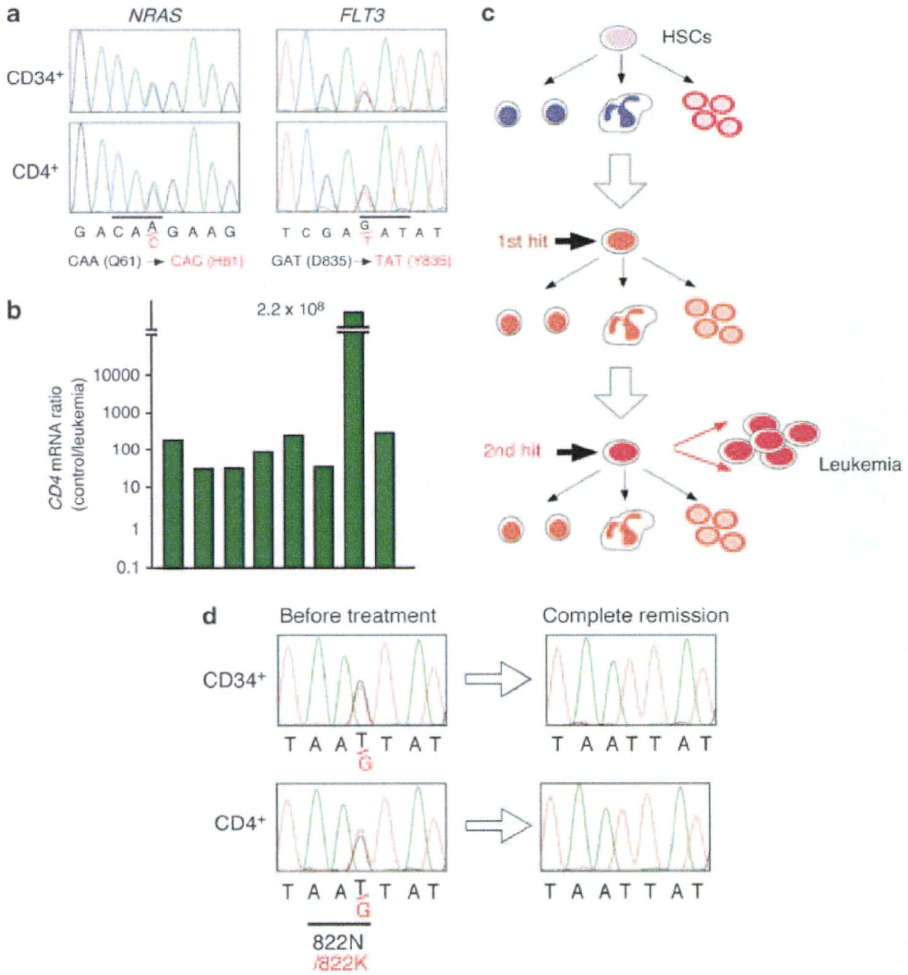

Figure 4 Proposed stepwise nature of leukemogenesis. (**a**) Sequencing electrophoretograms for the regions surrounding codon 61 of *NRAS* or codon 835 of *FLT3* in genomic DNA from the CD34$^+$ and CD4$^+$ fractions of patient IDs JM17 and JM08, respectively. Heterozygous nucleotide changes that give rise to NRAS(Q61H) or FLT3(D835Y) were detected in both fractions of the corresponding patients. (**b**) The amount of *CD4* mRNA in the CD4$^+$ (control) and CD34$^+$ (leukemia) fractions of leukemia patients (with a substantial amount of control *GAPDH* mRNA) was quantitated by reverse transcription and real-time PCR analysis and expressed as the control/leukemia ratio. (**c**) Hematopoietic stem cells (HSCs) give rise to a wide range of mature blood cells. Even after the first hit (mutation) of the genome, HSCs retain their full differentiation capacity, and therefore produce differentiated cells harboring this first hit. After the second hit, the affected cell fraction undergoes full transformation to leukemia. (**d**) Sequencing electrophoretograms for the genome of CD34$^+$ and CD4$^+$ fractions from patient ID JM03 showing a heterozygous mutation for KIT(N822K) before chemotherapy but not after.

between steps (Figure 4c). If a first hit occurs in the genome of hematopoietic stem (or progenitor) cells and if such a somatic change does not result directly in the generation of full-blown leukemia, the preleukemic clones may give rise to terminally differentiated blood cells (including CD4$^+$ cells). After a certain period, a second (or possibly a third) hit occurs in the immature cells and triggers the rapid growth of leukemic clones without differentiation. In such a scenario, terminally differentiated 'normal' cells may still harbor the first hit in their genome.

Support for this latter possibility was provided by patient ID JM03, who had AML (M2 subtype) with a t(8;21) chromosome anomaly. Before chemotherapy, the genomic DNA of both CD34$^+$ and CD4$^+$ fractions from this patient harbored a heterozygous mutation of *KIT* that results in the production of a constitutively activated mutant protein, KIT(N822K) (Shimada *et al.*, 2006) (Figure 4d). The same change was also detected in cDNA prepared from the CD34$^+$ fraction (data not shown). Leukemic blasts in this patient were sensitive to standard chemotherapeutic regimens, and the patient underwent complete remission. Examination of CD34$^+$ and CD4$^+$ fractions obtained during the remission period revealed that the N822K codon change was no longer detectable not only in the CD34$^+$ fraction but also in the CD4$^+$ fraction (Figure 4d). These data thus support the scenario shown in Figure 4c: The N822K

change represents the first hit and was present in differentiated blood cells, and the corresponding pre-leukemic clones were simultaneously eradicated together with the leukemic clones by chemotherapy.

On the other hand, as shown in Supplementary Tables S1 and S2, a heterozygous mutation for NRAS(G12S) was found only in the $CD34^+$ fraction, but not in the $CD4^+$ fraction of the patient ID JM16. Conventional chemotherapy for this patient eradicated the leukemic blasts carrying the mutation (Supplementary Figure S7), also confirming that a successful treatment results in the disappearance of cells with a (possible) 'second hit'.

Our hypothesis of the stepwise leukemogenesis is also consistent with the previous detection of the *RUNX1-CBFA2T1* oncogene in differentiated blood cells (Kwong *et al.*, 1996; Miyamoto *et al.*, 1996, 2000).

Discussion

Our large-scale genomic resequencing of human leukemia specimens with DNA microarrays has identified recurrent nucleotide changes responsible for the generation of JAK3 and DNMT3A mutants. Whereas JAK3 mutants were unexpectedly found in adult AML, their transforming ability, and possibly their contribution to leukemogenesis, varied substantially. However, our bone marrow transplantation experiments showed that at least one of these JAK3 mutants (M511I) directly participates in the development of leukemia. Identification of the M511I mutation of JAK3 in the leukemic fraction but not in the control fraction of patient ID JM07 suggests that this mutation may be the second hit triggering AML. Given that the blasts of this patient had a normal karyotype, it is likely that the first hit is present in the genome of both fractions. Karyotyping of other patients with *JAK3* mutations showed a total of three cases with a normal karyotype, one case with t(8;21), and one case with a numerical anomaly of several chromosomes (Supplementary Table S3), suggesting that *JAK3* mutations may be preferentially associated with leukemia with a normal karyotype.

Although JAK3(M511I) was identified in AML, our bone marrow transplantation experiments with hematopoietic stem cells expressing this mutant yielded T-cell acute lymphoblastic leukemia. In contrast to human leukemia, in which JAK3 changes may constitute a second hit (probably in progenitor cells), JAK3(M511I) may have been expressed in all hematopoietic cells of the recipient mice. JAK3(M511I) thus likely triggered leukemia within a T-cell fraction the intracellular context of which is optimized for JAK3 signaling.

It has been frequently observed that transgenic mouse or bone marrow transplantation experiments for leukemic oncogenes do not accurately recapitulate the original leukemia subtypes (Wong and Witte, 2001). Transgenic mice expressing $p210^{BCR\text{-}ABL1}$, for instance, usually develop T-cell lymphoma or acute lymphoblastic leukemia, not chronic myeloid leukemia. Furthermore, bone marrow transplantation with hematopoietic progenitor cells expressing $p210^{BCR\text{-}ABL1}$ often leads to development of lymphoma, AML, acute lymphoblastic leukemia or macrophage tumors. Generation of malignancy in such systems may, thus, be elaborately influenced by mouse strains, promoter fragments for artificial expression and/or cell types to be used for gene transduction.

Our detection of recurrent DNMT3A hypomorphic mutations in leukemia clones may indicate the presence of an abnormal methylation profile in the genome of such blasts. However, given the limited amount of the specimens available, we were able to investigate microsatellite stability only at certain loci (Koinuma *et al.*, 2005), revealing no apparent microsatellite instability (data not shown). We also generated BA/F3 cells expressing wild-type or R882H forms of DNMT3A to compare the methylation status of some CpG islands in the genome; again, we detected no discernable differences between the two cell preparations (data not shown). However, given that BA/F3 cells contained two copies of wild-type *Dnmt3a* in addition to multiple copies of mutant *DNMT3A*, whereas the leukemic blasts likely harbor one copy each of the wild-type and mutant *DNMT3A* alleles, the clinical relevance of the R882 mutant requires further examination under the latter condition. Cell proliferation/differentiation is indeed influenced substantially by the copy number of *DNMT3* genes (Okano *et al.*, 1999; Ehrlich, 2003).

Our observations indicate the importance of preparing paired normal fractions in large-scale resequencing projects, but they also reveal a difficulty in the preparation of *bona fide* 'normal' fractions in the case of leukemic disorders. Our data thus indicate that nonleukemic blood cells may harbor early genomic hits, rendering them inappropriate as controls. Furthermore, a substantial proportion of fingernail DNA was recently shown to be derived from donor cells among recipients of allogeneic stem cell transplants (Imanishi *et al.*, 2007), indicating that nonblood cells may contain DNA derived from transplanted cells. Therefore, it is possible that buccal, fingernail or even hair cells may not be suitable as normal cell controls. In contrast to solid tumors, for which blood cells are appropriate as paired normal fractions, leukemic disorders require that caution be taken to discriminate somatic nucleotide changes from germline polymorphisms.

Materials and methods

Wafer sequencing

$CD34^+$ and $CD4^+$ fractions were isolated from leukemic individuals using CD34microbeads and CD4microbeads, respectively, and a MidiMACS separator (Miltenyi Biotec, Gladbach, Germany). All clinical specimens were obtained with written informed consent, and the study was approved by the ethics committees of both the Jichi Medical University and the Nagasaki University. DNA sequencing wafers were designed and processed at Perlegen Sciences. Genes to be interrogated on the wafers were selected from the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene)

by searching with various keywords characteristic to each subcategory (such as DNA repair, regulation of chromatin structure, etc.), followed by manual inspection. The final gene list for the wafers is shown in Supplementary Table S6. Construction of the wafers, quality control analysis and data processing are described in Supplementary Text.

JAK3 analysis

Complementary DNAs for JAK3 mutants were generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and ligated into the pMX retroviral vector (Onishi *et al.*, 1996). Ecotropic recombinant retroviruses encoding each mutant were produced in BOSC23 cells transfected with the corresponding pMX-based plasmid and were used to infect BA/F3 or 32D cells as described previously (Choi *et al.*, 2007). Both types of cell were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (both from Life Technologies, Carlsbad, CA, USA) and mouse IL-3 (Sigma, St Loius, MO, USA) at 10 Units/ml; differentiation of 32D cells was induced by culture in the presence of serum and mouse granulocyte colony-stimulating factor (Sigma) at 0.5 ng/ml. A concentrated preparation of a retrovirus with a VSV-G envelope and encoding both JAK3(M511I) and enhanced green fluorescent protein was used to infect $CD34^-$ $c\text{-}Kit^+$ $Sca\text{-}1^+$ $Lineage\text{-}marker^-$ (CD34 KSL) hematopoietic stem cells isolated from the bone marrow of C57BL/6 mice, and the infected cells were transplanted into lethally irradiated mice congenic for the *Ly5* locus (Iwama *et al.*, 2004). *CD4*, *JAK2* and *JAK3* mRNAs were quantitated by reverse transcription and real-time PCR analysis using an ABI7900HT system (Life Technologies) and with the primers 5′-CTGGAATCCAACATCAAGGTTCTG-3′ and 5′-AATTGTAGAGGAGGCGAACAGGAG-3′ for CD4, 5′-CTCCAGAATCACTGACAGAGAGCA-3′ and 5′-CCAC TCGAAGAGCTAGATCCCTAA-3′ for *JAK2* and 5′-GAGC TCTTCACCTACTGCGACAAA-3′ and 5′-AGCTATGAAA AGGACAGGGAGTGG-3′ for JAK3; the cDNA for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was also amplified with the primers 5′-GTCAGTGGTGGACC TGACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′. The relative abundance of the cDNAs of interest was calculated from the threshold cycle (C_T) for each cDNA and that for *GAPDH* cDNA.

DNMT3A analysis

Recombinant His_6-tagged DNMT3A or DNMT3A(R882H) was expressed in SF9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA), and each protein was purified by stepwise column chromatography as described previously (Suetake *et al.*, 2003). The enzymatic activity of each protein was assayed with -*S*-adenosyl-L-methionine (GE Healthcare, Waukesha, WI, USA) and dIdC or dGdC as substrates (Suetake *et al.*, 2003). The association between Myc epitope-tagged human DNMT3L and wild-type or R882H forms of human DNMT3A in transfected HEK293 cells was examined by immunoprecipitation and immunoblot analyses.

Conflict of interest

The authors declare no conflict of interest.

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Molecular analysis of gastric differentiated-type intramucosal and submucosal cancers

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Carcinogenesis

Identification of the molecular characteristics of intramucosal (IMCs) and submucosal cancers (SMCs) is essential to our understanding of early gastric carcinogenesis. However, little is known regarding the differences between the 2 lesions. One hundred and forty-eight patients with primary early gastric cancer [IMC, 106; SMC, 42] were characterized for expression of cell cycle-related proteins and loss of heterozygosity (LOH). We also examined microsatellite instability (MSI) and methylation status. For LOH and methylation studies, we used a panel of 17 microsatellite markers (3p, 4p, 5q, 9p, 13q, 17p, 18q and 22q) and promoter regions of 9 genes (*MLH-1*, *RUNX3*, *p16*, *HPP1*, *RASSF2A*, *SFRP1*, *DKK-1*, *ZFP64* and *SALL4*) that are frequently altered or methylated in gastric cancers. Overexpression of p53 and cyclin D1 was observed in SMC. In addition, low expression of p27 was more frequent in SMC than in IMC. Frequencies of 4p, 9p, 13q and 22q were significantly higher in SMC than in IMC. The *SALL4* gene was frequently methylated in SMC compared with IMC. However, other gene methylations were common in both IMC and SMC. The frequency of LOH-high status/methylation-low status was significantly higher in SMC than in IMC. However, LOH-low status/methylation-high status in SMC was more frequently found in IMC. Our data confirm that methylation of cancer-related genes plays a major role in the development of IMCs. Importantly, the results also show that gastric submucosal progression is characterized by the accumulation of specific genetic alterations. In addition, changes of cell cycle-related proteins are associated with cancer progression.

Gastric adenocarcinoma is one of the most commonly diagnosed malignancies worldwide and is a leading cause of cancer mortality in Japan, Korea and South America. Although human tumor development has been analyzed at the molecular level,[1,2] the underlying molecular alterations that drive the neoplastic process in gastric cancers are not understood. In human tumors, impairment of the cell cycle is likely a critical mechanism underlying tumor development. G1 cyclins (cyclins D1 and A) and cyclin-dependent kinase (CDK) complexes play important roles in the transition through the G1 phase of the cell cycle, and their overexpression is implicated in neoplasia.[3–6] CDK inhibitors, including p27 and p21, negatively regulate G1 progression by binding to G1 cyclins/CDK complexes, thus inhibiting their activity and thereby preventing entry into the cell cycle.[3–5] In addition, nuclear accumulation of β-catenin plays an essential role in cell cycle progression as it induces cyclin D1, c-myc and MMP7.[7,8,9] Analysis of cell cycle-related protein expression is important for early gastric cancers (EGCs) to elucidate the mechanisms behind early gastric carcinogenesis.[3,4]

Many laboratories have studied genomic instability and the resulting allelic imbalance in gastric carcinomas through analyses of loss of heterozygosity (LOH) and comparative genomic hybridization.[10,11] Alterations most commonly found in gastric carcinogenesis include LOHs at 3p, 5q, 9p, 13q, 17p, 18q and 22q.[12–14] Thus, LOHs are useful markers for defining tumor aggressiveness. Accumulation of tumor LOHs (LOH-high status) is closely associated with tumor progression or tumor-invasive ability. Epigenetic aberrations are mechanistically important in human carcinogenesis.[15] A number of tumor suppressor genes are silenced by promoter methylation during gastric cancer development. Some gastric cancers undergo promoter methylation, which is referred to as the CpG island methylator phenotype (CIMP, methylation status).[15,16] In general, methylation-high (CIMP-high) tumors have distinct features, such as favorable tumor location (proximal location), greater predilection for females and specific genetic alterations (BRAF mutation and low incidence of

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Table 1. Clinicopathological findings of early gastric cancers

	Intramucosal cancer (%)	Submucosal cancer (%)
Total	106	42
Sex (man/woman)	70/36	32/10
Age (mean)	49–86 (71.6)	61–90 (73.6)
Size (mm, mean)	4–80 (17.1)	6–59 (21.8)
Location (P/D)	12/94	11/31
Macroscopic type		
Elevated type	73 (68.9)	18 (42.8)
Mixed type	7 (6.6)	12 (28.6)
Depressed type	26 (24.5)	12 (28.6)
Histological type		
WDA	94 (88.7)	28 (66.7)
MDA	9 (8.5)	13 (31.0)
PA	3 (2.8)	1 (2.3)
Histological grade		
Low	11 (10.3)	0 (0)
Intermediate	60 (56.7)	12 (28.6)
High	35 (33.0)	30 (71.4)

p53 alteration).[17] In addition, microsatellite instability (MSI) defines a novel molecular subtype of tumors.[17,18] In previous studies, MSI detected different changes compared with those observed in LOH-high status tissue. MSI overlaps with CIMP status (methylation-high status) in terms of clinicopathological and molecular features.[17–19] A molecular classification based on LOH status, methylation status and MSI is increasingly important in gastric carcinogenesis, because those alterations reflect global genomic or epigenetic aberrations.[15,17,18]

EGC is defined as a tumor that may invade into but is confined to the submucosa, irrespective of the presence of lymph node metastases.[19] Analysis of molecular alterations in EGC is important for understanding initial events in early gastric carcinogenesis. EGC is subclassified into intramucosal (IMCs) and submucosal (SMCs) cancers.[19] SMC could be regarded as an intermediate stage between IMC and advanced cancer. We hypothesize that submucosal tumors possess distinct molecular alterations compared with IMCs and that those alterations are critical for tumor progression. Thus, identifying the molecular differences between IMCs and SMCs is essential for understanding gastric carcinogenesis. In the present study, we attempted to identify these molecular differences.

Material and Methods

Patients

Detailed clinicopathological data derived from 106 IMCs and 42 SMCs are summarized in Table 1. The criteria used to diagnose IMC were based on Japanese histological criteria,[20] which differ from those used by western pathologists.[21,22]

Table 2. List of antibodies that we used

Antibody	Clone	Supplier	Dilution
p53	DO-7	Novocastra	1:100
p21	SX118	Dako	1:25
p27	SX53G8	Dako	1:50
β-Catenin	BD	Transduction Laboratories	1:200
Cyclin D1	SP4	Nichirei	1:100
Cyclin A	6E6	Novocastra	1:100
MLH-1	G168-15	BD Pharmingen	1:20
MMP7	141-7B2	Daiichi Finechemical	1:100
Ki-67	MIB1	Dako	1:100
MUC2	Ccp58	Novocastra	1:100
CD10	56C6	Novocastra	1:100
MUC5AC	CLH2	Novocastra	1:100
MUC6	CLH5	Novocastra	1:100

The nuclear grade was determined according to published criteria.[14]

For molecular investigations, tumor tissue was isolated from the resected stomach [samples from endoscopic submucosal dissection (ESD): 106 IMCs and 9 SMCs; samples from gastrectomy: 33 SMCs] using biopsy forceps within 30 min of resection. Most SMCs were obtained from gastrectomy, given that those SMCs were not an indication for ESD. The most distant normal gastric mucosa from the neoplasm was removed from the submucosa using scissors as a control for molecular analysis. Tissue for clinicopathological analysis was obtained from a region of the resected stomach adjacent to the region used for genetic analysis. All of the noncontrol tissue samples contained cancer tissue. Only tumor samples where the neoplastic cells accounted for at least 50% of the tissue cell population were selected (13 of 161 tumors were omitted due to failure to meet these criteria). In the SMCs, only tumor samples obtained from submucosal lesions were used for molecular analysis.

Immunohistochemical procedure

EGC specimens were fixed in buffered formalin and embedded in paraffin, according to routine procedures. For this study, 3-μm sections were prepared, dried, deparaffinized and rehydrated before microwave treatment (H2500, Microwave Processor, Bio Rad) in citrate buffer (pH 6.0) for 5 min. An automatic staining machine (DAKO Envision+ system) was used for the immunohistochemical procedure.[14] The slides were counterstained in hematoxylin, dehydrated and mounted. The antibody sources used in this study are shown in Table 2.

Immunohistochemical assessment

Nuclear immunostaining data for cyclin D1, cyclin A, p21, p27, p53, β-catenin, MLH-1 and ki-67 were expressed as the percentage of positive epithelial cells in relation to the total

number of cells encountered in at least 5–10 representative high-power fields (500–1,000 epithelial cells). The immunoreactivity was measured by means of light microscopic examination and evaluated independently by 2 experienced pathologists. (T.S. and N.U., not included in this manuscript). Differences in interpretation were reconciled by reviewing the slides separately. No necrosis was found in the tissue examined. Only nuclear staining was regarded as significant, and tumor cells showed a range of staining intensities. Cytoplasmic staining was disregarded. Tumor cells with weak staining were not considered positive.

Cutoffs for positive expression of p21 and p27 were defined as >10%, while for cyclin D1 and cyclin A, positivity was >30% as conducted by previous studies.[6,23,24] In the evaluation of p53 overexpression, samples showing >10% staining were considered positive.[14] More than 20% of cells showing β-catenin nuclear expression were regarded as positive.[25] Expression of MLH-1 was interpreted and compared with the surrounding tissue. If expression of MLH-1 in the tumor cell was considered to be low compared with its surrounding nonneoplastic tissue, the tumor tissue was regarded as low/negative staining. Finally, ki-67 staining >30% was judged as a highly proliferative tumor.

Mucin phenotypes of tumor cells were subclassified as follows: gastric, intestinal, mixed and unclassified, according to previous criteria.[14] In brief, the gastric phenotype included tumors having predominantly intracytoplasmic expression of gastric mucin, as determined by immunostaining of human gastric mucin (MUC5AC) or (and) pyloric gland mucin (MUC6), but with no MUC-2 positive cells. The intestinal phenotype was constituted by MUC2-positive cells or (and) CD10-positive cells (along with a brush border). The mixed-type contained immunostaining features of both gastric type as well as intestinal type. Finally, tumors lacking gastric or intestinal staining patterns were termed "unclassified." Immunopositivity of >5% of the tumor cells was regarded as positive according to the guidelines established by a previous report.[26]

PCR analysis

PCR reactions were performed using a thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer, CA) with 50–100 ng of genomic DNA as template, 25 pM of each primer, 0.2 mM deoxynucleotide triphosphate (dNTP), 1× reaction buffer containing 1.5 mM $MgCl_2$, and 1.5 u Taq polymerase (Boehringer Mannheim Co., Germany) in a final reaction volume of 25 μL. Samples were processed for 25–30 cycles, with each cycle consisting of 30 sec at 94°C, 1 min at 55–58°C and 2 min at 72°C, followed by a final 10-min extension at 72°C. For quantitative detection of the allelic loss at each locus, PCR-LOH analysis and MSI were performed as described previously.[27,28] A 1-μL aliquot of the PCR product was added to 3 μL formamide and a 0.5 μL TAMRA 500 size standard (Applied Biosystems, CA), loaded on a 6% polyacrylamide-8 M urea gel, and run for 2–6 hr in a 373A Automated Sequencer (Applied Biosystems) at a constant power of 30 W.

Assessment of LOH by polymerase chain reaction

Allelic losses on chromosomes 3p, 4p, 5q, 9p, 13q, 17p, 18p and 22q were examined in paired tumor and normal DNA samples obtained from 148 EGC patients using 17 highly pleomorphic microsatellite markers (D3S2402, D3S1234, D4S2639, D4S1601, D5S107, D5S346, D5S299, D5S82, D9S171, D9S1118, D13S162, TP53, D18S487, D18S34, D22S274, D22S1140 and D22S1168). These microsatellite markers have been used frequently in studies of gastric carcinomas.[10,12–14] In addition, a variable number of tandem repeat polymorphisms at the DCC locus were tested. Microsatellite sequences were obtained from specific primers reported in the Genome Database (http://gdbwww.gdb.org/gdb/).

Determination of LOH

The peaks produced by the normal DNA sample were used to determine whether the cancerous sample was homozygous (1 peak) or heterozygous (2 peaks). The allelic ratio was calculated as described by Habano *et al.*[27] A tumor was considered to have allelic loss if the allele peak ratio was less than or equal to 0.60, representing an allelic signal reduction of at least 40%. We interpreted this allelic imbalance as allelic loss (LOH) with the provision that, in some cases, the changes in the allele peak ratio may have resulted from allelic amplification. Tumors exhibiting MSI at a given locus were not evaluated for allelic loss. The data were collected automatically and analyzed using GeneScan software (Applied Biosystems) to determine the allele score and to assess the possibility of allelic loss.

Scoring of LOH

LOH status was scored according to the following criteria. A tumor sample was considered to be LOH-high if 3 or more of the markers showed allelic loss. When data showed that 1 or 2 markers were lost, the tumor was designated as LOH-low.[19]

Assessment of MSI

Six different loci were considered for MSI assessment, including all those recommended by the Bethesda panel for colon cancer (BAT25, BAT26, D5S346, D2S123 and D17S250).[29] A tumor was defined as MSI-positive when a novel, abnormal-sized band occurred in the tumor sample compared with the corresponding normal DNA sample. MSI-positive colorectal carcinomas were used as controls in the study and were divided into 2 groups, those with high-level instability (*i.e.*, MSI at [dbmtequ]33% of loci) and those with low-level instability (*i.e.*, MSI at [dbltequ]17% of loci), as described previously. However, tumors with only 1 alteration of the marker examined using the above criteria and those previously

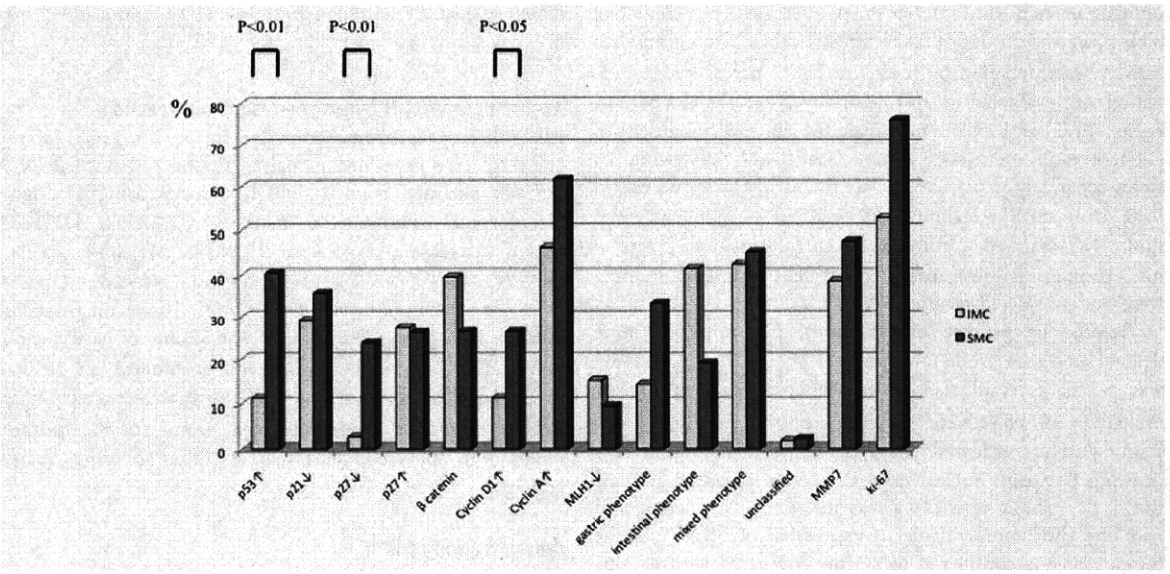

Figure 1. Frequencies of expression of cell cycle-related proteins and mucins in IMCs and SMCs. Sample numbers are as follows: IMC, 106 and SMC, 42 for all parameters.

categorized as MSI-low were considered MSI-negative tumors in this study.

Confirmation of methylation

Bisulfite treatment of genomic DNA was carried out as described previously.[30] For examination of methylation status, we used a combined bisulphate restriction analysis (COBRA) as described previously.[29,30] The COBRA of *MLH-1*, *RUNX3*, *p16*, *HPP1*, *RASSF2A*, *SFRP1*, *DKK-1*, *ZFP64* and *SALL4* genes were determined using primers, restriction enzyme and conditions as previously described.[39–37]

The colon cancer cell lines RKO and SW48 (American Tissue Culture, Manassas, VA) and water were used as positive and negative controls, respectively. After amplification, the PCR products were digested with restriction enzymes and electrophoresed on 3% agarose gels. The gels were stained with ethidium bromide, and the proportion of methylated alleles was visually compared with unmethylated alleles. Cleavage fragments were quantified by densitometry.

Tumors were classified as methylation-negative/low (methylation-low) if 1 or 2 loci were methylated and methylation-high if 3 or more were methylated.

Statistical analysis

The data were analyzed using a chi-squared test with the aid of StatView-IV software (Abacus Concepts, Berkeley, CA). Samples were determined to be significantly different when the p value was less than 0.05.

Results

Expression of cell cycle-related proteins in gastric IMCs and SMCs

The results of the survey are depicted in Figure 1. Overexpression of the p53 gene product was significantly more frequent in SMCs (40.5%) than in IMCs (11.3%; $p < 0.01$). Conversely, the frequency of low expression of the p27 product was statistically higher in SMCs (23.8%) than in IMCs (2.8%; $p < 0.01$). In addition, cyclin D1 overexpression was more frequently found in SMCs (26.5%) compared with IMCs (11.3%; $p < 0.05$). The Ki-67 positivity rate for SMCs (76.2%) was higher than that of IMCs (52.8%; $p < 0.05$). There were no significant differences in low expression of p21 and MLH-1 between IMCs (29.2 and 15.1%, respectively) and SMCs (35.7 and 9.5%, respectively). Overexpression of cyclin A and nuclear accumulation of β-catenin were commonly observed in IMCs (46.2 and 39.6%, respectively) and SMCs (61.9 and 26.5%, respectively). Although MMP7 was primarily expressed at the invasive front of the submucosal lesion, no significant difference of MMP7 was observed between IMCs (38.7%) and SMCs (47.6%). As for mucin phenotype, the gastric phenotype was found significantly more often in SMCs (33.3%) than in IMCs (14.2%) ($p < 0.05$). By contrast, the intestinal phenotype was expressed more in IMCs (41.5%) than in SMCs (19%).

Analysis of LOHs at multiple chromosomal loci in IMCs and SMCs

LOH data are summarized in Figure 2. Allelic loss of 5q was a common alteration in IMCs (38.5%) and SMCs (39.5%).

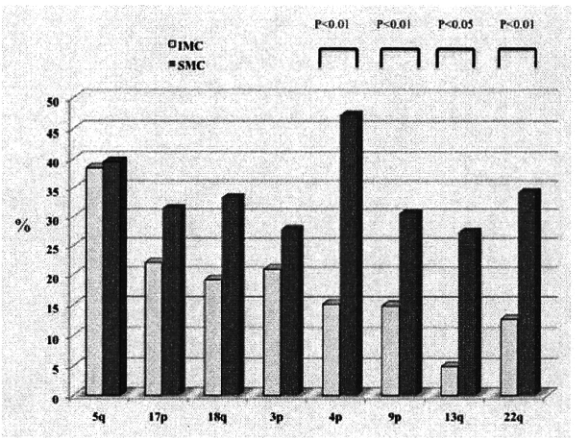

Figure 2. Frequencies of LOH at multiple cancer-related chromosomal loci in IMCs and SMCs. IMC, 5q, 35/91, 38.5%; 17p, 18/82, 22%; 18q, 17/89, 19.1%; 3p, 19/91, 20.8%; 4p, 14/93, 15.1%; 9p, 13/88, 14.8%, and 22q, 11/87, 12.6%. SMC, 5q, 15/38, 39.5%; 17p, 11/35, 31.4%; 18q, 12/36, 3p, 33.3%; 10/36, 27.8%; 4p, 17/36, 47.2%, 9p, 11/36, 30.6% and 22q, 13/38, 34.2%.

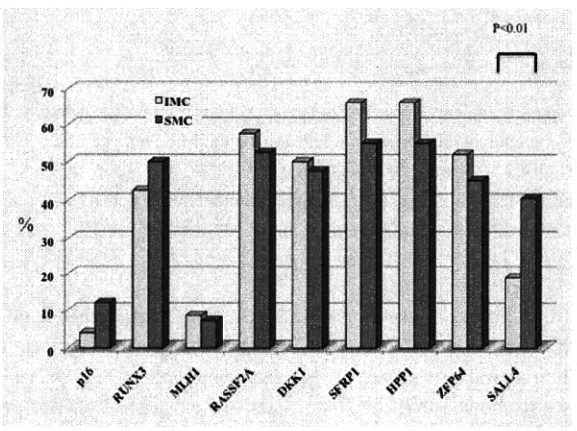

Figure 3. Frequencies of multiple gene promoter methylations in IMCs and SMCs. Sample numbers are as follows: IMC, 106 and SMC, 42 for all genes.

Although allelic losses of 17p, 18q and 3p were more frequent in SMCs (31.4, 33.3 and 27.8%, respectively) than in IMCs (22, 19.1 and 20.8%, respectively), no significant differences were found. The frequencies of 4p, 9p and 22q allelic losses were significantly higher in SMCs (47.2, 30.6, and 34.2%, respectively) than in IMCs (15.1, 14.8 and 12.6%, respectively) ($p < 0.01$). Finally, allelic loss at 13q was more frequently found in SMCs (27.3%) than in IMCs (4.9%) ($p < 0.05$).

Analysis of methylation in IMCs and SMCs

The associations between the 2 lesions are shown in Figure 3. High frequencies of methylation of *RUNX-3*, *RASSF2A*,

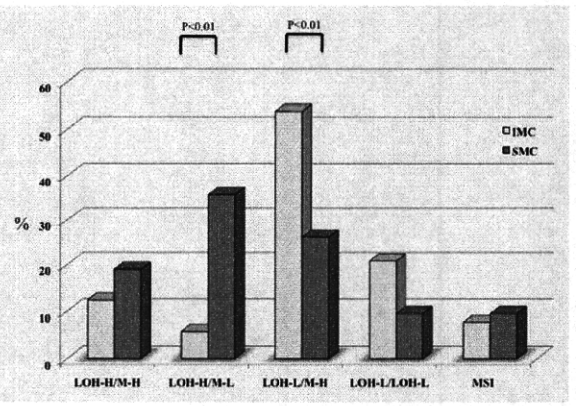

Figure 4. Frequencies of LOH and methylation status in IMCs and SMCs. Sample numbers are as follows: IMC, 106 and SMC, 42.

DKK1, *SFRP1*, *HPP1* and *ZFP64* were commonly observed in both IMCs (42.7, 57.5, 50, 66, 66 and 51.9%, respectively) and SMCs (50, 52.4, 47.6, 54.8, 54.8, and 45.2%, respectively). However, only *SALL4* methylation was significantly more frequent in SMCs (40.5%) compared with IMCs (18.9%) ($p < 0.01$). Although p16 was frequently methylated in SMCs (11.9%), when compared with IMCs (3.8%), no significant difference was detected between them. The frequencies of *MLH-1* methylation were low in IMCs (8.5%) and SMCs (7.1%).

Analysis of molecular status of IMCs and SMCs

The molecular status of intramucosal and submucosal tumors is displayed in Figure 4. The frequency of LOH-high/methylation-low status was significantly higher in SMCs (35.7%) than in IMCs (5.7%; $p < 0.01$). On the other hand, LOH-L/methylation-H status was more frequently found in IMCs (53.8%) than in SMCs (26.2%; $p < 0.01$) Although LOH-L/methylation-L status was found more frequently in IMCs (20.8%) compared with SMCs (9.5%), the difference did not reach a statistically significant level. There were no significant differences of LOH-H/methylation-H status between IMCs and SMCs. The frequencies of MSI in IMCs and SMCs were 7.6 and 9.5%, respectively. Finally, the frequency of p53 overexpression of LOH-high status cancers (47.6%) was significantly higher than that of LOH-L status (9.6%; $p < 0.01$).

Representative histological and molecular examples of SMC are shown in Figures 5 and 6.

Discussion

This study was conducted to examine the differences in molecular alterations between IMCs and SMCs in early gastric carcinogenesis. Identifying the molecular differences between the 2 lesions is important in understanding carcinogenetic mechanisms involving SMCs. This study successfully documented a number of differences in the molecular status of IMCs and SMCs.

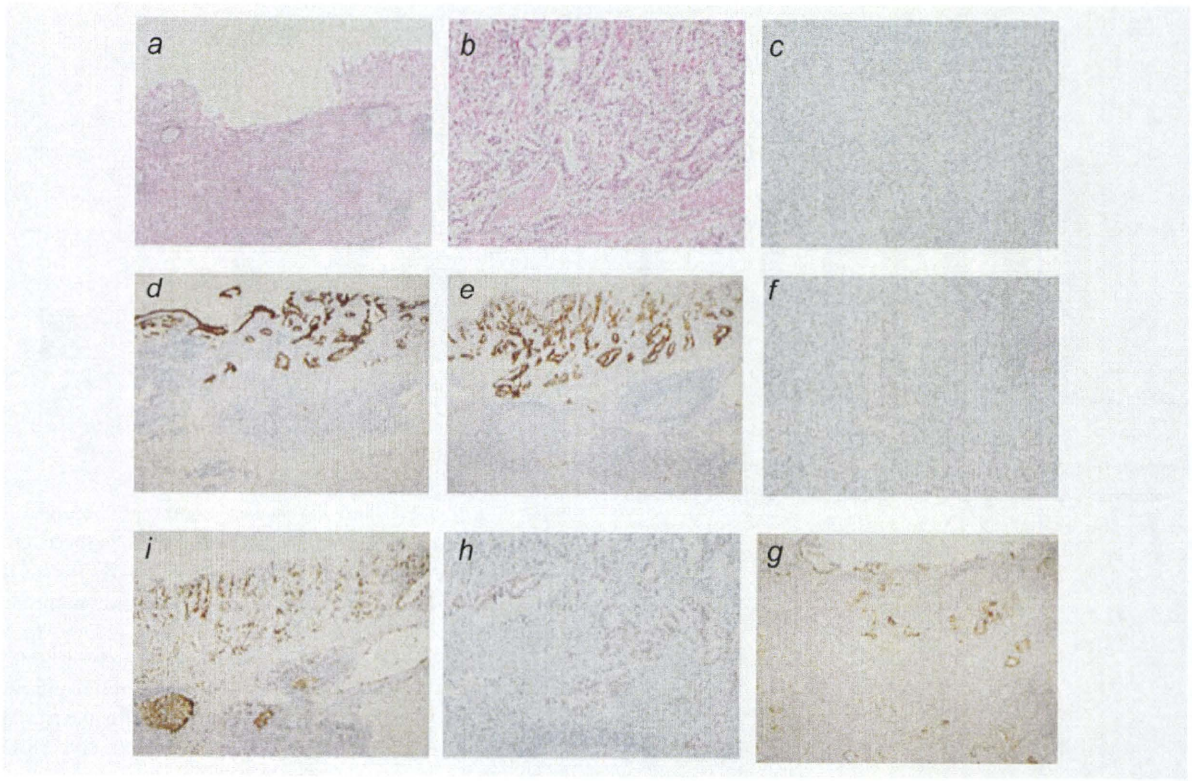

Figure 5. Representative example of the immunohistochemical study of early (intramucosal) cancer. (*a*) Low-power view of the SMC section. (*b*) High-power view of the cancer section. The tumor was diagnosed as a moderately differentiated adenocarcinoma. However, the SMC was poorly differentiated and also had massive lymphocytic infiltrates. (*c*) MUC2 stain was negative. (*d* and *e*) MUC5AC and MUC6 showed positive staining in the primary mucosal lesion, respectively. (*f*) Expression of MLH-1 was low. (*g*) Although high proliferative activity was seen in the mucosal lesion, low expression of ki-67 was found in the submucosal lesion. (*h*) Overexpression of p53 was not seen. (*i*) MMP-7 is positive in cancer tissue.

Recent studies have shown that mucin expression by tumor cells is closely associated with gastric tumorigenesis.[38] In fact, the mucin phenotype of tumor cells correlates with clinicopathological findings and molecular alterations.[14,38] In the present study, although the intestinal phenotype was primarily found in IMCs, the gastric phenotype was frequently detected in SMCs. On the other hand, Wakatsuki *et al.* indicated that tumors with the intestinal phenotype had the worst prognosis.[39] While this finding seems to conflict with our data, patient prognosis is often determined by a multitude of factors. Our finding suggests that tumor cells with a gastric phenotype are more likely to invade into the submucosa in gastric cancers.

In the present study, p53 overexpression was more frequently found in SMCs than in IMCs. This finding suggests that p53 overexpression plays a major role in submucosal invasion of IMC and represents a novel predictive marker for SMC. In addition, this finding implies that p53 overexpression is not associated with the mucosal onset of gastric carcinogenesis. In our data, the frequency of p53 overexpression by SMC was ~40%, consistent with previous reports ranging from 35.7 to 57.1% in gastric SMCs.[40,41] On the other hand, it is well known that p53 overexpression is correlated with tumor nuclear grade or mucin phenotype.[22] The present study showed that the frequencies of both gastric phenotype and tumor cells of high nuclear grade were greater in SMCs than in IMCs. These findings suggest that both gastric mucin phenotype and tumor nuclear grade reflect p53 overexpression in gastric SMCs.

Previous studies have shown that cyclins are useful immunohistochemical markers when evaluating the aggressiveness of tumor cells.[4,6] In the present study, cyclin D1 was upregulated in SMCs compared with IMCs. *Cyclin D1*, a target gene of Wnt signal transduction, is an essential molecule in human tumorigenesis. Cyclin D1 induces significant changes in gene expression in human tumors, such as phosphorylated Rb, myc and cell adhesion-related genes.[8,42] This finding suggests that *cyclin D1* overexpression may play an important role in the progression from IMC to SMC. With regard to cyclin A, however, no difference was found between IMCs and SMCs, suggesting that upregulation of cyclin A is an early event in the development of differentiated-type gastric cancers.

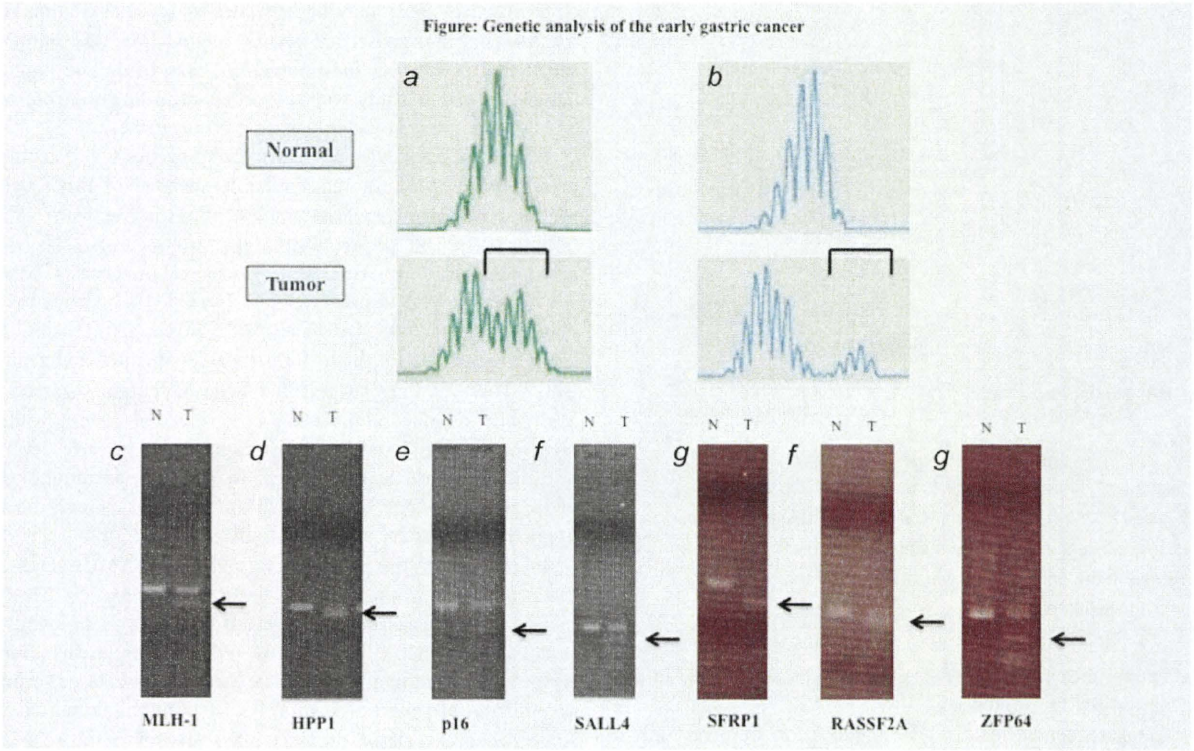

Figure 6. Representative example of the molecular study of early (intramucosal) cancer. Additional peaks of BAT 25 and BAT 26 were positive. Multiple methylations were seen in c to g. Brackets and arrow heads indicate MSI and methylated bands, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Low or nonexistent expression of p27 was associated with patient outcome and prediction of tumor aggressiveness,[43] although opposing data have been reported.[44] However, the point at which low p27 expression occurs in the progression of gastric cancers remains unknown. We found that low expression of p27 was associated with submucosal invasion in differentiated-type gastric cancers. However, Oya *et al.* indicated that reduced expression of p27 occurred more frequently in carcinoma than in adenoma.[45] Although the reason for these differences remains unknown, they could be due to the number of samples examined, differences in cutoff values for the examined proteins, the sources of antibodies, or the tumor grade examined in the study. In addition, our survey demonstrated that the reduction of p21 was a common alteration in both IMCs and SMCs. This finding suggests that p21 reduction is closely associated with early tumorigenesis of gastric cancers.

Numerous publications have reported regions of LOH that occurred frequently in gastric carcinogenesis, particularly those at 5q, 17p, 18q, 3p, 4p, 9p, 13q and 22q, including *APC* and *p53* genes.[10–14] One previous study showed that aggressive tumor cells tend to acquire LOH and thus LOH, in some cases, proves to be a novel marker for predicting tumor-invasive or aggressive activities.[11] In the present study, 5q allelic loss was a common alteration in both IMCs and SMCs. In contrast, the frequencies of 4p, 9p, 13q and 22q allelic losses were significantly higher in SMCs than in IMCs. These findings suggest that although 5q allelic loss plays a major role in early gastric carcinogenesis, 4p, 9p, 13q and 22q allelic losses are associated with submucosal invasion from mucosal lesions. The frequencies of 17p, 18q and 3p allelic losses in IMCs were low in the present study. We suggest that those allelic losses also contribute to the early development of gastric carcinogenesis, because the frequencies of those allelic losses differed among mucin phenotypes of the differentiated EGCs. That is, the frequencies of those allelic losses were low in intestinal phenotype cancers, but high in gastric phenotype cancers.

We demonstrated that gastric cancer cells are heavily methylated in the early stages of gastric tumorigenesis. This finding suggests that a progressive increase in methylation of most of those genes does not occur during the progression of EGCs. Rather, the data indicate that methylation is an early and essential event in gastric carcinogenesis. In addition, we found that methylation of *RUNX3*, *HPP1*, *RASSF2A*, *SFRP1*, *DKK-1* and *ZFP64* gene promoters plays an important role in early gastric carcinogenesis. In particular, methylation of the *ZFP64* gene was described in the first report identifying frequent alterations in gastric cancers, although its function

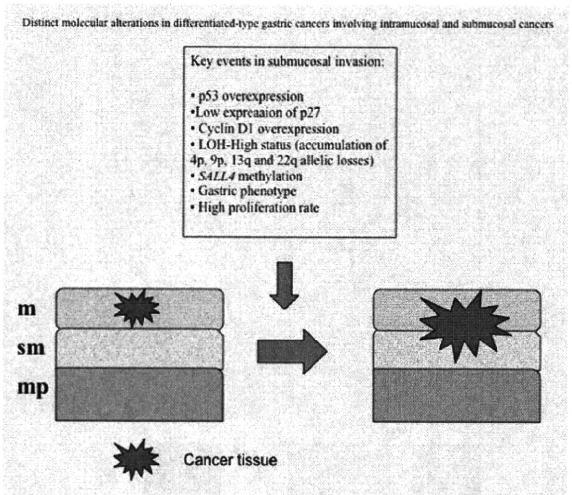

Figure 7. Distinct molecular alterations in differentiated-type gastric cancers involving IMCs and SMCs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

remains unclear. The *ZFP64* gene may be a key gene in gastrointestinal tumorigenesis.

The frequencies of *p16* gene methylation were very low in both lesions. This suggests that inactivation of the p16 gene plays a minor role in EGC. According to our data, only *SALL4* methylation frequency was higher in SMCs than in IMCs. *SALL4* methylation may therefore be associated with submucosal invasion of IMC. Although the function of the *SALL4* gene remains unknown,[46] its expression is linked to Wnt signal transduction.[47] Our finding implies that submucosal invasive ability may be activated by the methylation of the *SALL4* gene, given that *SALL4* methylation was significantly higher in aneuploid populations than in diploid populations.[36] Further study will be needed regarding the role of *SALL4* gene methylation in gastric carcinogenesis.

MSI is an early event in gastric carcinogenesis.[46–48] In the present study, MSI showed similar frequencies in IMCs and SMCs, supporting previous studies.[48,49] However, our data indicate that MSI is involved in the progression of gastric carcinogenesis. Furthermore, in gastric carcinogenesis, MSI accelerates neoplastic progression.[50] Here, LOH-high/methylation-low status was associated with SMCs. In contrast, a LOH-low/methylation-high status was frequently found in IMCs. These findings suggest that LOH-high status is associated with submucosal invasion of a mucosal lesion, while high methylation status plays an essential role in early development of gastric carcinogenesis. In addition, accumulation of LOHs (LOH-high status) in the mucosal lesion may be a predictive marker for submucosal invasion.

In conclusion, the present data suggest that SMCs acquire distinct molecular alterations in gastric carcinogenesis. These molecular characteristics are depicted in Figure 7. This illustration shows that although IMC is caused by methylation (epigenetic) dominant alterations, genetic alterations must accumulate for progression to SMC. In addition, alteration of some cell cycle-related proteins plays an important role in cancer evolution. Further studies will be required to clarify the origins of human early gastric carcinogenesis.

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