Materials and methods

Cell lines DNA transfection, and cell cloning. Primary Cas −/− fibroblasts were maintained as described earlier [21] and spontaneous cell lines were obtained. Cas-re-expressing fibroblasts were obtained by transfecting pSSRαbsr-Cas [21] using Lipofectamine (Gibco), followed by selection in 10 μg/ml blasticidin hydrochloride (Kaken Seiyaku). For the Cas mutant stable cell lines, plasmids for HA-tagged Cas mutants [23] together with pSV2bsr (Kaken Seiyaku) were transfected to Cas-deficient fibroblasts using Lipofectamine (Gibco), followed by selection in 10 μg/ml blasticidin hydrochloride (Kaken Seiyaku). Primary Cas +/+ fibroblasts, Cas −/− and a-Src cells, and Cas+/+ and a-Src cells were described earlier [21]. Briefly, activated Src (a-Src) with Myc-tag was created by replacing aa 527–536 of rat Src, which include the negative-regulatory tyrosine residues, by c-Myc epitope tag. The plasmid was introduced to Cas −/− or Cas +/+ cells using Lipofectamine (Gibco) with puromycin-resistance gene.

Microarray analysis. Total RNAs were extracted from Cas-re-expressing fibroblasts or vector control-transfected Cas −/− fibroblasts as described [24]. Poly(A) RNAs were purified using OligoTex (Roche). One μg poly(A) RNAs was sent to Incyte Genomics, and MOUSE GEM 1 microarray analysis was performed.

Western blot analysis. Proteins were extracted by lysing cells in 1% Triton buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, and 1 mM Na_3VO_4). Protein aliquots were separated by SDS–PAGE, blotted to Immobilon PVDF membranes (MILLIPORE), and probed with 1:2500 diluted anti-Cas2 antibody [2]. Positive signals were visualized using the ProtoBlot Western AP system (Promega).

Northern blot analysis. Total RNAs or poly(A) RNAs were separated on a 1% agarose gel by electrophoresis and transferred to a Hybond N nylon filter (Amersham). The cDNAs for COL1A1 and TSC-36 are gifts from Dr. Masaki Noda with the permission of Dr. Barbara E. Kream and from Dr. Kiyoshi Nose, respectively. The cDNA for Cas was described previously [2]. The other cDNAs were purchased from Incyte Genomics. For TSC-36 probe, pGEM/TSC-36 [25] was digested with *Eco*RI and the 550 bp fragment was purified using QIAquick (Qiagen). For COL1A1 probe, pBR322-rat COL1A1 was digested with *Eco*RI and the 1.4 kb fragment was purified. For COL3A1 probe, W89883 was digested with *Eco*RI and *Hin*dIII and the 450 bp fragment was purified. For COL11A1 probe, AI89400 was digested with *Eco*RI and *Pst*I and the 350 bp fragment was purified. For elastin probe, AA048878 was digested with *Eco*RI and *Hin*dIII and the 450 bp fragment was purified. For periostin probe, W82878 was digested with *Eco*RI and *Bst*XI and the 330 bp fragment was purified. For MARCKS probe, AI32268 was digested with *Eco*RI and *Pst*I and the 400 bp fragment was purified. For caveolin-1 probe, AA138963 was digested with *Eco*RI and *Bst*XI and the 360 bp fragment was purified. For heme oxygenase 1 probe, AA213167 was digested with *Eco*RI and *Hin*dIII and the 400 bp fragment was purified.

The cDNAs were labeled by a Multiprime DNA Labeling System (Amersham) and used as probes. Hybridization was carried out at 42 °C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, denatured salmon sperm DNA (20 μg/ml), and a ^{32}P-labeled probe. Hybridized filters were washed twice in 2× SSC/0.1% SDS for 20 min at 55 °C, followed by exposure to BAS-III imaging plate (Fuji Film).

Results

Establishment of Cas-re-expressing fibroblasts

The spontaneous Cas-deficient fibroblast cell line was transfected with pSSRαbsr-Cas or with pSSRαbsr. The transfected cells were subjected to selection with blasticidin and the selected colonies were collected together and analyzed. Re-expression of Cas was confirmed by Western blot with anti-Cas2 antibody [2] (Fig. 1B).

Result of microarray analysis and verification by Northern blot analysis

Poly(A) RNAs were extracted from Cas-re-expressing fibroblasts or vector-control-transfected fibroblasts, and analyzed by Incyte Genomics, using MOUSE GEM 1 microarray system that includes approximately 8900 EST clones from approximately 5000 genes. Genes that showed more than 2.5-fold change in expression were presented in Table 1. All the cDNAs except for the unknown genes and for the duplicates were prepared for probes. The poly(A) RNAs used in the microarray analysis were electrophoresed, transferred to filters, and hybridized with the probes. Many of them (caveolin-1, heme oxygenase 1, procollagen type 1 α 1 (COL1A1), procollagen type 3 α 1 (COL3A1), procollagen type 11 α 1 (COL11A1), elastin, periostin, TSC-36, MARCKS, Slim1, protease nexin 1, and lumican) showed clear

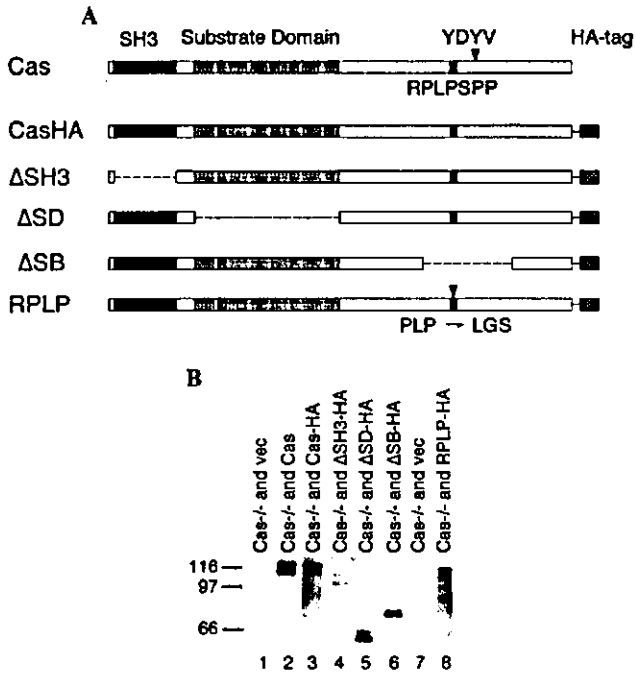


Fig. 1. (A) Schematic diagram representing the various Cas constructs used [23]. (B) Western blot for detecting the re-expression of Cas and Cas mutants. Fifty μg cell lysates extracted from Cas-deficient vector control-transfected (vec) (lane 1), Cas-re-expressing (Cas) (lane 2), CasHA-re-expressing (CasHA) (lane 3), ΔSH3-re-expressing (ΔSH3) (lane 4), ΔSD-re-expressing (ΔSD) (lane 5), ΔSB-re-expressing (ΔSB) (lane 6), vector-control-transfected (vec) (lane 7), and RPLP-re-expressing (RPLP) (lane 8) fibroblasts was separated by 7.5% SDS–PAGE, transferred to PVDF membranes, and probed with anti-Cas2 antibody [2]. The position of protein markers is shown on the left.

Table 1
Genes differentially expressed by >2.5-fold, among approximately 8900 ESTs examined

Genes down-regulated in Cas-deficient cells		
3.1	AA260520	TEL
3.1	AA260248	GRB10
2.6	AA213167	Heme oxygenase (decycling) 1
2.5	AA138693	Caveolin
Genes up-regulated in Cas-deficient cells		
Extracellular matrix		
−4.5	AA242611	TSC-36, FRP
−3.9	AA048878	Elastin
−3.7	AI894006	Procollagen, type 11, α 1 (COL11A1)
−3.4	W89883	Procollagen, type 3, α 1 (COL3A1)
−3.3	AA268082	Lumican
−3.0	AA073604	Procollagen, type 1, α 1 (COL1A1)
−3.0	W81878	Periostin (osteoblast-specific factor 2)
−2.6	AA239171	Elastin[a]
Cytoskeletal and signaling		
−3.8	AI322868	MARCKS
−3.2	AA175094	MARCKS[a]
−3.0	AA000799	Similar to synaptopodin
−2.6	AA047966	FHL/SLIM1
Metabolic		
−3.1	AI892192	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1
−2.8	AA139624	CytoC oxidase V1B
−2.6	AI322570	COQ7, CLK-1
−2.5	AA521758	MIPP65
Other known genes		
−3.3	AA051654	Metallothionein 1
−3.0	AA543573	Dactylin, hagoromo
−2.9	AA068750	Sdf-1 β
−2.7	AA218279	Spi4, PN-1 (protease-nexin1)
−2.7	AA110791	Similar to p37NB
Unknown		
−2.8	AA119804	ESTs
−2.7	W14925	ESTs
−2.6	AA266229	Public domain EST
−2.5	AA185701	ESTs

[a] There are duplicates, because several ESTs were derived from the same genes.

change of expression (Fig. 2 and data now shown). However, in the comparison between Cas −/− cells and wild-type Cas +/+ cells (Fig. 4, lanes 1 and 3), we detected no change in the expression of slim1, protease nexin 1, and lumican (data not shown). SDF1β, Dad1, COQ7, Akt, Hagoromo, cyto C oxidase, synaptopodin, p37NB, GRB10, and TEL showed only small differences in expression (data not shown). MIPP65 and metallothionein had differences of background or cross-reactive bands (data not shown). We could not detect Hmgcs1 (3-hydroxy-3-methylglutaryl-coenzyme A synthase 1) band.

Based on the above results, we picked up nine genes (caveolin-1, heme oxygenase 1, COL1A1, COL3A1, COL11A1, elastin, periostin, TSC-36, and MARCKS) in the following study. The characteristics of these nine gene products are summarized below.

Caveolin-1 is an integral membrane protein and is a major structural component of caveolae but is also found in the cytoplasm [26]. In response to integrin stimulation, caveolin-1 is known to recruit Fyn to phosphorylate Shc [27,28]. Heme oxygenase 1 is an inducible enzyme that catalyzes the conversion of heme to biliverdin, carbon monoxide and iron [29]. Heme oxygenase 1 is induced by heme products, hydrogen peroxide, ultraviolet A radiation, heavy metals, endotoxin, and oxidant stress. Collagens and elastin are extracellular matrix proteins. ECM triggers the tyrosine phosphorylation of Cas through the stimulation of integrins [15,16]. Periostin is a secreted factor and is thought to be a homolog of insect adhesion molecule fasciclin I [30]. Periostin is reported to support the attachment and spreading of MC3T3-E1 cells [31] and is expressed in the heart, playing important roles in ECM deposition following myocardial infarction and heart valve formation [32,33]. TSC-36 is a TGF-β inducible extracellular glycoprotein that has a follistatin mod-

ule [25] and is down-regulated in K-ras-transformed fibroblasts [34]. Overexpression of TSC-36 is known to cause growth inhibition of lung cancer cell lines [35] and inhibits invasion [36]. MARCKS (myristoylated alanine-rich C kinase substrate) is a substrate for PKC (protein kinase C) and is implicated in the regulation of brain development, cell migration, and adhesion as well as endo-, exo-, and phago-cytosis [37]. MARCKS is found associated both with plasma membrane and in the cytosol. Recently, MARCKS is reported to be involved in the regulation of the actin cytoskeleton and the control of lipid second messengers [37]. Overexpression of MARCKS in melanoma cells restores focal contacts [38] and decreases the cell proliferation [39,40].

The substrate domain is necessary for periostin repression by Cas

To characterize the domain necessary for this transcriptional change, we expressed a series of Cas mutants [23] (Fig. 1A) in Cas −/− fibroblasts (Fig. 1B), although we could not obtain comparable expression of ΔSH3 mutant of Cas (Fig. 1B, 3J).

Caveolin-1 and heme oxygenase 1 showed enhanced expression with the re-introduction of Cas, ΔSD, ΔSB, and RPLP but not with that of ΔSH3 (Fig. 3A and B). In contrast, COL3A1, COL11A1, and elastin showed reduced expression with the re-introduction of Cas, ΔSD, ΔSB, and RPLP but not with that of ΔSH3 (Fig. 3D–F). Periostin showed reduced expression with the re-introduction of Cas, ΔSH3, ΔSB, and RPLP but not with that of ΔSD (Fig. 3G). COL1A1 showed reduced expression with the re-expression of Cas, ΔSB, and RPLP but not with that of ΔSH3 nor ΔSB (Fig. 3C). TSC-36 and MARCKS showed reduced expression with all the mutants (Fig. 3H and I). COL3A1, COL11A1, periostin, and MARCKS showed significant down-regulation with the introduction of ΔSB or RPLP (Fig. 3C, D, G, and I).

These results indicate that the domains necessary for transcriptional change are different depending on the genes regulated, suggesting that multiple signaling pathways triggered by Cas are involved.

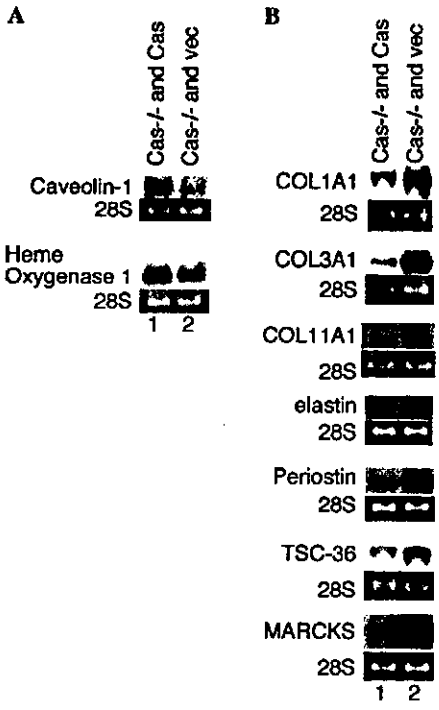


Fig. 2. Northern blots confirming altered expression of target genes. Poly(A) RNA applied to microarray analysis was used for Northern blots. Ethidium bromide-stained 28S RNA levels demonstrate similar loading in each lane. (A) Genes down-regulated in Cas −/− cells, caveolin-1 (upper), and heme oxygenase 1 (lower). (B) Genes up-regulated in Cas −/− cells, COL1A1, COL3A1, COL11A1, elastin, periostin, TSC-36, and MARCKS.

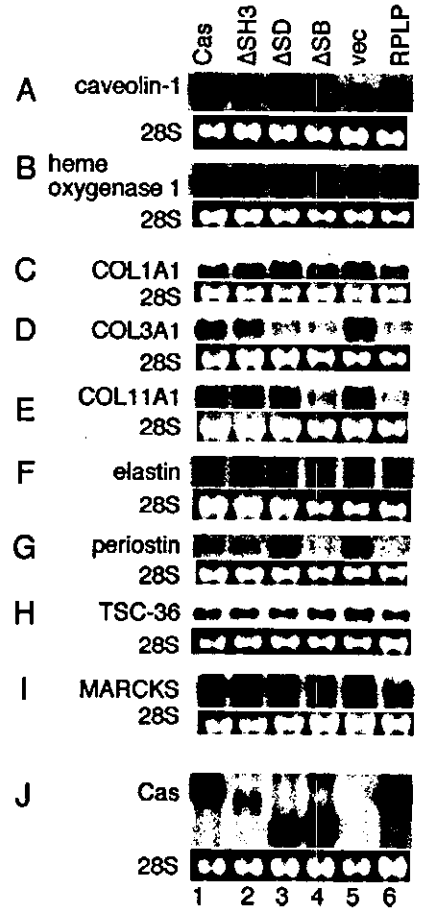


Fig. 3. Northern blots showing expression of target genes in Cas-mutant-re-expressing cells. Total RNAs harvested from Cas-HA- (lane 1), ΔSH3- (lane 2), ΔSD- (lane 3), ΔSB- (lane 4), vec- (lane 5), and RPLP (lane 6) expressing cells were used for Northern blots. Ethidium bromide-stained 28S RNA levels are shown. The probes used are for caveolin-1 (A), heme oxygenase 1 (B), COL1A1 (C), COL3A1 (D), COL11A1 (E), elastin (F), periostin (G), TSC-36 (H), MARCKS (I), and Cas (J).

Activated Src-mediated reduction of gene expression is not totally dependent on Cas

Cas was originally cloned as a tyrosine phoshorylated protein in v-Crk- and v-Src-transformed cells [2] and Cas is reported to mediate transcriptional activation of the serum response element by Src [41]. Therefore we investigated the transcriptional change of these proteins in v-Src- or v-Crk-transformed rat 3Y1 cells [2] (Fig. 4, lanes 1–3). COL1A1, COL3A1, COL11A1, elastin, TSC-36, MARCKS, and caveolin-1 showed reduced expression with the transformation by v-Src (Fig. 4, lanes 1 and 2), but they showed slightly reduced expression with the transformation by v-Crk (Fig. 4, lanes 1 and 3). In contrast, the expression of heme oxygenase 1 was enhanced with the transformation by v-Src and was slightly up-regulated with the transformation by v-Crk (Fig. 4B, lanes 1–3). We could not detect the expression of periostin in these cells (data not shown).

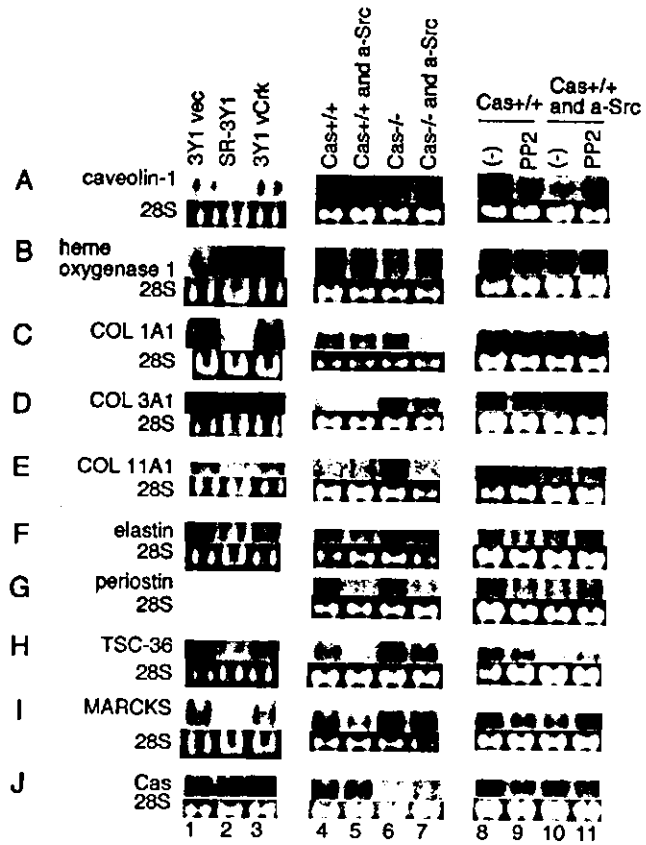


Fig. 4. Northern blots showing the regulation of target genes by activated Src expression. Total RNAs were harvested from 3Y1-vec (lane 1), SR-3Y1 (lane 2), 3Y1-vCrk (lane 3) [2], Cas +/+ cells (lane 4), Cas +/+ cells expressing a-Src (lane 5), Cas −/− cells (lane 6), Cas −/− cells expressing a-Src (lane 7) [21], Cas +/+ cells treated with DMSO as vehicle (lane 8), Cas +/+ cells treated with PP2, Src inhibitor (lane 9), Cas +/+ cells expressing a-Src treated with DMSO (lane 10), and Cas +/+ cells expressing a-Src treated with PP2 (lane 11). Ethidium bromide-stained 28S RNA levels are shown. The probes used are for caveolin-1 (A), heme oxygenase 1 (B), COL1A1 (C), COL3A1 (D), COL11A1 (E), elastin (F), periostin (G), TSC-36 (H), MARCKS (I), and Cas (J).

We next checked whether these Src-induced changes are dependent on Cas. RNAs were extracted from Cas −/− fibroblasts, Cas +/+ wild-type sibling-derived fibroblasts, Cas −/− fibroblasts transfected with a-Src, and Cas +/+ fibroblasts transfected with a-Src [21]. Both in Cas −/− cells and in Cas +/+ cells, the introduction of a-Src reduced the expression of the seven genes that were up-regulated in Cas −/− cells (Fig. 4C–I, lanes 4–7). However, COL3A1, elastin, TSC-36, and MARCKS showed higher expression in Cas −/− and a-Src cells than that of Cas +/+ cells (Fig. 4D, F, H, I, lanes 4–7). Caveolin-1 and heme oxygenase 1 showed reduced expression by a-Src in Cas +/+ cells, but their expression was increased by a-Src in Cas −/− cells (Fig. 4A, B, lanes 4–7).

To ascertain that the a-Src-induced change is dependent on tyrosine kinase activities, we treated the cells with 10 μM Src kinase inhibitor, PP2 (Wako) for 4 h. In Cas +/+ and a-Src-transformed cells, the expression was increased by PP2 (Fig. 4, lanes 10–11), confirming that a-Src-induced reduction of expression is kinase dependent. In contrast, untransformed Cas +/+ cells showed reduced expression when treated with PP2 (Fig. 4, lanes 8 and 9).

Discussion

In this report, we compared the expression profiles between Cas −/− fibroblasts and Cas-re-expressing fibroblasts. To avoid the clonal differences, we collected the polyclonal blasticidin-resistant clones. Among the genes differentially expressed in Cas −/− cells, nine genes (caveolin-1, heme oxygenase 1, COL1A1, COL3A1, COL11A1, elastin, periostin, TSC-36, and MARCKS) were selected for further analyses. These nine genes showed clear difference of expression not only between Cas −/− fibroblasts and Cas-re-expressing fibroblasts but also between Cas −/− fibroblasts and Cas +/+ fibroblasts.

Among the seven genes that are up-regulated in Cas −/− fibroblasts, six genes (COL1A1, COL3A1, COL11A1, elastin, periostin, and TSC-36) encode the extracellular matrix proteins and the other one, MARCKS, is known to regulate actin cytoskeleton [37]. Since Cas is known to be a signal transducer from integrin stimulation by ECM [15,16], the transcriptional reduction by Cas might be a result of negative feedback. Caveolin-1, which is up-regulated with expression of Cas, is also involved in integrin signaling [27,28] and is reported to reduce the Src kinase activity [42]. Although heme oxygenase 1 is not reported to be involved in integrin signaling, the oxidative stress that induces heme oxygenase 1 is reported to tyrosine phosphorylate Cas

[43]. These facts suggest that the nine genes are functionally related to Cas signaling.

In the case of caveolin-1, heme oxygenase 1, COL1A1, COL11A1, and elastin, the ΔSH3 mutant did not change the expression of them. This result could be attributed to the lower expression of the ΔSH3 mutant compared to other mutants, or the failure of this mutant to localize to focal adhesions [23]. However, it is also possible that signaling through the SH3 domain of Cas regulates the expression of these genes. Among the SH3 binding partners of Cas, CIZ is reported to regulate type I collagen [44,45]. The other binding partners such as C3G, PTP-PEST might play roles in this regulation. On the other hand, deletion of the substrate domain clearly abolished the change of expression of periostin (Fig. 3G). The signaling through the substrate domain is mediated by c-Crk that leads to DOCK180/Rac1/JNK pathway [17,18] and the expression of periostin might be regulated by this pathway. These results of the deletion study suggest multiple signaling pathways are involved in the gene regulation by Cas.

Reduction of expression of caveolin-1, COL1A1, and MARCKS [46–48] and enhancement of heme oxygenase-1 expression [49] by v-Src-induced transformation are reported. In this report, we added COL3A1, COL11A1, elastin, periostin, and TSC-36 as another down-regulated genes by v-Src (Fig. 4). However, TSC-36 is reported to be unchanged by v-Src transformation in NIH3T3 cells [34]. The discrepancy might be attributed to the difference of cell lines (we used 3Y1 cells and primary MEF) and suggests a limited role of TSC-36 in a-Src-mediated cell transformation. In addition, with the transformation by activated Src, the expression of heme oxygenase 1 showed reduction of expression in primary Cas +/+ MEFs (Fig. 4B, lanes 4 and 5) but enhancement in 3Y1 cells (Fig. 4B, lanes 1 and 2) and in CEFs [49] by v-Src. This differential regulation of heme oxygenase 1 by a-Src might suggest the irrelevancy of this gene in a-Src-mediated cell transformation.

In Cas −/− context, a-Src expression also reduced the expression of seven genes that were up-regulated in Cas −/− cells (Fig. 4C–J, lanes 6 and 7). Furthermore, the expression of the other two genes (caveolin-1 and heme oxygenase 1) that were down-regulated in Cas −/− cells were down-regulated or differentially regulated by a-Src expression. These facts indicate at least the existence of Cas-independent pathway that causes these regulations, or might suggest the possibility that Cas and a-Src independently regulate the expression of these genes, although the involvement of Cas in a-Src-mediated gene regulation was not excluded. In any case, we can at least say that Cas affects the expression of these genes that are regulated by a-Src-mediated transformation. The expression of COL3A1, elastin, TSC-36, and MARCKS in Cas −/− cells expressing a-Src is still higher than that in Cas +/+ cell transformed by a-Src and is even higher than that in Cas +/+ cells (Fig. 4D, F, H, I, lanes 4–7). Considering the fact that TSC-36 and MARCKS are reported to inhibit growth of cancer cells [35,39,40], the impaired transformation of Cas −/− MEF cells by v-Src [21] can be in part attributed to the overexpression of TSC-36 and MARCKS in these cells.

Since PP2 inhibition in Cas +/+ transformed by a-Src up-regulates the expression of caveolin-1, COL3A1, elastin, periostin, TSC-36, and MARCKS, the down-regulation by a-Src would be caused by the kinase activity of a-Src (Fig. 4, lanes 10 and 11). In contrast, PP2 reduced the expression in non-transformed Cas +/+ cells (Fig. 4, lanes 8 and 9). This reduction might be non-specific down-regulation including the expression of Cas (Fig. 4J, lanes 8 and 9). Otherwise, the role of non-transforming c-Src in the regulation of these genes might be different from that of a-Src and be related to the stronger down-regulation by ΔSB and RPLP mutants than by wild-type Cas (Fig. 3).

Overexpression of Cas in breast cancer was associated with tamoxifen resistance and with poor prognosis [50,51]. The transcriptional changes induced by Cas, especially those of TSC-36 and MARCKS, might contribute to the progression and invasion of carcinoma cells. Recently, another Cas family protein, HEF1/CasL, is reported to induce transcriptional change of matrix metalloproteinases and MLCK, p160Rock, which are believed to enhance cell migration and invasion [52]. On the other hand, TGF-β is known to regulate ECM proteins, periostin, and TSC-36 [25,31] and is implicated in fibrotic change in diseases. Considering the report that Cas is tyrosine phosphorylated by TGF-β stimulation [53], the results described here might suggest the possibility of the involvement of Cas in the deposition of ECM by TGF-β. Among the genes that showed differential expression in response to myocardial infarction included are collagens, caveolin family proteins, periostin, and ANP (atrial natriuretic peptide), which is reported to be mediated by Cas in cardiac cells [32,54]. The role of Cas and Cas-mediated transcriptional change in cancer progression, myocardial infarction, and other pathological processes would be a target for future study.

Acknowledgments

We thank Drs. Masaki Noda, Barbara E. Kream, and Kiyoshi Nose for generously providing probes. This work was in parts supported by Grants-in-Aids from the Ministry of Education, Science and Culture of Japan.

References

[1] R. Sakai, A. Iwamatsu, N. Hirano, S. Ogawa, T. Tanaka, J. Nishida, Y. Yazaki, H. Hirai, J. Biol. Chem. 269 (1994) 32740–32746.

[2] R. Sakai, A. Iwamatsu, N. Hirano, S. Ogawa, T. Tanaka, H. Mano, Y. Yazaki, H. Hirai, EMBO J. 13 (1994) 3748–3756.
[3] T. Nakamoto, R. Sakai, K. Ozawa, Y. Yazaki, H. Hirai, J. Biol. Chem. 271 (1996) 8959–8965.
[4] T. Gotoh, D. Cai, X. Tian, L.A. Feig, A. Lerner, J. Biol. Chem. 275 (2000) 30118–30123.
[5] A. Sakakibara, S. Hattori, J. Biol. Chem. 275 (2000) 6404–6464 (see also p. 6410).
[6] T.R. Polte, S.K. Hanks, Proc. Natl. Acad. Sci. USA 92 (1995) 10106–10678 (see also p. 10682).
[7] F. Liu, D.E. Hill, J. Chernoff, J. Biol. Chem. 271 (1996) 31290–31295.
[8] A.J. Garton, M.R. Burnham, A.H. Bouton, N.K. Tonks, Oncogene 15 (1997) 877–885.
[9] K.H. Kirsch, M.M. Georgescu, H. Hanafusa, J. Biol. Chem. 273 (1998) 25673–25679.
[10] K.H. Kirsch, M.M. Georgescu, S. Ishimaru, H. Hanafusa, Proc. Natl. Acad. Sci. USA 96 (1999) 6211–6216.
[11] T. Nakamoto, T. Yamagata, R. Sakai, S. Ogawa, H. Honda, H. Ueno, N. Hirano, Y. Yazaki, H. Hirai, Mol. Cell. Biol. 20 (2000) 1649–1658.
[12] D.D. Schlaepfer, M.A. Broome, T. Hunter, Mol. Cell. Biol. 17 (1997) 1702–1713.
[13] J. Yi, S. Kloeker, C.C. Jensen, S. Bockholt, H. Honda, H. Hirai, M.C. Beckerle, J. Biol. Chem. (2002).
[14] E. Li, D.G. Stupack, S.L. Brown, R. Klemke, D.D. Schlaepfer, G.R. Nemerow, J. Biol. Chem. 275 (2000) 14729–14735.
[15] Y. Nojima, N. Morino, T. Mimura, K. Hamasaki, H. Furuya, R. Sakai, T. Sato, K. Tachibana, C. Morimoto, Y. Yazaki, H. Hirai, J. Biol. Chem. 270 (1995) 15398–15402.
[16] K. Vuori, E. Ruoslahti, J. Biol. Chem. 270 (1995) 22259–22262.
[17] F. Dolfi, M. Garcia-Guzman, M. Ojaniemi, H. Nakamura, M. Matsuda, K. Vuori, Proc. Natl. Acad. Sci. USA 95 (1998) 15394–15399.
[18] E. Kiyokawa, Y. Hashimoto, S. Kobayashi, H. Sugimura, T. Kurata, M. Matsuda, Genes Dev. 12 (1998) 3331–3336.
[19] L.A. Cary, D.C. Han, T.R. Polte, S.K. Hanks, J.L. Guan, J. Cell. Biol. 140 (1998) 211–221.
[20] R.L. Klemke, J. Leng, R. Molander, P.C. Brooks, K. Vuori, D.A. Cheresh, J. Cell. Biol. 140 (1998) 961–972.
[21] H. Honda, H. Oda, T. Nakamoto, Z. Honda, R. Sakai, T. Suzuki, T. Saito, K. Nakamura, K. Nakao, T. Ishikawa, M. Katsuki, Y. Yazaki, H. Hirai, Nat. Genet. 19 (1998) 361–365.
[22] H. Honda, T. Nakamoto, R. Sakai, H. Hirai, Biochem. Biophys. Res. Commun. 262 (1999) 25–30.
[23] T. Nakamoto, R. Sakai, H. Honda, S. Ogawa, H. Ueno, T. Suzuki, S. Aizawa, Y. Yazaki, H. Hirai, Mol. Cell. Biol. 17 (1997) 3884–3897.
[24] P. Chomczynski, N. Sacchi, Anal. Biochem. 162 (1987) 156–159.
[25] M. Shibanuma, J. Mashimo, A. Mita, T. Kuroki, K. Nose, Eur. J. Biochem. 217 (1993) 13–19.
[26] E.J. Smart, G.A. Graf, M.A. McNiven, W.C. Sessa, J.A. Engelman, P.E. Scherer, T. Okamoto, M.P. Lisanti, Mol. Cell. Biol. 19 (1999) 7289–7304.
[27] K.K. Wary, F. Mainiero, S.J. Isakoff, E.E. Marcantonio, F.G. Giancotti, Cell 87 (1996) 733–743.
[28] K.K. Wary, A. Mariotti, C. Zurzolo, F.G. Giancotti, Cell 94 (1998) 625–634.
[29] M.D. Maines, Annu. Rev. Pharmacol. Toxicol. 37 (1997) 517–554.
[30] S. Takeshita, R. Kikuno, K. Tezuka, E. Amann, Biochem. J. 294 (Pt 1) (1993) 271–278.
[31] K. Horiuchi, N. Amizuka, S. Takeshita, H. Takamatsu, M. Katsuura, H. Ozawa, Y. Toyama, L.F. Bonewald, A. Kudo, J. Bone Miner. Res. 14 (1999) 1239–1249.
[32] L.W. Stanton, L.J. Garrard, D. Damm, B.L. Garrick, A. Lam, A.M. Kapoun, Q. Zheng, A.A. Protter, G.F. Schreiner, R.T. White, Circ. Res. 86 (2000) 939–945.
[33] A. Kruzynska-Frejtag, M. Machnicki, R. Rogers, R.R. Markwald, S.J. Conway, Mech. Dev. 103 (2001) 183–188.
[34] J. Mashimo, R. Maniwa, H. Sugino, K. Nose, Cancer Lett. 113 (1997) 213–219.
[35] K. Sumitomo, A. Kurisaki, N. Yamakawa, K. Tsuchida, E. Shimizu, S. Sone, H. Sugino, Cancer Lett. 155 (2000) 37–46.
[36] I.M. Johnston, H.J. Spence, J.N. Winnie, L. McGarry, J.K. Vass, L. Meagher, G. Stapleton, B.W. Ozanne, Oncogene 19 (2000) 5348–5358.
[37] A. Arbuzova, A.A. Schmitz, G. Vergeres, Biochem. J. 362 (2002) 1–12.
[38] S. Manenti, F. Malecaze, J.M. Darbon, FEBS Lett. 419 (1997) 95–98.
[39] G. Brooks, S.F. Brooks, M.W. Goss, Carcinogenesis 17 (1996) 683–689.
[40] S. Manenti, F. Malecaze, H. Chap, J.M. Darbon, Cancer Res. 58 (1998) 1429–1434.
[41] Y. Hakak, G.S. Martin, Mol. Cell. Biol. 19 (1999) 6953–6962.
[42] S. Li, J. Couet, M.P. Lisanti, J. Biol. Chem. 271 (1996) 29182–29190.
[43] M. Yoshizumi, J. Abe, J. Haendeler, Q. Huang, B.C. Berk, J. Biol. Chem. 275 (2000) 11706–11712.
[44] K. Furuya, T. Nakamoto, Z.J. Shen, K. Tsuji, A. Nifuji, H. Hirai, M. Noda, Exp. Cell Res. 261 (2000) 329–335.
[45] P. Thunyakitpisal, M. Alvarez, K. Tokunaga, J.E. Onyia, J. Hock, N. Ohashi, H. Feister, S.J. Rhodes, J.P. Bidwell, J. Bone Miner. Res. 16 (2001) 10–23.
[46] J.A. Engelman, X.L. Zhang, B. Razani, R.G. Pestell, M.P. Lisanti, J. Biol. Chem. 274 (1999) 32333–32341.
[47] B.J. Frankfort, I.H. Gelman, Biochem. Biophys. Res. Commun. 206 (1995) 916–926.
[48] C.K. Joseph, S.A. Qureshi, D.J. Wallace, D.A. Foster, J. Biol. Chem. 267 (1992) 1327–1330.
[49] J.G. Hoey, J. Summy, D.C. Flynn, Cell. Signal. 12 (2000) 691–701.
[50] A. Brinkman, S. van der Flier, E.M. Kok, L.C. Dorssers, J. Natl. Cancer Inst. 92 (2000) 112–120.
[51] S. van der Flier, A. Brinkman, M.P. Look, E.M. Kok, M.E. Meijer-van Gelder, J.G. Klijn, L.C. Dorssers, J.A. Foekens, J. Natl. Cancer Inst. 92 (2000) 120–127.
[52] S.J. Fashena, M.B. Einarson, G.M. O'Neill, C. Patriotis, E.A. Golemis, J. Cell Sci. 115 (2002) 99–111.
[53] M.C. Riedy, M.C. Brown, C.J. Molloy, C.E. Turner, Exp. Cell. Res. 251 (1999) 194–202.
[54] B. Kovacic-Milivojevic, F. Roediger, E.A. Almeida, C.H. Damsky, D.G. Gardner, D. Ilic, Mol. Biol. Cell. 12 (2001) 2290–2307.

International Congress Series 1246 (2002) 251–260

Identification of candidate tumor suppressor genes from critical deletions of long arm of chromosome 6 in hematopoietic neoplasm

Seishi Ogawa*, Akira Hangaishi, Hisamaru Hirai

Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan

Abstract

Deletion of the long arm of chromosome 6 has been frequently described in human lymphoid neoplasms as well as in other solid tumors, implicating the presence of tumor suppressor genes (TSGs) within this chromosomal segment. To isolate the relevant TSGs from the 6q14–23 region, we performed deletion analysis for a large panel of hematopoietic tumor samples using fluorescent in situ hybridization (FISH), employing more than 50 PAC/BAC clones mapped in 6q14–23 as probes. At least six critical regions (D1 to D6) existed within 6q14–23 that underwent overlapping deletions, but were discretely separated from each other, indicating multiple tumor suppressor loci might reside in this region. Deletions were most frequently observed in the D2 region that was flanked by the genetic markers D6S449 and AFMA084ZE9, followed by the D3, the region between D6S447 and WI-5694. Although attempts to identify candidate TSGs from the latter two regions involving sequencing analysis of both regions failed, we found another locus showing homozygous deletion telomeric to the D3 region by FISH analysis using additional probes. Of 106 cell lines examined, 3 had homozygous deletion in this locus, and finally, the Blimp1 gene was identified as the only gene that existed within the minimum overlapping homozygous deletion. Mutation analysis revealed that Blimp1 was mutated in 3 out of 106 primary lymphoid tumors. Our findings indicated that Blimp1 might be one of the target TSGs within 6q that was inactivated in human lymphoid neoplasms.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Tumor suppressor genes; Chromosome 6; Hematopoietic neoplasm

1. Introduction

During the past several decades, there has been a great deal of progress in the field of cancer research. It has been well established that cancer develops through a number

* Corresponding author.
E-mail address: sogawa-tky@umin.ac.jp (S. Ogawa).

0531-5131/02 © 2002 Elsevier Science B.V. All rights reserved.
PII: S0531-5131(02)01128-7

of congenital or acquired genetic changes, and one of the most powerful approaches to identify these genetic changes is to molecularly characterize chromosomal abnormalities found in cancers, especially for leukemias and lymphomas. Several kinds of chromosomal abnormalities have been described in leukemias and lymphomas, including translocations, deletions, insertions, and duplications [1]. Among these, the chromosomal translocations have been most extensively investigated, and dozens of the target genes, which are involved in the major recurrent translocations, have been identified from their breakpoint analysis; and this latter process seems to be facilitated, in this genomic era, for the remaining, molecularly still undefined translocations which might be more minor in frequency, but still have potential importance in understandings of leukemogenesis [2].

Deletion of a chromosomal segment is the other type of chromosomal abnormality, which is frequently found in hematopoietic neoplasms as well as in other solid tumors. There are diverging types of chromosomal deletions, which have been described in almost all kinds of human cancers, and it is presumed that tumor suppressor genes (TSGs) may reside within these deletions [3]. In contrast to translocations, however, the target TSGs for these deletions have mostly remained to be isolated because exploration of such chromosomal deletions has been frequently hampered by their large size and due to the high complexity of the human genome. The situation, however, seems to have improved in recent years, as the human genome project has progressed rapidly. In addition to the primary sequences of the human genome, it also provides a well-organized set of ordered clones that consist of arrays of contigs, a large number of STS markers, EST clone information, as well as tools for genome informatics [4]. With these progresses in mind, we approached, in this study, one of the chromosomal deletions recurrently observed in human hematopoietic tumors.

Deletion of the long arm of human chromosome 6 (6q −) was first described in acute lymphoblastic leukemia (ALL), and since then, it has been reported in a wide variety of human hematopoietic neoplasms including non-Hodgkin's lymphomas (NHL) and lymphoproliferative disorders [5–8]. The long arm of chromosome 6 is one of the most frequently deleted regions in acute lymphoblastic leukemia and in non-Hodgkin's lymphoma. Moreover, the 6q deletion has been reported in a wide variety of solid tumors including breast, ovarian, and prostatic cancers [9–11], indicating that this chromosomal region is likely to harbor one or more TSGs, inactivation of which contributes to development of these cancers. In order to identify the candidate gene for the target of this deletion, we performed deletion-mapping analysis of 6q using classical fluorescent in situ hybridization (FISH) technique, revealed several critical regions of deletion, and isolated a candidate for the target genes of the 6q deletion.

2. Materials and methods

2.1. Cell lines and patients

In total, 352 hematopoietic tumor samples were tested, including 137 cell lines established from leukemia or lymphoma patients and 215 primary tumor samples from

patients with a variety of hematopoietic neoplasms, after obtaining their informed consent. Diagnoses of the patients and origins of cell lines include ALL, adult T-cell leukemia (ATL), chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), myelodysplastic syndrome (MDS) with or without acute transformation, multiple myeloma (MM), NHL, and acute myelocytic leukemia (AML). After briefly cultured in RPMI 1640, samples were treated with colcemid followed by hypotonic treatment, fixed in methanol/acetic acid (3:1), and stored at −20 °C for subsequent FISH analysis. Among these samples, 54 cell lines were used for Southern blot analysis, and 106 samples of primary lymphoid malignancies, including 66 NHL, 19 ALL, and 21 ATL, were selected for mutation analysis of the Blimp1 gene. Samples were bone marrow or peripheral blood mononuclear cells separated on Ficoll–Hypaque density gradient, lymph node biopsy specimens, and tumor cells obtained from ascites. Genomic DNA and total RNA were extracted as described previously [12].

2.2. FISH probes

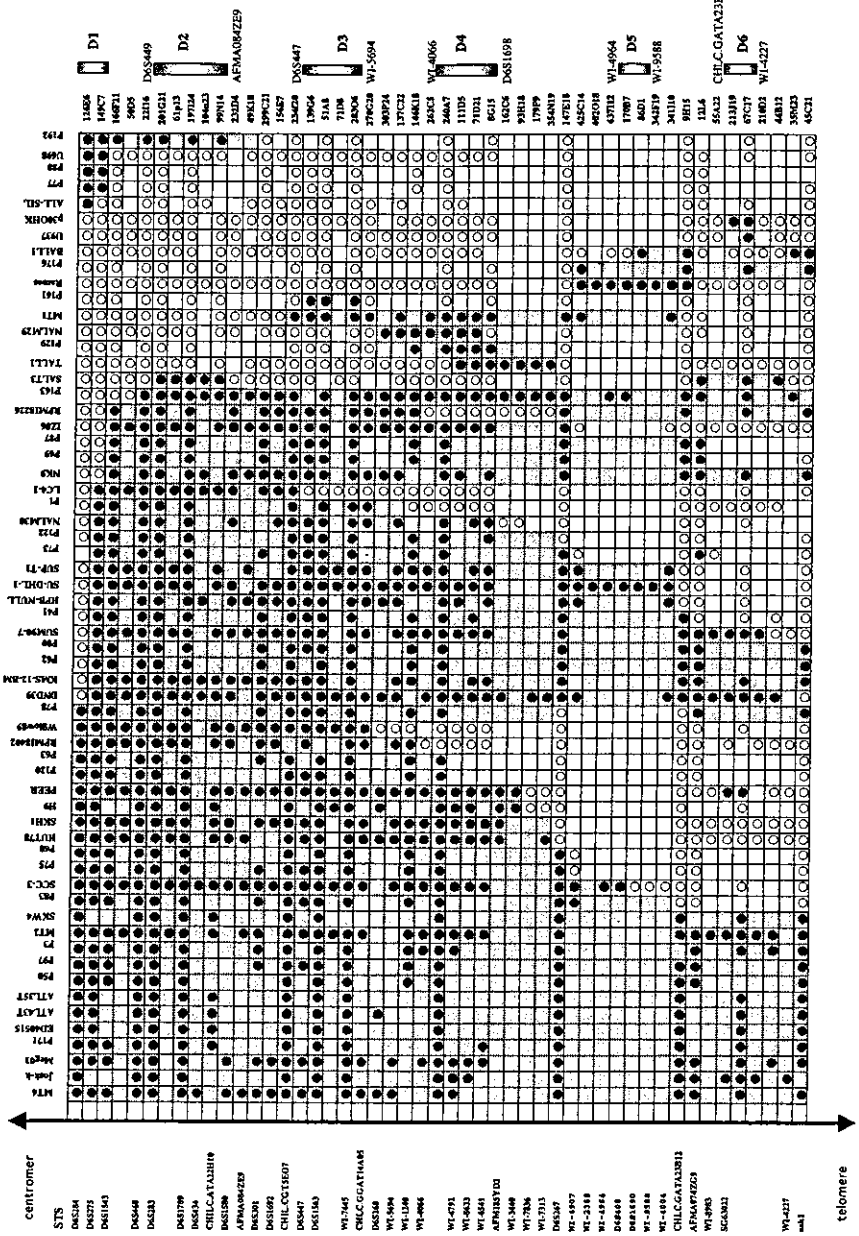
According to the genetic map that is published from the Whitehead Institute/MIT (http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map), we isolated a number of PAC or BAC clones corresponding to the STS markers that spans from 6q14 to 6q23 by PCR-screening of PAC/BAC libraries available from the Incyte Genomics (Palo Alto, CA), and used as FISH probes. In total, 63 PAC/BAC clones were isolated and used for FISH analysis. Representative probes are listed in Fig. 1 together with the corresponding STS makers. We used D6Z1 as a reference probe to identify chromosome 6.

2.3. Two-color FISH analysis

FISH experiments on metaphase chromosomes were performed according to the previously described method [13] with the following modifications of post hybridization washes: in 2 × SSC/50% formamide at 37 °C for 15 min followed by washes in 2 × SSC and 1 × SSC at room temperature for 15 min each. D6Z1 was labeled with digoxigenin-11-dUTP and test probes, by which the presence of deletion was examined, were with biotin-16-dUTP. The biotin- and digoxigenin-labeled probes were detected with avidin-FITC (Roche, Mannheim, Germany) and anti-digoxigenin-rhodamine Fab fragments (Roche), respectively. Metaphase chromosomes were counter-stained with 4,6-diamidino-2-phenylinodole (DAPI). Deletions were judged to be present if the number of signals from the test probe was less than that from D6Z1.

2.4. Construction of PAC/BAC contigs and genome sequencing

When the deletion mapping analysis predicts the presence of a relatively small overlapping deletion, we tried to make a contig of PAC and BAC clones that covers the entire overlapping deletion by end-sequencing of the seeding PAC/BAC clones followed by PCR-based gene walking. Briefly, PCR-primers were designed from the end sequences of the prior clone, and the next overlapping clones were isolated by PCR screening of appropriate PAC/BAC libraries using the primers. Each PAC/BAC clone was shotgun-


D1
D2
D3
D4
D5
D6
centromer
telomere
STS

sequenced according to the usual method with the phred/phrap as basecaller/assembler, which were kindly provided by Gordon et al. [14].

2.5. Southern blot analysis and mutation analysis

Southern blot analysis was performed as described previously [12]. Briefly, 10 μg of genomic DNAs extracted from patients' samples and cell lines were digested with an appropriate endonuclease to completion, separated on 1% agarose gel, and transferred to nylon membranes. The membranes were hybridized with 25 ng of denatured probe DNA labeled with 32P-dCTP in 5 × SSC, 50% formamide, 1 × Denhardt's solution, and 0.2 mg/ml of denatured salmon sperm DNA at 42 °C overnight. After high stringency wash with 0.1 × SSC and 0.1% SDS at 65 °C for 30 min, they were subjected to autoradiogram. Mutations were detected by direct sequencing of PCR-amplified target DNAs with or without prior screening of PCR products by SSCP analysis [15].

3. Results

3.1. Deletion mapping of 6q14–23 and identification of the critical deletions

Among 352 hematopoietic tumor samples examined, 61 samples had some deletions within 6q14–23 (Fig. 1). The deletions are distributed over the entire 6q14–23 segment. In the current analysis, there existed at least six discrete regions showing isolated overlapping deletions, designated as D1 to D6: a region from 6q16 to centromere (D1), and those flanked by D6S449 and AFMA084ZE9 (D2; 6q16.3–6q21), by D6S447 and WI-5694 (D3; 6q21–22), by WI-4066 and D6S1698 (D4; 6q22), by WI-4964 and WI-9588 (D5; 6q22–23), and by CHLC.GATA23B17 and WI-4227 (D6; 6q23). Deletions were most frequently observed in the D2 (50/372, 13.4%), followed by the D3 (49/372, 13.2%).

3.2. Sequencing analysis of the critical deletions

Since the critical deletions thus recognized have been the primary candidate loci for which putative TSGs are to be explored, we constructed contigs of PAC/BAC clones that spanned two of these critical regions, D2 and D3, for sequencing. The contig that covers the D2 deletion consists of five PAC/BAC clones. We determined the sequences of these five clones by the shotgun method. Within this region is encoded part of the glutamate kinate receptor 6 gene (GluR6), which is expressed mainly in neural tissues and is known

Fig. 1. A summary of deletion analysis for 61 hematopoietic tumor samples having deletion within 6q14–21 region. The presence or absence of deletion is designated by closed or open circles, respectively. PAC and BAC probes used for FISH analysis are listed in the right end column with their corresponding STS in the left end column. Cell lines and patients' samples are shown at the top of the figure. The regions expected to harbor deletion are indicated by shadows and the minimum regions showing overlapping deletions (D1 to D6) are depicted in the rightmost end with their demarcating STSs.

to mediate neural cell death following ischemic insults. Therefore, it was thought to be a candidate for TSG, and we examined a panel of tumor samples for possible mutations of this gene. Unfortunately, however, we could not find any tumor specific mutations of GluR6. We also examined the D2 region for the presence of small deletions by Southern blot analysis using a series of probes prepared at about 5-kb intervals within the region, but we did not find any homozygous deletions. Similarly, we sequenced the contig for the D3 deletion consisting of three PAC clones. In spite of the search for ESTs as well as inquiring the coding sequences with exon-prediction programs, we failed to identify any definitive transcriptomes from this region.

3.3. Identification of a candidate target gene Blimp1 from the minimum region undergoing homozygous deletions

Because our attempt to isolate a target gene from sequencing analysis of the D2 and D3 regions failed, we extended our FISH analysis to the outside of the D2 region using additional probes, and found a novel region showing homozygous deletion in a lymphoma-derived cell line (SKW4) which could be identified with PR1-101M23, located far telomeric to the D2 region. According to the published sequence of PR1-101M23 and its annotation information, it contains the Blimp1 gene near its centromeric end. Southern blot screening with a Blimp1 cDNA probe revealed that the other two cell lines (IZ86 and

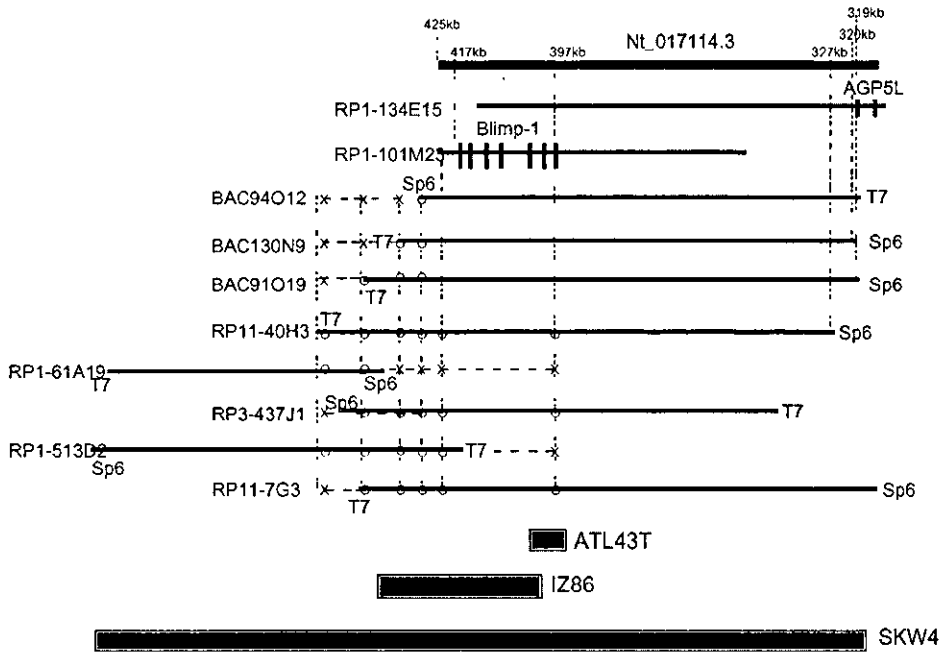


Fig. 2. A deletion map around the Blimp1 locus with overlapping PAC or BAC clones mapped to this locus. Black bars at the bottom indicate the extent of the homozygous deletion for each cell line as designated.

ATL-43T) also carried homozygous deletion at this locus. We mapped these homozygous deletions in more detail using a number of probes around the Blimp1 locus. As shown in Fig. 2, the homozygous deletions found in these three cell lines are mutually overlapped, and Blimp1 is the only gene that is commonly deleted in these three cell lines, implicating that Blimp1 is a feasible candidate for the TSG at this locus.

3.4. Mutation/deletion analysis of Blimp1 gene in primary hematopoietic tumor samples

In order to obtain further evidence that Blimp1 is the real target of this newly identified deletion, we performed mutation analysis of the Blimp1 gene using 106 primary lymphoid tumor samples in search of tumor-specific mutations. The presence of homozygous deletion was also examined for these samples by Southern blot analysis. Of the 106 samples, we found three cases carrying a point mutation of Blimp1 that accompanied amino acid conversion, while none of the mutations was identified in 100 samples from normal volunteers. There were no homozygous deletions of Blimp1 in these primary tumor samples.

4. Discussion

Chromosomal deletion is one of the most commonly observed cytogenetic abnormalities in human cancers [3]. It has long been postulated that it represents a general mechanism through which one or more TSGs are inactivated. According to this hypothesis, we have made an attempt to isolate TSGs from one of these deletions, the deletion of the long arm of chromosome 6, and identified the Blimp1 gene as a candidate. It was homozygously deleted in three lymphoma-derived cell lines, and was the only gene found in the common deletion among these. Moreover, mutation analysis of 106 primary hematopoietic tumor samples disclosed three tumor-specific mutations of Blimp1.

Blimp1, also known as PRDM-1 or PRDI-BF1, is a 98 KD zinc finger protein originally isolated as a master regulator of the terminal differentiation of B-lymphocytes to immunoglobulin-producing plasma cells [16]. It is ubiquitously expressed in a wide variety of human tissues, and presumed to function as a transcriptional repressor. Recently, it has been functionally related to Bcl-6 and c-*myc* proto-oncogenes, which are overexpressed in B-cell lymphomas (Fig. 3). Blimp1 potently represses expression of c-*myc*,

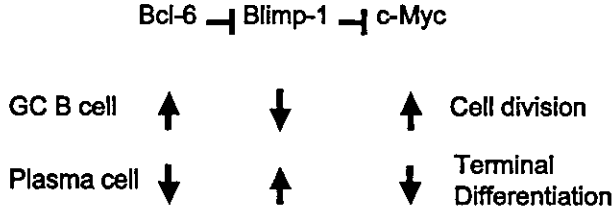


Fig. 3. Regulation of Blimp1 and c-*myc* expression in normal germinal center (GC) B-cells and plasma cells. Deregulated overexpression of Bcl-6 and c-*myc* proteins as well as loss of Blimp1 expression would lead to stalled differentiation of B-cells to plasma cells and continued cell divisions.

which is one of the critical events for terminal differentiation of B-cells [17]. On the other hand, Bcl6 has been shown to directly repress Blimp1 transcription. It is expressed normally proliferating germinal centers, represses Blimp1, and maintains c-*myc* expression that is required for B-cell proliferation. In contrast, down-regulation of Bcl6 results in Blimp1 expression, which shuts off the c-*myc* expression and leads to terminal differentiation [18,19]. Moreover, recent data suggests that Blimp1 is also involved in the terminal differentiation of myeloid cells [20]. Because overexpression of Bcl6 and c-*myc*, as a result of chromosomal translocations, is a well-known feature of B-cell lymphomas [21], it may be postulated that the loss of Blimp1 plays a role in lymphomagenesis. There is, however, for the time being, no direct evidence that the loss of Blimp1 is involved in tumorigenesis. Because Blimp1-null mice are known to be fatal during embryogenesis, construction of conditionally Blimp1-targeted mice is in progress.

We revealed several non-overlapping critical deletions (D1 to D6, and Blimp1 loci) within 6q14–23 among 61 hematopoietic tumor samples through extensive deletion analysis, implicating that multiple tumor suppressor loci might exist within this chromosomal region. Some of these deletions were closely overlapped with the regions previously described, through different approaches, as the targets for 6q-deletion in leukemias and lymphomas [22–24]. However, a candidate for the target TSG has been successfully identified from only one of these regions. Since the margins of these critical regions had been defined based on the deletions in only one or two cell lines, we could not exclude a possibility that such deletions were merely a chance product, which might impose an intrinsic limitation of exploring chromosomal deletion through the current approach: defining a minimally overlapping deletion through deletion mapping among different samples, and then isolating a target gene from the minimum deletion. It will require many samples showing relatively small deletion in order to reliably pinpoint the critical regions, whereas most of the deletions thus far detected by FISH analysis involved a very large chromosomal segment. This might be partly because the number of probes used for our deletion mapping was very small relative to the large size of chromosomal bands to be investigated. For more reliable mapping, hundreds of genetic markers should be included in the deletion study by introducing more efficient methods for detecting deletions such as microarray-based CGH analysis [25].

It is a challenging trial to find out a TSG from a very large segment of chromosomal deletion. We need to bear in mind that even "haplo-insufficiency" of some genes can predispose to cancer development. Abnormal hypermethylation of the CpG island of the promoter has also been proposed as a novel mechanism of gene inactivation. With these mechanics being at work, the current approach to chromosomal deletion, including ours, does not seem clear enough to be more effective. A more sophisticated strategy should be contrived for the future investigations.

Acknowledgements

We thank Ying Qiao, Lili Wang, Masahiro Kami (Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, Tokyo), Hiroyuki Aburatani (Genome Science Division, Research Center for Advanced Science and

Technology, University of Tokyo, Tokyo), and Johji Inazawa (Department of Molecular Cytogenetics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo) for their technical collaborations. Cell lines and patients' samples were kindly provided by Yoshinobu Matsuo (Fujisaki Cell Centre, Hayashibara Biochemical Laboratories, Okayama), Kazuma Ohyashiki (First Department of Internal Medicine, Tokyo Medical University, Tokyo), Yasunori Kawaji (Asahikawa City Hospital, Asahikawa), Yasuhide Hayashi (Department of Pediatrics, Graduate School of Medicine, University of Tokyo, Tokyo), Yasusada Miura (Social Insurance Chuo General Hospital, Tokyo), Tatsuo Abe (Department of Hygiene, Kyoto Prefectural University of Medicine, Kyoto), Nanao Kamada (Department of Cancer Cytogenetics, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima), Kenshi Suzuki (Department of Hematology, Japanese Red Cross Medical Center, Tokyo), Takanori Ueda (First Department of Internal Medicine, Fukui Medical University, Fukui), Norio Asou (Second Department of Internal Medicine, Kumamoto University School of Medicine, Kumamoto), Yasunobu Kuraishi (Department of Internal Medicine, Jikei University School of Medicine, Tokyo), Eiji Tatsumi (International Center for Medical Research, Kobe University School of Medicine, Kobe), Michiyuki Maeda (Institute for Frontier Medical Science, Kyoto University, Kyoto), Ryuzo Ueda (Second Department of Internal Medicine, Nagoya City University School of Medicine, Nagoya), and Ryuzo Ohno (Aichi Cancer Center Hospital, Nagoya). This study was supported in part by the Uehara Memorial Foundation, Fellowships in Cancer Research of the Japan Society for the Promotion of Science for Young Scientists, and Japan Adult Leukemia Study Group.

References

[1] F. Mitelman, Catalog of Chromosome Aberration in Cancer '98, Wiley-Liss, New York, 1998.
[2] J. Scandura, P. Boccuni, J. Cammenga, S. Nimer, Transcription factor fusions in acute leukemia: variations on a thema, Oncogene 21 (2002) 3422–3444.
[3] S. Thiagalingam, R. Foy, K. Cheng, H. Lee, A. Thiagalingam, J. Ponte, Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence, Current Opinion in Oncology 14 (1) (2002) 65–72.
[4] R. Strausberg, K. Buetow, M. Emmert-Buck, R. Klausner, The cancer genome anatomy project: building an annotated gene index, Trends in Genetics 16 (3) (2000) 103–106.
[5] J. Kowalczyk, M. Grossi, A. Sandberg, Cytogenetic findings in childhood acute lymphoblastic leukemia, Cancer Genetics and Cytogenetics 15 (1–2) (1985) 47–64.
[6] Y. Hayashi, S.C. Raimondi, A.T. Look, F.G. Behm, G.R. Kitchingman, C.H. Pui, G.K. Rivera, D.L. Williams, Abnormalities of the long arm of chromosome 6 in childhood acute lymphoblastic leukemia, Blood 76 (8) (1990) 1626–1630.
[7] G. Gaidano, R.S. Hauptschein, N.Z. Parsa, K. Offit, P.H. Rao, G. Lenoir, D.M. Knowles, R.S. Chaganti, F.R. Dalla, Deletions involving two distinct regions of 6q in B-cell non-Hodgkin lymphoma, Blood 80 (7) (1992) 1781–1787.
[8] A. Jackson, P. Carrara, V. Duke, P. Sinclair, M. Papaioannou, C.J. Harrison, L. Foroni, Deletion of 6q16–q21 in human lymphoid malignancies: a mapping and deletion analysis, Cancer Research 60 (11) (2000) 2775–2779.
[9] K.A. Cooney, J.C. Wetzel, C.M. Consolino, K.J. Wojno, Identification and characterization of proximal 6q deletions in prostate cancer, Cancer Research 56 (18) (1996) 4150–4153.
[10] V. Orphanos, G. McGown, Y. Hey, J.M. Boyle, K.M. Santibanez, Proximal 6q, a region showing allele loss in primary breast cancer, British Journal of Cancer 71 (2) (1995) 290–293.

[11] K.J. Rodabaugh, G. Blanchard, W.R. Welch, D.A. Bell, R.S. Berkowitz, S.C. Mok, Detailed deletion mapping of chromosome 6q in borderline epithelial ovarian tumors, Cancer Research 55 (10) (1995) 2169–2172.
[12] S. Ogawa, N. Hirano, N. Sato, T. Takahashi, A. Hangaishi, K. Tanaka, M. Kurokawa, T. Tanaka, K. Mitani, Y. Yazaki, et al., Homozygous loss of the cyclin-dependent kinase 4-inhibitor (p16) gene in human leukemias, Blood 84 (8) (1994) 2431–2435.
[13] S. Ogawa, M. Kurokawa, T. Tanaka, K. Mitani, J. Inazawa, A. Hangaishi, K. Tanaka Y. Matsuo, J. Minowada, T. Tsubota, Y. Yazaki, H. Hirai, Structurally altered Evi-1 protein generated in the 3q21q26 syndrome, Oncogene 13 (1) (1996) 183–191.
[14] D. Gordon, C. Abajian, P. Green, Consed: a graphical tool for sequence finishing, Genome Research 8 (3) (1998) 186–194.
[15] A. Hangaishi, S. Ogawa, N. Imamura, S. Miyawaki, Y. Miura, N. Uike, C. Shimazaki, N. Emi, K. Takeyama, S. Hirosawa, N. Kamada, Y. Kobayashi, Y. Takemoto, T. Kitani, K. Toyama, S. Ohtake, Y. Yazaki, R. Ueda, H. Hirai, Inactivation of multiple tumor-suppressor genes involved in negative regulation of the cell cycle, MTS1/p16INK4A/CDKN2, MTS2/p15INK4B, p53, and Rb genes in primary lymphoid malignancies, Blood 87 (12) (1996) 4949–4958.
[16] C.A. Turner Jr., D.H. Mack, M.M. Davis, Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells, Cell 77 (2) (1994) 297–306.
[17] Y. Lin, K. Wong, K. Calame, Repression of c-*myc* transcription by Blimp-1, an inducer of terminal B cell differentiation, Science 276 (5312) (1997) 596–599.
[18] G. Cattoretti, C.C. Chang, K. Cechova, J. Zhang, B.H. Ye, B. Falini, D.C. Louie, K. Offit, R.S. Chaganti, R. Dalla-Favera, BCL-6 protein is expressed in germinal-center B cells, Blood 86 (1) (1995) 45–53.
[19] A.L. Shaffer, X. Yu, Y. He, J. Boldrick, E.P. Chan, L.M. Staudt, BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control, Immunity 13 (2) (2000) 199–212.
[20] D.H. Chang, C. Angelin-Duclos, K. Calame, BLIMP-1: trigger for differentiation of myeloid lineage, Nature Immunology 1 (2) (2000) 169–176.
[21] U. Vitolo, G. Gaidano, B. Botto, G. Volpe, E. Audisio, M. Bertini, R. Calvi, R. Freilone, D. Novero, L. Orsucci, C. Pastore, D. Capello, G. Parvis, C. Sacco, V. Zagonel, A. Carbone, U. Mazza, G. Palestro, G. Saglio, L. Resegotti, Rearrangements of bcl-6, bcl-2, c-*myc* and 6q deletion in B-diffuse large-cell lymphoma: clinical relevance in 71 patients, Annals of Oncology 9 (1) (1998) 55–61.
[22] L.P. Menasce, V. Orphanos, K.M. Santibanez, J.M. Boyle, C.J. Harrison, Deletion of a common region on the long arm of chromosome 6 in acute lymphoblastic leukemia, Genes, Chromosomes and Cancer 10 (1) (1994) 26–29.
[23] M. Merup, T.C. Moreno, M. Heyman, K. Ronnberg, D. Grander, R. Detlofsson, O. Rasool, Y. Liu, S. Soderhall, G. Juliusson, G. Gahrton, S. Einhorn, 6q deletions in acute lymphoblastic leukemia and non-Hodgkin's lymphomas, Blood 91 (9) (1998) 3397–3400.
[24] T. Sherratt, C. Morelli, J.M. Boyle, C.J. Harrison, Analysis of chromosome 6 deletions in lymphoid malignancies provides evidence for a region of minimal deletion within a 2-megabase segment of 6q21, Chromosome Research 5 (2) (1997) 118–124.
[25] A. Snijders, N. Nowak, R. Segraves, S. Blackwood, N. Brown, J. Conroy, G. Hamilton, A. Hindle, B. Huey, K. Kimura, S. Law, K. Myambo, J. Palmer, B. Ylstra, J. Yue, J. Gray, A. Jain, D. Pinkel, D. Albertson, Assembly of microarrays for genome-wide measurement of DNA copy number, Nature Genetics 29 (3) (2001) 263–264.

based on the presence of multilineage (2 or 3 myeloid cell lines) or unilineage (mainly affecting the erythroid series) dysplasia.

Unfortunately the reference quoted[3] for the WHO classification did not provide sufficient information on the precise criteria described in detail in the recently published WHO manual,[4] and, in addition, the authors do not reference the Germing et al paper,[5] which confirms these new proposals. An additional review of the new WHO classification was published before the final criteria of the precise percent of dysplastic cells and the consideration of merging the dysplastic and proliferative forms of CMML were agreed upon.[6]

The major difficulty we have with the Nösslinger et al study is the adoption of the "50%" criteria for dysplasia in 2 or more cell lines for refractory cytopenia with multilineage dysplasia (RCMD). In the WHO proposals the threshold of 50% dysplasia has been utilized only in identifying AML with multilineage dysplasia[7] but not for the MDS category of RCMD. In fact, in the WHO proposals RCMD is defined as an MDS subgroup with fewer than 5% blasts in the bone marrow, and dysplasia in 10% or more of the cells of 2 or more myeloid lineages (erythroid, granulocytic, and/or megakaryocytic). These criteria were adapted in the study of 1600 patients with MDS by Germing et al,[5] although they did elect to use a 40% threshold for megakaryocytes. Germing et al and others[8,9] have confirmed the worse prognosis of RCMD compared to RA or RARS. To accurately evaluate the WHO proposals it will be necessary to reassess the "unclassified" group in the Nösslinger et al study utilizing these criteria. It is very likely that the "unclassified" category (MDS-U) would diminish considerably, impacting the survival results.

In addition others have demonstrated that the survival of CMML is dependent on the bone marrow blast percentage[10] and that CMML is much more heterogeneous than other subtypes of MDS. In order to emphasize the prognostic importance of the blast percentage in CMML the WHO classification divides CMML into 2 categories, CMML-1 and CMML-2, depending on the blast count in the peripheral blood and the bone marrow. It does not subdivide CMML according to the white blood cell count. In Table 1 of the Nösslinger et al article,[1] CMML resembles RAEB in the International Prognostic Scoring System[11] (IPSS) distribution. The separation of RAEB into 2 types (5%-9% blasts and 10%-19% blasts) is of importance as the authors demonstrate with a significant difference in IPPS distribution. A similar analysis of their patients with CMML should be performed. Confirmation of the similarities in outcome for RAEB-T and AML in the Nösslinger et al study provides further evidence in support of allowing such patients (20%-30% marrow blasts) to enter AML trials where appropriate.

In summary it is our hope that Nösslinger and colleagues will consider reviewing their data using the recently published WHO criteria. Such an effort would be important, because, although the Nösslinger et al study is interesting, it does not justify any statement about the validity of the WHO classification. We anticipate that a new look at the data of Germing et al would confirm the conclusion that the WHO system does provide improved and relevant guidelines for the classification of patients with MDS.

John M. Bennett, Richard D. Brunning, and James W. Vardiman

Correspondence: John M. Bennett, James P. Wilmot Cancer Center, University of Rochester Medical Center, 601 Elmwood Ave, Box 704, Rochester, NY 14642

References

1. Nösslinger T, Reisner R, Koller E, et al. Myelodysplastic syndromes, from French-American-British to World Health Organization: comparison of classifications on 431 unselected patients from a single institution. Blood. 2001;98: 2935-2941.
2. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the myelodysplastic syndromes. Br J Haematol. 1982;51:189-199.
3. Harris NL, Jaffe ES, Diebold J, et al. World Health Organization of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the clinical advisory committee meeting—Airlie House, Virginia, November 1997. J Clin Oncol. 1999:17:3835-3849.
4. Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. World Health Organization classification of tumours: pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon, France: IARC Press; 2001:47-73.
5. Germing U, Gattermann N, Strupp C, et al. Validation of the WHO proposals for a new classification of the myelodysplastic syndromes: a retrospective analysis of 1600 patients. Leuk Res. 2000;24:983-992.
6. Bennett JM. World Health Organization Classification of the acute leukemias and myelodysplastic syndrome. Int J Hematol. 2000;72:131-133.
7. Gahn B, Haase D, Unterhalt M, et al. *De novo* AML with dysplastic hematopoiesis: cytogenetic and prognostic significance. Leukemia. 1996;10:946-951.
8. Rosati S, Mick R, Xu F, et al. Refractory cytopenia with multilineage dysplasia: further characterization of an 'unclassifiable' myelodysplastic syndrome. Leukemia. 1996;10:20-26.
9. Balduini CL, Guarnone R, Pecci A, et al. Multilineage dysplasia without increased blasts identifies a poor prognosis subset of myelodysplastic syndromes. Leukemia. 1998;12:1655-1656.
10. Storniolo AM, Moloney WC, Rosenthal DS, Cox, and Bennett JM. Chronic myelomonocytic leukemia. Leukemia. 1990;4:766-770.
11. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood. 1997;89:2079-2088.

To the editor:

Mutations of Chk2 in primary hematopoietic neoplasms

Chk2 is a novel checkpoint kinase isolated as a human homologue of yeast Cds1/Rad53.[1] Recent analyses have revealed that it is among key molecules signaling DNA damage via the ATM protein kinase to p53.[1,2] Of great interest is the report that germ line mutations of the *Chk2* gene are found in a fraction of Li-Fraumeni syndrome (LFS),[3] a hereditary cancer-susceptibility syndrome originally linked with germ line *p53* mutations, suggesting that *Chk2* is a tumor suppressor gene whose functional deficit will lead to development of human cancers. Given that the *p53* and *ATM* genes are inactive in leukemias and lymphomas, it is intriguing to investigate whether or not somatic mutations of *Chk2* are also responsible for leukemias and lymphomas. To address this point, we screened for mutations of *Chk2* in a variety of human hematopoietic neoplasms.

A total of 109 tumor specimens of hematopoietic malignant disorders were examined for mutations of *Chk2* using reverse transcriptase–polymerase chain reaction/single strand conformational polymorphism (RT-PCR/SSCP) analysis. Numbers and diagnoses of these patients are listed in Table 1. Two samples showed abnormally migrating bands on RT-PCR/SSCP analysis of the *Chk2* transcripts (patient 1375 and patient 154), and the nucleotide alterations were further confirmed by sequencing analysis in both cases (Figure 1 and Table 2).

Table 1. Frequency of *Chk2*, *p53*, and *p16* alterations in primary hematopoietic neoplasms

Diagnosis	No. of patients	No. of mutations		
		Chk2	*p53*	*p16*
ALL	14	0	0	5
AML	55	1	5	1
CML	12	0	0	0
CLL	5	0	1	1
PLL	2	0	1	0
NHL	7	1	0	1
ATL	4	0	0	1
MM	2	0	0	0
MDS	8	0	0	0
Total	109	2	7	9

ALL indicates acute lymphocytic leukemia; AML, acute myelocytic leukemia; CML, chronic myelocytic leukemia; CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia; NHL, non-Hodgkin lymphoma; ATL, adult T-cell lymphoma/leukemia; MM, multiple myeloma; MDS, myelodysplastic syndrome.

Patient 1375 was diagnosed with acute myeloid leukemia (AML), French-American-British subtype M1, and had a 7-bp insertion at the boundary of exons 10 and 11 of *Chk2* (Figure 1A and Table 2), which caused a frameshift of the coding sequence and resulted in premature truncation of the protein at codon 424. Sequencing analysis of the corresponding genomic sequence revealed an A>G substitution at the splicing acceptor site of the intron 10, 8 bp before exon 11, suggesting that the mutation created an alternative splicing acceptor site 7 bp upstream from the original one and resulted in the 7-bp insertion between exons 10 and 11. Because a DNA sample from his normal skin showed an A/A genotype at this position, this is really a somatic mutation (Figure 1A). Because the RT-PCR/SSCP analysis showed exclusively abnormally migrating bands, function of Chk2 is expected to be lost in patient 1375.

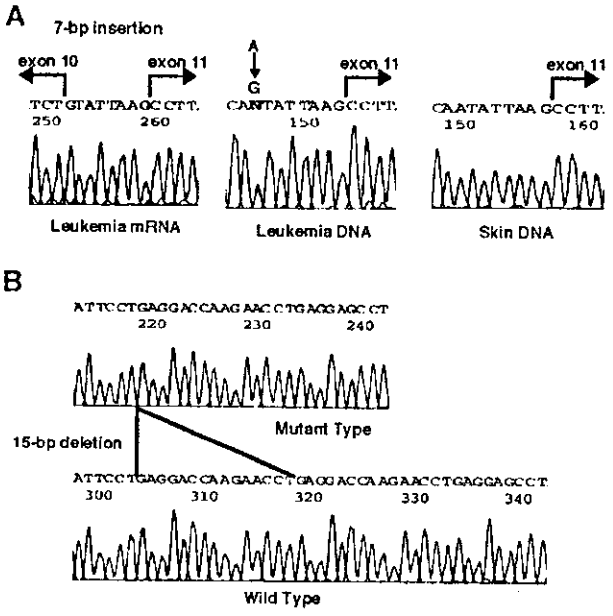


Figure 1. Sequencing analysis of the *Chk2* gene. (A) Direct sequencing of the message of patient 1375's leukemia cells showed a 7-bp insertion between exons 10 and 11 (left). Sequencing of the Chk2 exon 11–F/R–amplified PCR products of patient 1375's DNAs revealed that a A>G nucleotide change in the consensus splice-acceptor site of intron 10 was only shown in leukemia DNA (center) but not in germ-line DNA from patient 1375's skin (right). (B) Patient 154 showed a 15-bp deletion between codons 75 and 79 of *Chk2* (upper) and wild-type sequence of *Chk2* (lower).

Table 2. Summary of *Chk2* alterations in primary hematopoietic neoplasms

Patient	Diagnosis	DNA position	Alteration	Transcript
154	NHL	223-237	15-bp deletion	5 amino acids' deletion
1375	AML(M1)	intron 10 8-bp before exon 11	A > G transition	7-bp insertion between exons 10 and 11 stop codon at 424

The other patient, patient 154, was diagnosed with non-Hodgkin lymphoma (NHL), with mantle cell morphology. Direct sequencing of the abnormal bands on the SSCP analysis revealed a 15-bp deletion between codons 75 and 79. The 15-bp deletion resulted in loss of 5 amino acids as shown in Figure 1B and Table 2. The deleted 15 nucleotides are a half of the two 15-bp repeats between codons 75 and 84. Because genomic sequences of both tumor and normal samples also had the 15-bp deletion, this deletion was most likely to be a germ-line mutation. In this case, normally migrated bands were also detected. But because this sample was apparently contaminated by normal bone marrow cells, we could not determine whether it represented a residual allele in tumor cells or it was derived from the contaminated normal cells and the tumor cells themselves lacked a wild-type allele.

We compared the mutation rate of *Chk2* with those of other well-known tumor-suppressor genes in the same panel of 109 hematopoietic neoplasms. *p53* was mutated in 7 samples (6.4%), while homozygous deletion of *p16* was identified in 9 samples (8.3%). There appeared to be a tendency that more *p53* mutations were found in AML and *p16* deletions occurred preferentially in acute lymphoid leukemia (ALL). Distributions of these mutations are summarized in Table 1. There were no overlapping mutations of *Chk2*, *p53*, and *p16*, except for in patient 1375, in whom, in addition to the *Chk2* mutation described above, a missense mutation (TGT>TAT, Cys→Tyr) at codon 238 in *p53* existed. On the other hand, patient 154 also had an additional genetic alteration that may affect cell-cycle regulation. Because her lymphoma cells had a t(11;14)(q13;q32) translocation with rearrangement between the cyclin D1 gene and the J_H region of immunoglobulin heavy chain causing overexpression of cyclin D1.

It is noteworthy that both *Chk2* mutations were compounded with other genetic alterations that were presumed to disrupt the G1 checkpoint mechanism. The first patient (patient 1375) had a point mutation in *p53*, and the second (patient 154) carried a t(11;14)(q13;q32) translocation with overexpression of cyclin D1. In this context, it may be worth mentioning that the other case of *Chk2* mutation thus far reported in a case with small-cell lung cancer also carried mutation of *p53*.[4] In these cases, both G1 and G2 checkpoint regulations are simultaneously abrogated; *p53* mutation and overexpression of cyclin D1 will affect G1 regulation, and the *Chk2* mutations will be associated with compromised G2 checkpoint. The *Chk2* mutation associated with mantle cell lymphoma (MCL) carrying t(11;14)(q13;q32) may be in parallel with a recent observation that *ATM*, an upstream regulator of Chk2 kinase, is frequently inactivated in MCL.[5] Mice null for both *p53* and *ATM* genes show accelerated tumor growth as compared with mice null only for either *p53* or *ATM* alone.[6] Thus compounded G1 and G2 checkpoint abnormalities might confer more proliferative or anti-apoptotic advantages upon the tumor cells.

In conclusion, sporadic mutation of *Chk2* is rare in hematopoietic neoplasms. Recently Hofmann et al also reported a similar observation in myelodysplastic syndrome (MDS) and AML, where

only one MDS case had a *Chk2* mutation.[7] *Chk2* is rarely mutated in sporadic cases of small-cell lung cancers and tumor-derived cell lines.[3,4] While germ-line mutations of *Chk2* predispose to several cancers in LFS, our and others' observations indicate that *Chk2* belongs to a tumor suppressor gene of a "caretaker" type, just like *hMLH1* and *BRCA1*.[8] Inactivation of *Chk2* itself may not be sufficient for tumorigenesis but could induce a kind of genetic instability, which will facilitate the oncogenic processes in pathogenesis of sporadic cancers, including hematopoietic neoplasms.

Akira Hangaishi, Seishi Ogawa, Ying Qiao, Lili Wang, Noriko Hosoya, Koichiro Yuji, Yoichi Imai, Kengo Takeuchi, Shuichi Miyawaki, and Hisamaru Hirai

Correspondence: Hisamaru Hirai, Department of Hematology & Oncology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; e-mail: hhirai-tky@umin.ac.jp

Supported in part by Fellowships in Cancer Research of the Japan Society for the Promotion of Science for Young Scientists.

References

1. Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science. 1998;282:1893-1897.
2. Hirao A, Kong YY, Matsuoka S, et al. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science. 2000;287:1824-1827.
3. Bell DW, Varley JM, Szydlo TE, et al. Heterozygous germ line hChk2 mutations in Li Fraumeni syndrome. Science. 1999;286:2528-2531.
4. Haruki N, Saito H, Tatematsu Y, et al. Histological type-selective, tumor-predominant expression of a novel CHK1 isoform and infrequent in vivo somatic Chk2 mutation in small cell lung cancer. Cancer Res. 2000;60:4689-4692.
5. Schaffner C, Idler I, Stilgenbauer S, Dohner H, Lichter P. Mantle cell lymphoma is characterized by inactivation of the ATM gene. Proc Natl Acad Sci U S A. 2000;97:2773-2778.
6. Westphal CH, Rowan S, Schmaltz C, Elson A, Fisher DE, Leder P. atm and p53 cooperate in apoptosis and suppression of tumorigenesis, but not in resistance to acute radiation toxicity. Nat Genet. 1997;16:397-401.
7. Hofmann WK, Miller CW, Tsukasaki K, et al. Mutation analysis of the DNA-damage checkpoint gene CHK2 in myelodysplastic syndromes and acute myeloid leukemias. Leuk Res. 2001;25:333-338.
8. Kinzler KW, Vogelstein B. Cancer-susceptibility genes: gatekeepers and caretakers [news; comment]. Nature. 1997;386:761,763.

Oncogene (2002) 21, 2695–2703
© 2002 Nature Publishing Group All rights reserved 0950–9232/02 $25.00
www.nature.com/onc

The t(3;21) fusion product, AML1/Evi-1 blocks AML1-induced transactivation by recruiting CtBP

Koji Izutsu[1], Mineo Kurokawa[1], Yoichi Imai[1], Motoshi Ichikawa[1], Takashi Asai[1], Kazuhiro Maki[2], Kinuko Mitani[2] and Hisamaru Hirai*[,1]

[1]*Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan;*
[2]*Department of Hematology, Dokkyo University School of Medicine, Tochigi 321-0293, Japan*

AML1/Evi-1 is a chimeric protein that is derived from t(3;21), found in blastic transformation of chronic myelogenous leukemia. It is composed of the N-terminal AML1 portion with the DNA-binding Runt domain and the C-terminal Evi-1 portion. It has been shown to dominantly repress AML1-induced transactivation. The mechanism for it has been mainly attributed to competition with AML1 for the DNA-binding and for the interaction with PEBP2β (CBFβ), a partner protein which heterodimerizes with AML1. It was recently found that Evi-1 interacts with C-terminal binding protein (CtBP) to repress TGFβ-induced transactivation. Here, we demonstrate that AML1/Evi-1 interacts with CtBP in SKH1 cells, a leukemic cell line which endogenously overexpresses AML1/Evi-1 and that AML1/Evi-1 requires the interaction with CtBP to repress AML1-induced transactivation. The association with CtBP is also required when AML1/Evi-1 blocks myeloid differentiation of 32Dcl3 cells induced by granulocyte colony-stimulating factor. Taken together, it is suggested that one of the mechanisms for AML1/Evi-1-associated leukemogenesis should be an aberrant recruitment of a corepressor complex by the chimeric protein.
Oncogene (2002) **21**, 2695–2703. DOI: 10.1038/sj/onc/1205356

Keywords: AML1/Evi-1; CtBP; AML1; dominant negative

Introduction

The *AML1* gene, which is located in human chromosome 21q22, was originally identified in the cells of acute myelogenous leukemia with t(8;21) (Miyoshi *et al.*, 1991). The AML1 protein (also called as PEBP2αB, CBFA2, or Runx1) is a member of the AML1 transcription factor family, which is also called as polyomavirus enhancer binding protein 2 (PEBP2), core binding factor (CBF) or most recently, Runx (Wheeler *et al.*, 2000). Runx proteins share the Runt domain, which has homology to a *Drosophila* protein, runt. Through this domain, Runx proteins heterodimerize with a β subunit, PEBP2β (CBFβ), which is common to each Runx family member. The heterodimers bind to the cognate sequence of DNA, which is called the PEBP2-binding site, in the regulatory region of target genes. This protein-DNA interaction is also mediated through the Runt domain. Three members of this family have been identified to date, each of which is reported to have a unique role in embryogenesis and development. AML1-null mice are not able to establish adult type hematopoiesis, and succumb to intracranial hemorrhage around embryonic day 12.5 (Okuda *et al.*, 1996). Thus, AML1 seems to be essential for establishing the hematopoietic system during embryonic ontogeny (Westendorf and Hiebert, 1999). It remains to be elucidated precisely what are the downstream targets of AML1 during induction of adult type hematopoiesis. Several hematopoietic lineage-specific genes, however, are shown to be regulated by AML1. Among them are the genes for myeloperoxidase (MPO), neutrophil elastase, granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor, macrophage colony-stimulating factor (M-CSF) receptor, and T-cell receptor β.

On the other hand, alterations in the *AML1* gene closely associate with the development of hematological disorders in humans. The *AML1* gene is recurrently involved in leukemia-associated chromosomal translocations (Mitani, 1997). The translocations generate fusion transcripts and the resulting chimeric proteins that consist of AML1 and a product from each partner gene. AML1/Evi-1, AML1/ETO(MTG8), and TEL/AML1 chimeric proteins are generated by t(3;21), t(8;21), and t(12;21), respectively (Golub *et al.*, 1995; Mitani *et al.*, 1994; Miyoshi *et al.*, 1993). Germ line mutations in the *AML1* gene are reported in the pedigrees of familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) (Song *et al.*, 1999). Somatic mutations in *AML1* have been repeatedly found in cases of acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS) (Imai *et al.*, 2000; Osato *et al.*, 1999). These

*Correspondence: H Hirai, Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan;
E-mail: hhirai-tky@umin.ac.jp
Received 18 September 2001; revised 21 January 2002; accepted 22 January 2002