Table 6 Life-table estimates of survival at 12 years by risk group for CNS regimens

	CNS regimens by risk group	*Measure of survival, % (s.e.)*			
		CNS relapse-free		*BM relapse-free*	
Low risk					
L874A	IT ×3+CRT (18 Gy)	97.2 (3.2)	ns	77.2 (6.8)	ns
L874B	IT ×13+HDMTX ×3	90.2 (5.4)		82.6 (6.5)	
L911	Same as in L874B	90.7 (4.5)		85.3 (5.6)	
Intermediate risk					
I841B	IT ×3+CRT (18 Gy)	87.5 (8.3)	ns	67.9 (11.1)	ns
I841C	IT ×1+HDMTX ×5	87.1 (5.4)		72.3 (6.9)	
I874A	IT ×3+HDMTX ×1+CRT (18 Gy)	100	$P=0.01$	68.1 (6.8)	ns
I874B	IT ×1+HDMTX ×4	79.3 (6.2)		70.6 (7.0)	
I911	TIT ×24	98.1 (1.9)		83.9 (1.9)	
High risk					
H851A	IT ×3+HDMTX ×6	74.7 (8.6)		31.9 (9.6)	
H851B	Same as in H851A	60.2 (9.6)		55.8 (9.8)	
H874A	TIT ×8+HDMTX ×3+CRT (18 Gy)	95.5 (2.5)		67.8 (5.4)	
H874B	Same as in H874A	88.3 (5.6)		43.3 (6.6)	
H911	TIT ×6+CRT (18 Gy)	92.4 (3.3)		77.4 (5.1)	
HH911	TIT ×6+CRT (24 Gy)	71.7 (11.1)		50.1 (11.0)	

Abbreviations: BM, bone marrow; CRT, cranial radiation; HDMTX, high-dose MTX; IT, intrathecal MTX; MTX, methotrexate; ns, not significant; TIT, triple IT.
HDMTX: 100 mg/kg for I841C; 2 g/m^2 for L874B/I874A/L911; 4.5 g/m^2 for I874B; 3 g/m^2 for H874A/B. Folinic acid (15 mg/m^2) was given orally every 6 h for a total seven doses. Rescue begins 36 h from the start of MTX infusion.

hepatocarcinoma and one further I874A patient developed a malignant fibrous histocytoma.

ALL911. A total of 230 children were enrolled in the ALL911 study, of which 223 patients were evaluable. Ages ranged from 0.7 to 15.8 years, with a median of 5.0. In this study, HHR patient outcome was extremely poor compared with that of HR patients. On the other hand, HR group outcomes compared favorably to those of both LR and IR groups. Unfavorable prognostic features included NCI high-risk, older age (>10 years) and very high WBC counts (≧100 × 10^9/l), in descending order (Table 5d). One HHR911 patient developed myelodysplatic syndrome and one IR911 patient developed chronic myelogenous leukemia.

Extramedullary relapse

Table 6 shows CNS relapse-free survival by the CNS prophylaxis regimen. In our four studies, more than half of the patients received prophylactic CRT and proportion of the patients in each study is shown in Table 2. From the three protocols including L874, I841 and I874, two regimens with or without CRT were compared with respect to their ability to prevent CNS leukemia and to improve the overall ALL outcome. The CRT regimens seem to be associated with better CNS-free survival outcome than the HDMTX regimen. However, the difference was significant in only I874 but not other studies (L874 and I841), and no significant difference in systemic survival rates was observed between the two CNS regimens in either risk group. The CNS remission rate of patients treated with the I911 protocol was significantly higher than that of patients given the HDMTX regimen in the I874B protocol ($P=0.0127$), and was comparable to that of patients given the CRT regimen in the I874A protocol. In HR groups, HDMTX regimens without CRT (H851A/B) showed very poor CNS-free survival outcomes.

The incidence of isolated testicular relapse was very different between maintenance regimens. In the randomized study of SR patients in ALL811, only one of 36 (2.8%) males who received an intermittent cyclic regimen of MTX and 6MP (S811A) developed testicular relapse, in contrast to seven of 31 (22.6%) males receiving continuous administration of low-dose MTX and 6MP for maintenance chemotherapy (S811B). In addition, from the 841 protocol, only seven additional testicular relapses were seen for 497 males (Table 4).

Late effects

Questionnaire results about late adverse effects in the four studies (ALL811, ALL841, ALL874 and ALL911) were obtained from 521 of 640 (81.4%) living patients and from 313 of 381 (82.1%) deceased patients. The results are shown in Table 7. Late effects were observed in 70/834 (8.4%) patients, of whom 17 had at least two late effects. Hepatitis and short stature were most commonly reported, followed by secondary malignancy, disturbed neurocognitive function, gonadal dysfunction and cardiomyopathy. There were no cases of osteoporosis or osteopenia. More than half the patients with short stature, leukoencephalopathy or visual disturbance received CRT.

Since late adverse effects are closely correlated with cumulative doses of cytotoxic drugs used in treatment protocols, we highlighted such cumulative doses of cytotoxic drugs and antimetabolites in each protocol (Table 8). In ALL811, CPM and DOX were used only for the HR protocols. In ALL841, CPM was used for only HR, but DOX was used for the HR and I841C protocols. In ALL874, CPM and anthracycline were used, as in ALL841. In ALL911, CPM was used for the IR and HR protocols and anthracycline was used for all the protocols, including SR patients. Consequently, cumulative doses of anthracycline exceeded 400 mg/m^2 in nine protocols (HR811B, IR841C, HR841B, IR874A and IR874B and 911LR/IR/HR/HHR) and

Table 7 Late adverse effects of patients in the JCCLSG ALL studies

Study	*ALL811*	*ALL841*	*ALL874*	*ALL911*	*Total*	*Relevant factors*	
No. of patients (alive/deceased)	*81/68*	*107/76*	*214/122*	*148/52*	*550/318*	*Relapse*	*CRT*
Short stature	2/0	4/0	7/0	7/0	20/0	5	12
Hepatitis	3/0	7/0	10/0	1/0	21/0	4	1
Leukoenchalopathy	0/0	0/2	1/1	1/0	2/3	4	4
Cardiomyopathy	2/0	0/1	1/0	0	3/1	4	3
MR/LD	1/0	1/0	3/0	1/0	6/0	2	3
Gonadal dysfunction	3/0	0	0	2/0	5/0	3	2
Liver dysfuction	0	0/1	1/0	0	1/1	0	0
Visual disturbance	0	0	0	3/0	3/0	2	2
EEG abnormality	0	0	0/1	0	0/1	0	1
DM	0	0	1/0	0	1/0	0	1
Sudden death	0	0	0	0/1	0/1	0	0
Secondary malignancy	1/1	0/1	1/1	2/0	4/3	1	5
Others	4/0	0	4/0	4/0	12/0	2	5
Subtotal	8/4	8/4	26/2	20/1	79/9	27	39

Abbreviations: ALL, acute lymphoblastic leukemia; CRT, cranial irradiation; DM, diabetes mellitus; JCCLSG, Japanese Childhood Cancer and Leukemia Study Group; LD, learning disturbance; MR, mental retardation.

Table 8 Cumulative doses of drugs used in the JCCLSG ALL studies

Study	*VCR (mg)*	*CPM (mg)*	*PSL (mg)*	*LASP (kg)*	*DOX (mg)*	*THP (mg)*	*6MP (mg)*	*MTX (mg)*	*HDMTX (g)*	*VP16 (mg)*	*Ara-C (mg)*
ALL811											
SR-A	86	0	25230	0	0	0	34125	8100	18	0	0
SR-B	86	0	25230	0	0	0	50750	2940	18	0	0
HR-A	60	1200	17280	0	300	0	22750	0	6.1	0	0
HR-B	110	7800	20490	0	540	0	23325	1500	0	0	0
ALL841											
LR-A	86	0	25230	0	0	0	34125	5850	30	0	0
LR/IR-B	86	0	25230	98	0	0	34125	5850	30	0	0
IR-C	108	0	16830	20	540	0	21875	0	10.5	0	0
HR-A	72	1200	17430	0	240	0	22750	0	183	0	0
HR-B	108	7800	14150	0	460	0	23100	1320	27	0	0
ALL874											
LR-A	86	0	25230	20	0	0	34125	8775	0	0	0
LR-B	86	0	25230	20	0	0	34125	8100	6	0	0
IR-A	94	0	15030	64	420	0	19250	225	92	0	0
IR-B	88	0	13680	64	450	0	18375	0	105.5	0	0
HR-A	84	2400	22270	38	225	0	31290	6750	7.5	0	3600
HR-B	84	3600	22270	38	225	0	26250	6750	7.5	0	18000
ALL911											
LR	60	0	17430	24	0	450	29250	6900	6	0	0
IR	92	1600	24370	44	0	450	33110	7650	0	0	2400
HR	88	2400	23470	44	225	240	33425	7200	0	0	3600
HHR	66	2400	16870	44	225	180	23415	4725	0	3600	10800

Abbreviations: ALL, acute lymphoblastic leukemia; Ara-C, cytarabine; CPM, cyclophosphamide; DOX, doxorubicin; HDMTX, high-dose MTX (≧500 mg/m^2); HR, high risk; HHR, high-high-risk; IR, intermediate risk; JCCLSG, Japanese Childhood Cancer and Leukemia Study Group; LASP, L-asparaginase; LR, low risk; 6MP, 6-mercaoutoprine; MTX, methotrexate; PSL, prednisolone; THP, pirarubicin; VCR, vincristine; VP16, etoposide. All doses are shown per square meter of body surface area.

cumulative doses of CPA exceeded 4 g/m^2 in two protocols (HR811B and HR851B).

Discussion

Our results reveal that the cure rate has gradually increased from 55% with ALL811 to 70% with ALL911 over an observation period of 15 years, while toxic death rates during remission were below 1% in all protocols except ALL911. These results are favorably comparable to those from large pediatric ALL trials by other study groups, and reflect the effectiveness of risk-directed therapy and improvements in supportive care of children with ALL.[2–4]

Improvement of outcome in each protocol study seems to be attributable to their respective aims. As shown in Table 4, better outcome in ALL841 as compared with that in the ALL811 is partly explained by a decrease in isolate testicular relapse.

This might be the result of the cyclic schedule of intermediate-dose MTX in maintenance therapy. Improvement in ALL874 as compared with ALL841 was mainly due to a decrease in isolated CNS relapse. This was achieved by the CRT regimen for HR patients and extended intrathecal treatment for LR patients. The better survival rates in ALL911 as compared with ALL874 were mainly due to improved outcomes of the HR patients. The intensified chemotherapy of this protocol decreased the incidence of bone marrow relapse, although therapy-related death during remission increased to 4%. More importantly, as shown in Tables 5a–5d, outcomes of patients with high ($50–99 \times 10^9$/l) WBC counts in ALL874 and ALL911 were markedly improved in comparison with those in the ALL811 and ALL841 studies. This is probably due to employment of consolidation therapy (CCM regimen) and reinduction therapy.[14]

Another major interest in our studies is a unique intermittent cyclic regimen for maintenance chemotherapy. In the ALL811 study, we showed that intermittent cyclic administration of intermediate-dose MTX (225 mg/m^2, intravenous) alternating biweekly 6MP (170 mg/day, orally for 5 days) was more effective than conventional administration of low-dose MTX (20 mg/m^2/week, orally) and 6MP (50 mg/m^2, orally, every day).[7] As a result of these data, intermittent cyclic administration of MTX and 6MP has become a standard regimen of maintenance chemotherapy in JCCLSG ALL protocols.

CNS protective chemotherapy without CRT for treatment of non-HR patients with ALL has been widely accepted.[15–19] In our study, the LR patients of L874B and L911 who received HDMTX therapy as CNS prophylaxis showed 7–9% cumulative incidence of isolated CNS relapse. However, BFM-based intensive chemotherapy using extended intrathecal chemotherapy has reported lower than 5% incidence of CNS relapse.[15,19–21] Similar results are seen in the I911 protocol, where an extended triple inrathecal MTX regimen with intensive systemic therapy achieved a 2% cumulative incidence of CNS relapse in the IR patients. Thus, it is likely that systemic intravenous infusions of HDMTX could not be substituted for intrathecal injections of MTX in the maintenance therapy for CNS protection. This is also supported by the results of meta-analysis of CNS-directed therapy, which show that radiotherapy can be replaced by long-term intrathecal therapy but not by intravenous MTX.[22]

Whether CRT can be excluded from preventive therapy for HR patients is still subject to controversy. In ALL851, we employed CNS chemoprophylaxis without CRT for the HR patients, but failed to prevent CNS relapse.[8] Since high incidence of CNS relapse is associated with high initial WBC count and T-cell phenotype,[23,24] development of a new strategy for these subgroups could overcome this difficult matter. In fact, a recent report from the Memphis group has shown that complete omission of prophylactic CRT without compromising OS can be achieved by using risk-adjusted chemotherapy based on minimal residual disease levels and pharmacogenetics.[25]

In the ALL841–911 studies, the incidence of isolated testicular relapse was 7 of 278 relapses (2.5%), which was considerably lower than the general rate of about 10%.[26] The cyclic schedule of MTX at an intermediate dose in our maintenance therapy may contribute to prevention of relapse in sanctuary sites, especially in the testes.

Development of curative therapy for pediatric ALL has produced a large population of childhood cancer survivors who face increased risk of a variety of health problems resulting from their cancer or its treatment. In particular, secondary malignancy by alkylating agents and anthracycline cardiotoxicity are the most serious late events in pediatric cancer treatment.[27,28] Fortunately, the incidences of secondary malignancies and cardiotoxicity were relatively small in our ALL studies. Although pirarubicin was chosen as an anthracyclin drug with less cardiotoxicity than DOX, it is unclear whether pirarubicin could reduce the incidence of cardiotoxicity without jeopardizing the overall outcome.[29,30] In fact, our observation period with a median of 13 years (range 8–22 years) after diagnosis is too short to estimate the true incidence of late adverse effects, because excess mortality continues at least as long as 25–30 years after treatment, for cancer survivors.[27] Therefore, establishment of a long-term, follow-up care system based on collaboration between clinical and laboratory investigators is our most urgent issue.[31,32]

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank the patients who enrolled in these studies and their families.

References

1 Pui CH, Evans WE. Acute lymphoblastic leukemia. *N Engl J Med* 1998; **339**: 605–615.
2 Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med* 2004; **350**: 1535–1548.
3 Schrappe M. Evolution of BFM trials for childhood ALL. *Ann Hematol* 2004; **83**: 121–123.
4 Chessells J. Recent advances in management of acute leukaemia. *Arch Dis Child* 2000; **82**: 438–442.
5 Fujimoto T, Hasegawa K, Take H, Miyazaki S, Kishida H, Shin H *et al.* Maintenance therapy and pharmacokinetics of intermittent high-dose methotrexate in acute childhood leukemia. *Acta Haematol Jpn* 1977; **40**: 119–132.
6 Hiyoshi Y, Fujimoto T, Kuriya N, Otani Y, Nibu K, Yanai M *et al.* Prognostic factors in children with acute lymphoblastic leukemia: II. Multivariate analysis. *Jpn J Clin Oncol* 1985; **15**: 13–23.
7 Koizumi S, Fujimoto T, Takeda T, Yatabe M, Utsumi J, Mimaya J *et al.* Comparison of intermittent or continuous methotrexate plus 6-mercaptopurine in regimens for standard-risk acute lymphoblastic leukemia in childhood (JCCLSG-S811). *Cancer* 1988; **61**: 1292–1300.
8 Koizumi S, Fujimoto T. Improvement in treatment of childhood acute lymphoblastic leukemia: a 10-year study by the Children's Cancer and Leukemia Study Group. *Int J Hematol* 1994; **59**: 99–112.
9 Koizumi S, Fujimoto T, Oka T, Watanabe S, Kikuta A, Tsuchiya T *et al.* Overview of clinical studies of childhood acute lymphoblastic leukemia for more than ten years by the Japanese Children's Cancer and Leukemia Study Group. *Pediatr Hematol Oncol* 1997; **14**: 17–28.
10 Tsurusawa M, Katano N, Yamamoto Y, Hirota T, Koizumi S, Watanabe A *et al.* Improvement in CNS protective treatment in non-high-risk childhood acute lymphoblastic leukemia: report from the Japanese Children's Cancer and Leukemia Study Group. *Med Pediatr Oncol* 1999; **32**: 259–266.
11 Okamoto T, Yokota S, Katano N, Seriu T, Nakao M, Taniwaki M *et al.* Minimal residual disease in early phase of chemotherapy reflects poor outcome in children with acute lymphoblastic leukemia—A retrospective study by the Children's Cancer and Leukemia Study Group in Japan. *Leuk Lymph* 2002; **43**: 1001–1006.
12 Hori T, Yamaji K, Yokota S, Okamoto T, Watanabe A, Kikuta A *et al.* Augmented therapy for MRD-positive patients with acute

lymphoblastic leukemia can reduce the MRD levels and the risk of relapse: a report from the Japanese Childhood Cancer and Leukemia Study Group ALL 2000 MRD pilot study. *Blood* 2008; **112**: 117 (abstract 301).

13 Smith M, Arthur D, Camitta B, Carroll AJ, Crist W, Gaynon P *et al.* Uniform approach to risk classification and treatment assignment for children with acute lymphoblastic leukemia. *J Clin Oncol* 1996; **14**: 4–6.

14 Childhood ALL Collaborative Group. Duration and intensity of maintenance chemotherapy in acute lymphoblastic leukaemia: overview of 42 trials involving 12000 randomised children. *Lancet* 1996; **347**: 1783–1788.

15 Tubergen DG, Gilchrist GS, O'Brien RT, Coccia PF, Sather NH, Waskerwitz MJ *et al.* Prevention of CNS disease in intermediate-risk acute lymphoblastic leukemia: comparison of cranial radiation and intrathecal methotrexate and importance of systemic therapy: a Children's Cancer Group report. *J Clin Oncol* 1993; **11**: 520–526.

16 Sullivan MP, Chen T, Dyment PG, Hvizdala E, Steuber CP. Equivalence of intrathecal chemotherapy and radiotherapy as central nervous system prophylaxis in children with acute lymphoblastic leukemia: a Pediatric Oncology Group study. *Blood* 1982; **60**: 948–958.

17 Pullen J, Boyett J, Shuster J, Crist W, Land V, Frankel L *et al.* Extended triple intrathecal chemotherapy trial for prevention of CNS relapse in good-risk and poor-risk patients with B-progenitor acute lymphoblastic leukemia: a Pediatric Oncology Group study. *J Clin Oncol* 1993; **11**: 839–849.

18 Pui CH, Simone JV, Hancock ML, Evans WE, Williams DL, Bowman WP *et al.* Impact of three methods of treatment intensification on acute lymphoblastic leukemia in children: long-term results of St Jude total therapy X. *Leukemia* 1992; **6**: 150–157.

19 Buhrer C, Henze G, Hofmann J, Reiter A, Schellonq G, Riehm H. Central nervous system relapse prevention in 1165 standard-risk children with acute lymphoblastic leukemia in five BFM trials. *Hematol Blood Transfus* 1990; **33**: 500–503.

20 Reiter A, Schrappe M, Ludwig WD, Hiddemann W, Sauter S, Henze G *et al.* Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. *Blood* 1994; **84**: 3122–3133.

21 Conter V, Arico M, Valsecchi MG, Rizzari C, Testi AM, Messina C *et al.* Extended intrathecal methotrexate may replace cranial irradiation for prevention of CNS relapse in children with intermediate-risk acute lymphoblastic leukemia treated with Berlin–Frankfurt–Munster-based intensive chemotherapy. *J Clin Oncol* 1995; **13**: 2497–2502.

22 Clarke M, Gaynon P, Hann I, Harrison G, Masera G, Peto R *et al.* CNS-directed therapy for childhood acute lymphoblastic leukemia: Childhood ALL Collaborative Group overview of 43 randomized trials. *J Clin Oncol* 2003; **21**: 1798–1809.

23 Steinherz PG. Radiotherapy vs intrathecal chemotherapy for CNS prophylaxis in childhood ALL. *Oncology* 1989; **3**: 47–53.

24 Cherlow JM, Steinherz PG, Sather HN, Gaynon PS, Grossman NJ, Kersey JH *et al.* The role of radiation therapy in the treatment of acute lymphoblastic leukemia with lymphomatous presentation: a report from the Children's Cancer Group. *Int J Radiat Oncol Biol Phys* 1993; **27**: 1001–1009.

25 Pui CH, Campana D, Deqing P, Bowman WP, Sandlund JT, Kaste SC *et al.* Treating childhood acute lymphoblastic leukaemia without cranial irradiation. *N Engl J Med* 2009; **360**: 2730–2741.

26 Chessells JM, Veys P, Kempski H, Henley P, Leiper A, Webb D *et al.* Long-term follow-up of relapsed childhood acute lymphoblastic leukaemia. *Br J Haematol* 2003; **123**: 396–405.

27 Davies SM. Subsequent malignant neoplasms in survivors of childhood cancer: Childhood Cancer Survivor Study (CCSS) studies. *Pediatr Blood Cancer* 2007; **48**: 727–730.

28 Steinherz LJ, Graham T, Hurwitz R, Sondheimer HM, Schaffer EM, Schwartz RG *et al.* Guidelines for monitoring of anthracycline cardiomyopathy: a rebuttal. *Pediatrics* 1994; **93**: 433–437.

29 Dantchev D, Paintrand M, Hayat M, Bourut C, Mathe G. Low heart and skin toxicity of a tetrahydropyranyl derivative of adriamycin (THPADM) as observed by electron and light microscopy. *J Antibiot (Tokyo)* 1979; **32**: 1085–1086.

30 Chauvergne J, Fumoleau P, Cappelaere P, Metz R, Armand JP, Chevallier B *et al.* Phase II study of pirarubicin (THP) in patients with cervical, endometrial and ovarian cancer: study of the Clinical Screening Group of the European Organization for Research and Treatment of Cancer (EORTC). *Eur J Cancer* 1993; **29A**: 350–354.

31 Oeffinger KC, Eshelman DA, Tomlinson GE, Buchanan GR, Foster BM. Grading of late effects in young adult survivors of childhood cancer followed in an ambulatory adult setting. *Cancer* 2000; **88**: 1687–1695.

32 Landier W, Bhatia S, Eshelman DA, Forte KJ, Sweeney T, Hester AL *et al.* Development of risk-based guidelines for pediatric cancer survivors: the Children's Oncology Group Long-Term Follow-up Guidelines from the Children's Oncology Group Late Effects Committee and Nursing Discipline. *J Clin Oncol* 2004; **22**: 4979–4990.

Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome–negative myeloproliferative neoplasms

*Nils H. Thoennissen,[1] *Utz O. Krug,[2] Dhong Hyun Tony Lee,[1] Norihiko Kawamata,[1] Gabriela B. Iwanski,[1] Terra Lasho,[3] Tamara Weiss,[4] Daniel Nowak,[1] Maya Koren-Michowitz,[1] Motohiro Kato,[5-7] Masashi Sanada,[5-7] Lee-Yung Shih,[8] Arnon Nagler,[9] Sophie D. Raynaud,[10] Carsten Müller-Tidow,[2] Ruben Mesa,[3] Torsten Haferlach,[4] D. Gary Gilliland,[11-13] Ayalew Tefferi,[3] †Seishi Ogawa,[5-7] and †H. Phillip Koeffler[1,14]

[1]Division of Hematology and Oncology, Cedars-Sinai Medical Center, University of California Los Angeles (UCLA) School of Medicine; [2]Department of Medicine, Hematology and Oncology, University of Münster, Münster, Germany; [3]Department of Hematology, Mayo Clinic, Rochester, MN; [4]MLL Munich Leukemia Laboratory, Munich, Germany; [5]Department of Hematology and Oncology Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [6]Department of Cell Therapy and Transplantation Medicine and the 21st century COE program, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [7]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Tokyo, Japan; [8]Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital, Taipei, Taiwan School of Medicine, Chang Gung University, Taoyuan, Taiwan; [9]Division of Hematology, Sheba Medical Center and Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel; [10]Genetics Laboratory, Archet Hospital, Nice, France; [11]Hematology, Brigham and Women's Hospital, Harvard University, School of Medicine, Boston, MA; [12]Harvard Stem Cell Institute, Boston, MA; [13]Howard Hughes Medical Institute, Harvard University, School of Medicine, Boston, MA; and [14]Department of Medicine, National University of Singapore, Singapore

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera, essential thrombocythemia, and primary myelofibrosis show an inherent tendency for transformation into leukemia (MPN-blast phase), which is hypothesized to be accompanied by acquisition of additional genomic lesions. We, therefore, examined chromosomal abnormalities by high-resolution single nucleotide polymorphism (SNP) array in 88 MPN patients, as well as 71 cases with MPN-blast phase, and correlated these findings with their clinical parameters. Frequent genomic alterations were found in MPN after leukemic transformation with up to 3-fold more genomic changes per sample compared with samples in chronic phase ($P < .001$). We identified commonly altered regions involved in disease progression including not only established targets *(ETV6, TP53,* and *RUNX1)* but also new candidate genes on 7q, 16q, 19p, and 21q. Moreover, trisomy 8 or amplification of 8q24 *(MYC)* was almost exclusively detected in *JAK2V617F*$^-$ cases with MPN-blast phase. Remarkably, copy number–neutral loss of heterozygosity (CNN-LOH) on either 7q or 9p including homozygous *JAK2V617F* was related to decreased survival after leukemic transformation ($P = .01$ and $P = .016$, respectively). Our high-density SNP-array analysis of MPN genomes in the chronic compared with leukemic stage identified novel target genes and provided prognostic insights associated with the evolution to leukemia. (*Blood.* 2010; 115(14):2882-2890)

Introduction

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) are defined as clonal hematopoietic stem cell disorders and characterized by increased proliferation of terminally differentiated myeloid cells. The tyrosine kinase *JAK2* is directly linked to the pathogenesis of MPN with the identification of *JAK2V617F* as a recurring gain-of-function mutation.[1,2] Almost all cases with PV, and roughly 50% of patients with ET and PMF, carry this specific mutation localized on chromosome 9p24.

The long-term outcome of patients with acute myeloid leukemia (AML) secondary to MPN, myelodysplastic syndrome (MDS), or treatment with cytotoxic agents is relatively poor compared with patients with de novo AML. Patients with de novo and secondary AML have a similar spectrum of cytogenetic abnormalities, but the occurrence of cytogenetic changes associated with unfavorable risk such as 5q−, −7/7q−, trisomy 8, or complex karyotype is higher in secondary AML.[3,4] However, so far only a small number of studies with limited number of cases have explored the chromosomal alterations and/or clinical markers associated with acceleration to blast phase of patients with MPN.

Previously, we developed the copy number analyzer for Affymetrix GeneChip (CNAG) program and the new algorithm allele-specific copy number analysis using anonymous references (AsCNAR).[5,6] These techniques in combination with high-density single nucleotide polymorphism (SNP) array provide a robust and

Submitted July 27, 2009; accepted December 8, 2009. Prepublished online as *Blood* First Edition paper, January 12, 2010; DOI 10.1182/blood-2009-07-235119.

*N.H.T. and U.O.K. contributed equally and should be considered as co–first authors.

†S.O. and H.P.K. contributed equally and should be considered as co–last authors.

An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. Clinical features of MPN/MPN-blast phase cases (unmatched and matched)

	Unmatched MPN	Unmatched MPN-blast phase	Matched MPN	Matched MPN-blast phase
All cases, no. (%)	77 (56)	60 (44)	11 (50)	11 (50)
MPN diagnosis no. (%)				
PV samples	21 (27)	17 (28)	2 (18)	2 (18)
ET samples	31 (40)	18 (30)	1 (09)	1 (09)
PMF samples	25 (33)	25 (42)	8 (73)	8 (73)
Sex, M:F				
PV samples	1:2	1:1	1:1	1:1
ET samples	1:2	1:1	0:1	0:1
PMF samples	2:1	2:1	2:1	2:1
Mean age at diagnosis, y, ± SD*				
PV	57 ± 5	68 ± 5	—	—
ET	59 ± 6	69 ± 7	—	—
PMF	57 ± 6	65 ± 9	59 ± 9	65 ± 7
Mean blast count in bone marrow, ± SD, no. (%)*				
PV samples	< 5%	70 ± 20	—	—
ET samples	< 5%	66 ± 23	—	—
PMF samples	< 5%	70 ± 21	< 5%	66 ± 24
***JAK2V617F* (+) no. (%)**				
PV samples	21/21 (100)	14/17 (82)‡	2/2 (100)	1/2 (50)
ET samples	18/31 (58)	6/18 (33)	0/1 (0)	0/1 (0)
PMF samples	16/25 (64)	12/25 (48)	5/8 (62.5)	4/8 (50)
***c-MPL* mutation positive, no. (%)**				
PV samples	1/21 (5)†	0/17 (0)	0/2 (0)	0/2 (0)
ET samples	0/31 (0)	1/18 (6)	0/1 (0)	0/1 (0)
PMF samples	3/25 (12)	2/25 (8)	1/8 (12.5)	1/8 (12.5)

MPN indicates myeloproliferative neoplasm; PV, polycythemia vera; ET, essential thrombocytosis; M, male; F, female; and PMF, primary myelofibrosis.

*Data are available for 27 unmatched MPN (10 PV, 10 ET, and 7 PMF) and 54 unmatched MPN-blast phase (15 PV, 18 ET, and 21 PMF) cases, and 8 matched MPN (PMF) cases.

†This *c-MPL* mutation in a PV patient has already been validated and reported by Kawamata et al.[8]

‡Significantly fewer cases with *JAK2V617F* in blast phase vs chronic phase ($P = .045$).

detailed approach to detect large and small copy number changes, as well as copy number–neutral loss of heterozygosity (CNN-LOH). To obtain a comprehensive profile of genomic alterations associated with leukemic transformation in MPN, we applied this interrogational method and performed a systemic analysis of 159 samples obtained from patients either in chronic phase or blast phase of MPN.

Methods

Patients and clinical samples

In total, samples from 148 patients were analyzed by SNP-array. One hundred fifty-nine samples were obtained, of which 88 (55%) were diagnosed with MPN in chronic phase (23 PV, 32 ET, 33 PMF) and 71 (45%), with MPN in blast phase (19 PV, 19 ET, 33 PMF). Diagnosis was based on the World Health Organization criteria,[7] and an overview of patients, including clinical data, is given in Table 1. This study received institutional review board approval from the Cedars-Sinai Medical Center, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Given the relatively high incidence of homozygous *JAK2V17F* patients diagnosed with ET (3/18 in chronic phase, 2/6 in blast phase), which is usually lower for this disorder,[9] we suggest that at least some cases diagnosed with ET may have been incorrect.

Samples were provided by (1) Department of Hematology, Mayo Clinic (n = 35); (2) Brigham and Women's Hospital, Harvard University, School of Medicine (n = 46); (3) Department of Hematology, Archet Hospital (n = 44); (4) MLL Munich Leukemia Laboratory, (n = 14); (5) Division of Hematology-Oncology, Chang Gung Memorial Hospital (n = 14); and (6) Division of Hematology, Sheba Medical Center and Sackler School of Medicine, Tel-Aviv University (n = 6).

SNP-Chip analysis

A total of 159 tumor specimens (MPN and/or MPN-blast phase) were analyzed on GeneChip SNP genotyping microarrays (GeneChip Mapping 50K and/or 250K arrays; Affymetrix) as described previously.[5,6] After appropriate normalization of mean array intensities, signal ratios were calculated between tumors and anonymous normal references in an allele-specific manner. Genome-wide determination of allele-specific copy numbers (AsCNs) and detection of CNN-LOH at each SNP were inferred from the observed signal ratios based on the hidden Markov model using CNAG/AsCNAR algorithms (http://www.genome.umin.jp).[5,6] For clustering of patient samples with regard to the status of copy number changes, as well as CNN-LOH, CNAG-Graph software (Tokyo University) was used. Size, position, and location of genes were identified with the University of California, Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu)[10] and Ensemble Genome Browser (http://www.ensembl.org).[11] Germline copy number changes previously described as copy number variants at Database of Genomic Variants (http://projects.tcag.ca/variation)[12] and UCSC Genome Browser were excluded. SNP-array data used in this study are available in the Gene Expression Omnibus (GEO) database under accession number GSE19647.[13]

Comparison of 50K versus 250K SNP-Chip analysis in MPN chronic phase

SNP-array analysis of 46 of our MPN samples (10 PV, 20 ET, 16 PMF; kindly provided by D.G.G. at Brigham and Women's Hospital, Harvard University) has already been reported by our group.[8] At that time, only 50K arrays were available, whereas later in this study, the 250K arrays were accessible and used to analyze additionally 42 MPN and 71 MPN-blast phase samples. Because no significant differences in either number of deletions, duplications/amplifications, or CNN-LOH per case were found as analyzed by the 50K compared with 250K array (supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), we combined the analysis of both platforms in our

results. Supplemental Table 2 lists all individual samples and the array that was used.

Cytogenetics

Routine cytogenetic analysis with conventional banding techniques was performed in 35 of 88 MPN (10/23 PV, 10/32 ET, 15/33 PMF) and 63 of 71 MPN-blast phase (15/19 PV, 18/19 ET, 30/33 PMF) cases according to standard procedures as previously described.[14] No routine fluorescent in situ hybridization (FISH) panel was applied, but in some cases, however, FISH analysis was performed to supplement conventional cytogenetic analysis (supplemental Table 2).

Allele-specific PCR for *JAK2V617F* mutation

For the detection of *JAK2V617F*, allele-specific polymerase chain reaction (PCR) was performed according to the previously reported method.[15]

Direct mutation screening

Primers were designed to amplify and sequence coding exons and splice junctions of the following genes: *TET2*, *c-CBL*, *TP53*, and *RUNX1*. We screened only the 11 matched samples that showed genomic changes in the particular gene regions. Primer details are available from the corresponding author (N.H.T.).

We evaluated all MPN and MPN-blast phase patients with 1pCNN-LOH for the *MPLW515* mutation (exon 10) by direct sequencing. If no mutation was detected in this cohort, we also screened the other coding exons of the *c-MPL* gene previously shown to be mutated in MPN.[8]

Validation of acquired genomic copy number changes including CNN-LOH

To confirm the somatic origin of genomic copy number changes, quantitative genomic real-time (QG RT)–PCR was performed on the genomic DNA from the hybridized MPN and matched MPN-blast phase samples according to the calculation method described by Weksberg et al.[16] For example, we used primers for the *RUNX1* gene (21q22.12; supplemental Figure 1A) as well as *TET2* gene (4q24; data not shown) and a random region on chromosome 21q21.1 and 4p15.1, respectively, as a reference in patient 121.

Detection of acquired CNN-LOH was also validated by QG RT-PCR and subsequently by nucleotide sequencing. Three SNP sequences (rs919275, rs10854117, and rs10854117) on chromosome 19p in case 36 at diagnosis of PV, as well as at leukemic transformation, were determined (supplemental Figure 1B). The genomic region of each SNP site was amplified, and products were purified and sequenced (supplemental Figure 1C). In addition, we confirmed loss of CNN-LOH on 9p after leukemic transformation in matched case 120 using SNP sequences rs3858029, rs1360461, and rs10818814 on chromosome 9 (data not shown).

Homozygous deletions of *CUTL1* and *SH2B2* (case 138) as well as *PIG-A* (case 121) in both MPN and/or MPN-blast phase samples were also confirmed by QG RT-PCR (supplemental Figure 2). Primers for these experiments will be provided upon request.

Statistical analysis

Wilcoxon rank sum tests were used to assess differences in continuous variables, and categoric variables were assessed using chi-square tests, all with a significance level of $\alpha = .05$. The methods of estimations included the standard deviation ($\pm$ SD) of the sampling distribution. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition ($*P < .05$; $**P < .001$). Survival analysis was performed using the Kaplan-Meier method, and survival curves were compared using the log-rank test.

Results

Lower frequency of *JAK2V617F* and 9p alterations after leukemic transformation

In the present study, we examined 159 samples (88 MPN and 71 MPN-blast phase) from a total of 148 patients. An overview of the clinical features of matched and unmatched cases including sex, age, leukemic blast infiltration, and mutational status (*JAK2V617F, c-MPL*) is provided in Table 1. The sex ratio of male and female patients in chronic phase was 1:2 for PV and ET, whereas after transformation, the ratio was balanced with 1:1. For PMF patients, the male-to-female ratio was 2:1 in both MPN chronic and blast phase.

Overall, the incidence of *JAK2V617F* was almost 20% less in the blast phase compared with the chronic phase for both the matched and unmatched MPN cases (unmatched cases: $P < .05$; Figure 1A). Cases that were negative for *JAK2V617F* were also exclusively negative for 9p duplication, trisomy 9, or 9pCNN-LOH in the chronic as well as leukemic stage of MPN. 9pCNN-LOH was noted approximately 3 to 4 times more often than 9p duplication and/or trisomy 9 in *JAK2V617F*$^+$ MPN cases during either the chronic or blast phase (Figure 1A), but the frequency of 9pCNN-LOH was significantly less in the blast crisis compared with the chronic phase of unmatched PMF and PV patients (supplemental Table 3). In contrast, unmatched ET cases had about the same frequency of 9pCNN-LOH in the chronic phase versus the blast phase of the disease. Furthermore, in the analysis of the 11 matched MPN cases, 7 were positive for *JAK2V617F* (64%), 4 had 9p CNN-LOH (37%), and 1 had 9p duplication (9%) at first diagnosis (Figure 1A). In comparison, 2 of these patients were *JAK2V617F*$^+$ with either trisomy 9 or 9pCNN-LOH during their chronic phase (1 PV, 1 PMF), but no longer had detectable *JAK2V617F* with a normal chromosome 9 after leukemic evolution (Figure 1B).

JAK2V617F mutational status had no impact on time to transformation or survival

In the evaluation of clinical data for MPN-blast phase patients, no significant correlation was noted between the prevalence of *JAK2V617F* at transformation and either age, percentage of leukemic blast cells in the marrow, or pretreatment with alkylating agents and/or hydroxyurea (data not shown). Moreover, we found no statistical association between either time to leukemic transformation or overall survival and the *JAK2V617F* status at transformation in PV, ET, or PMF patients. The overall survival of MPN-blast phase patients with *JAK2V617F* versus blast phase patients without this mutation is provided in Figure 2A ($P = .6$). In addition, with respect to the comparably low frequency of *MPLW515*-positive MPN-blast phase patients (6%), we noted no impact of the *c-MPL* mutational status on either time to transformation (data not shown) or the overall survival in MPN patients who underwent leukemic transformation ($P = .5$; Figure 2B).

However, regardless of the mutational status of MPN-blast phase patients, we noted that the time from diagnosis of MPN to leukemic transformation was significantly shorter in those with pre-existing PMF (median, 58 months) compared with patients with either prior PV (median, 98 months) or ET (median, 110 months; $P = .01$). This earlier transformation resulted in a decreased overall survival from the time of diagnosis of the underlying MPN in leukemic patients with preceding PMF patients compared with preceding PV or ET ($P = .02$; Figure 2C), which is congruent with previously published results.[17]

Increased number of additional genomic changes after leukemic transformation

Altogether, a relatively low number of genomic alterations was found by SNP-array analysis in the chronic phase of the MPN samples (Figure 3A). In contrast, 2 to 3 times more abnormalities per sample were detected after leukemic evolution in both matched and unmatched cases with MPN (both $P < .001$; Figure 3A). We

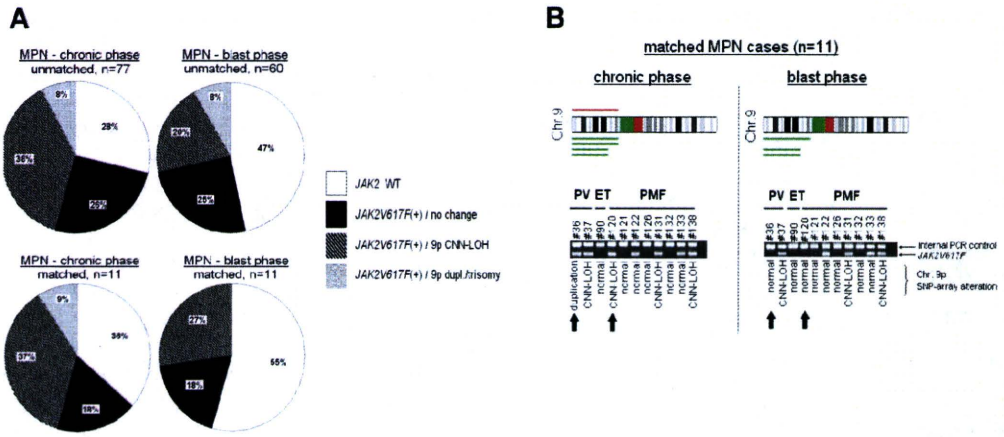


Figure 1. Frequency of *JAK2V617F* and associated alterations on chromosome 9. (A) Diagrams represent matched and unmatched MPN cases in chronic versus blast phase. Indicated are frequencies of *JAK2V617F* and association to 9p duplication (dupl)/trisomy 9 or 9pCNN-LOH. Data and statistical evaluation for underlying MPN subgroups are shown in supplemental Table 3. (B) CNAG software represents duplication (red) and CNN-LOH (green) on 9p detected in 11 patients with matched samples (chronic MPN vs MPN-blast phase). In addition, allele-specific PCR for the detection of *JAK2V617F* was performed in these samples. Arrows indicate 2 MPN patients who were initially positive for *JAK2V617F* in association with 9p imbalances; leukemic transformation was accompanied with loss of *JAK2V617F* and a normal chromosome 9.

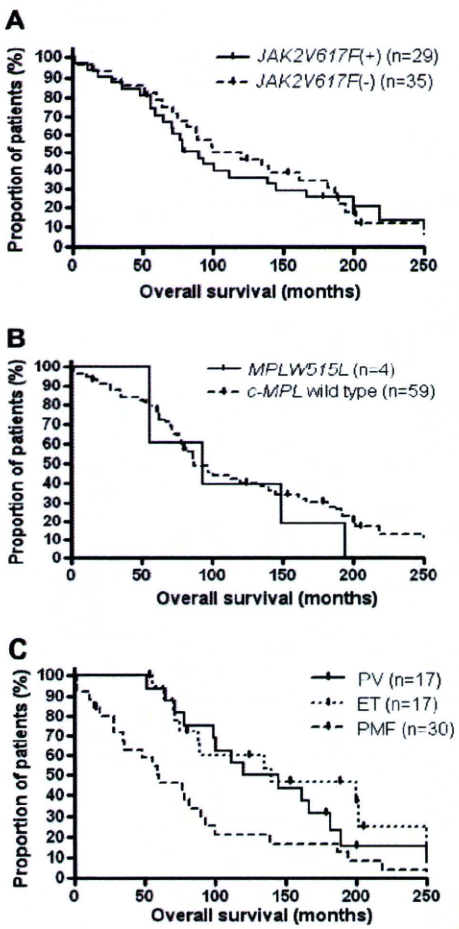


Figure 2. Overall survival of MPN patients with subsequent transformation to blast crisis. Kaplan-Meier plots of all MPN-blast phase patients from the diagnosis of pre-existing MPN were stratified for (A) the presence or absence of a *JAK2V617F* mutation at transformation, (B) the presence or absence of a *MPLW515L* mutation at transformation, and (C) the underlying type of MPN.

found no statistical relationship between the *JAK2V617F* status and the number of genomic changes in matched as well as unmatched samples (data not shown). However, samples from ET patients had fewer copy number changes than those from either PV or PMF patients in the chronic phase, which was highly significant in the unmatched cases ($P < .001$; Figure 3A, supplemental Figure 3A). After leukemic transformation, a similar number of SNP-array changes occurred in cases with prior ET compared with those with pre-existing PV and PMF (unmatched cases: $P = .59$). Statistical evaluation of the matched samples divided into each subentity was not possible because of the small number of cases (Figure 3Aii and supplemental Figure 3B). A subanalysis of the number of either deletions, duplications/amplifications, or CNN-LOH per case, matched and unmatched, is shown in supplemental Figure 3.

Compared with the cytogenetic data, SNP-array analysis detected more than 2-fold of additional chromosomal changes in the MPN samples of either chronic or blast phase, whereas SNP-array practically captured all cytogenetic abnormalities (Figure 3B).

Candidate genes involved in leukemic transformation of MPN patients

SNP-chip analysis detected several additionally altered regions in patients after leukemic evolution compared with the MPN chronic phase in both unmatched (Figure 4; supplemental Figure 4) and matched (Figure 5A) cases. The altered regions included chromosome 8q *(MYC)*, 12p *(ETV6)*, 17p *(TP53)*, and 21q *(RUNX1)*, which are already known to be involved in leukemogenesis.[18-22] Trisomy 8 was detected in 12% of unmatched and 9% of matched cases in MPN-blast phase; interestingly, almost all these samples were negative for *JAK2V617F*. PMF patient 148, who was also *JAK2V617F*$^-$, showed amplification of 8q24.21 in blast crisis involving the *MYC* gene. MPN-blast phase patients with trisomy 8 did not show an inferior outcome compared with cases without this abnormality ($P = .11$; data not shown).

In 20% of unmatched cases in MPN-blast phase, deletions (12%) or CNN-LOH (8%) occurred on chromosome 17 including *TP53* at p13.1. Deletions on the short arm of chromosome 17 were detected significantly often in MPN-blast phase patients who received prior treatment with hydroxyurea with or without the addition of alkylating agents ($P = .035$, Table 2). Supplemental

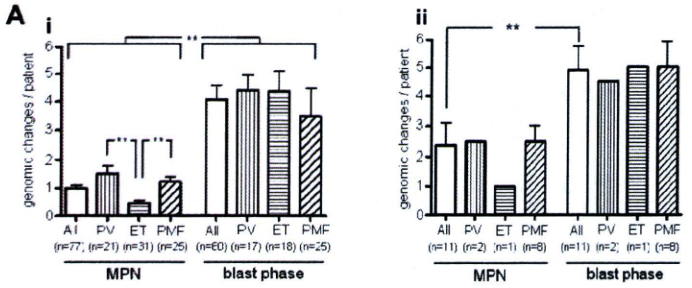
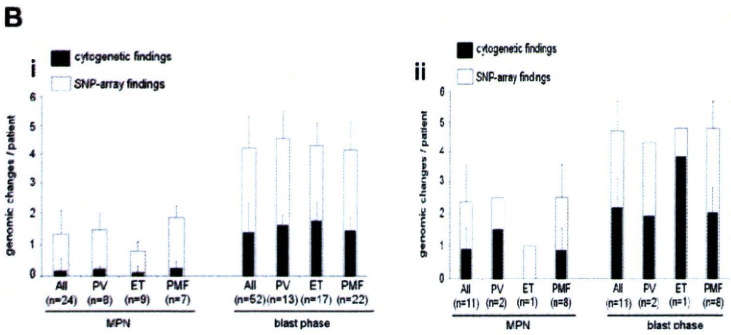


Figure 3. Genomic alterations per MPN patient in chronic versus blast phase. (A) Mean of SNP-array alterations per patient in MPN versus MPN-blast phase with (i) unmatched samples and (ii) matched samples (± SD); $^{**}P < .001$. (B) Mean of SNP-array aberrations compared with cytogenetic alterations per patient in chronic versus blast phase with (i) unmatched samples and (ii) matched samples (± SD).



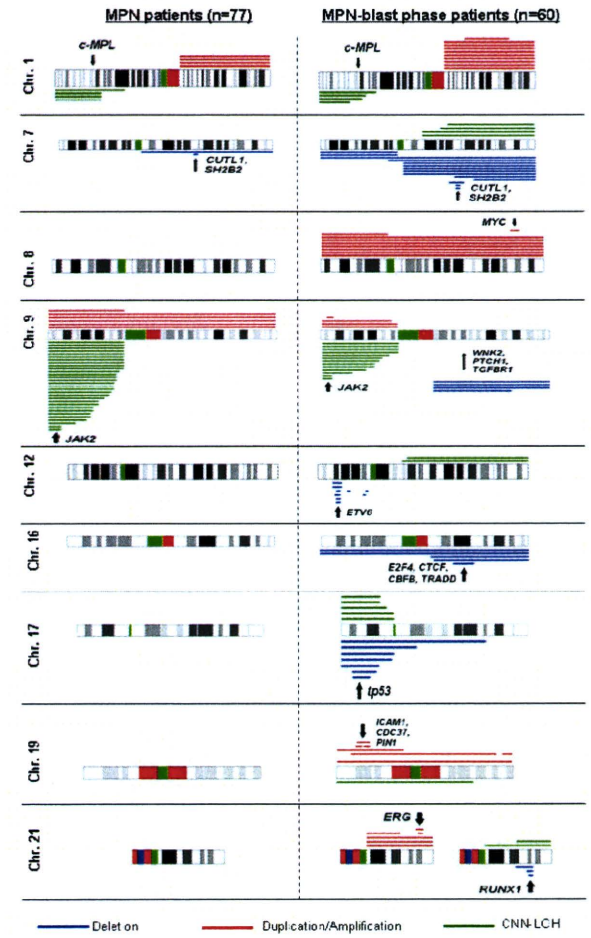


Figure 4. Overview of gains and losses detected by CNAG software. Indicated are the most common altered regions in unmatched MPN-blast phase patients (n = 60; right-sided cytobands) compared with unmatched MPN patients (n = 77; left-sided cytobands). Each line represents 1 sample with either deletion (blue), duplication/amplification (red), or CNN-LOH (green). Candidate genes of the minimal altered regions are highlighted by arrows.

Table 4 indicates pretreatment (hydroxyurea and/or alkylating agents) of 47 MPN-blast phase patients and their individual SNP-array findings. Deletion or CNN-LOH on 17p in unmatched blast phase cases was associated with either complex karyotype or isochromosome 17 ($P = .01$), and significantly decreased survival (with 17p deletion: $P = .012$; with 17p CNN-LOH: $P = .018$). One of the 11 matched MPN samples (case 120) acquired a 17p deletion at diagnosis of blast phase (Figure 5A), resulting in a hemizygous mutant *TP53* (M133K; Figure 5Bi).

On chromosome 21, SNP-chip analysis revealed either deletions or CNN-LOH in 8% of unmatched cases in MPN-blast phase involving the transcription factor *RUNX1* at q22.12. Patient 121 acquired a small deletion of that locus in the leukemic sample (Figure 5A) associated with a mutation of the Runt domain of the *RUNX1* gene on the remaining allele (Q392Stop codon; Figure 5Bii).

Deletion or CNN-LOH on 4q24 spanning the *TET2* gene was detected in 6% of unmatched blast phase cases and 1% in chronic phase. One *TET2* mutation was found by nucleotide sequencing in the matched MPN samples. *JAK2V617F*$^{-}$ case 121 had no genomicimbalances on 4q at diagnosis of PMF, but acquired a microdeletion (1 Mbp) on 4q24 (*TET2*) after leukemic evolution 1 year later (Figure 5A). The remaining allele had a *TET2* frameshift mutation (N486T; Figure 5Biii), and the mutation was absent in the matched PMF sample.

CNN-LOH involving 11q23.3, which has been shown to be strongly associated with *c-CBL* mutations,[23] had an even lower frequency, with only 2% of unmatched MPN cases in either chronic phase or blast crisis. The *JAK2V617F*$^{-}$ patient 132 had 11q CNN-LOH with a homozygous *c-CBL* missense mutation (Y371H) in the MPN-blast phase sample. Both the CNN-LOH and the mutation were absent in the corresponding chronic phase, 2 years before disease progression (Figure 5A-Biv).

Besides these already well-known targets, SNP-array analysis detected commonly altered regions on chromosomes 1, 7, 16, 19, and 21 encompassing potentially new candidate genes involved in MPN transformation. These imbalances were either absent or at least very infrequent in the chronic phase of the disease (Figures

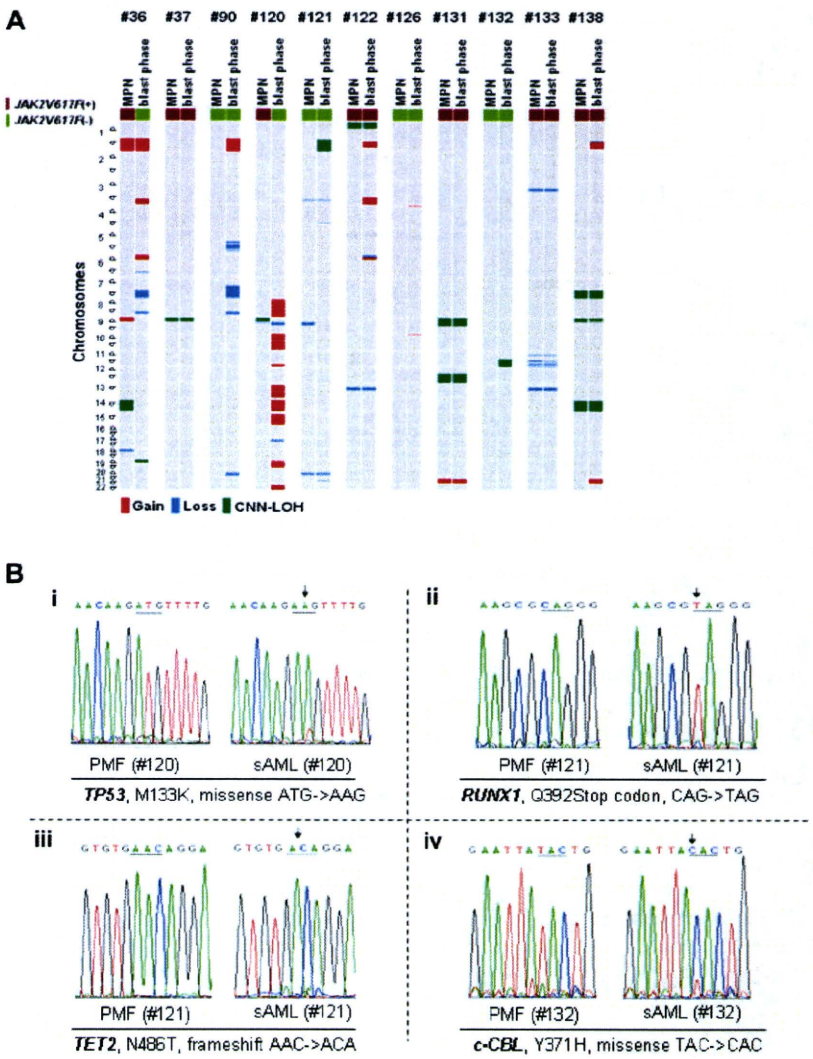


Figure 5. Gains and losses in matched MPN samples and mutational analysis. (A) Most commonly altered genomic regions in MPN samples (left sample column) compared with matched blast phase samples (right sample column) evolved from 11 patients (2 PV, 1 ET, 8 PMF). Each line represents 1 sample with either deletion (blue), duplication/amplification (red), or CNN-LOH (green). (Bi) Hemizygous *TP53* mutation detected in MPN-blast phase sample of case 120 associated with acquired 17q deletion, which was not present in the MPN phase of case 120. (ii) Hemizygous *RUNX1* mutation detected in MPN-blast phase sample (case 121) associated with acquired deletion at 22q22.1, which was not present in the MPN phase of case 121. (iii) Hemizygous *TET2* mutation detected in MPN-blast phase sample of case 121 associated with acquired cryptic deletion on 4q24, which was not present in the MPN phase of case 121. (iv) Homozygous *c-CBL* mutation detected in MPN-blast phase sample of case 132 associated with acquired 11q CNN-LOH, which was not present in the MPN phase of case 132.

4 and 5A, supplemental Figure 4). Ten percent of unmatched and 18% of matched MPN-blast phase cases had either duplication/amplification or CNN-LOH on 19p. The commonly involved region spanned a small locus (2 Mbp) at 19p13.2, where, among others, the genes *PIN1, ICAM1,* and *CDC37,* which have been associated with carcinogenesis, are located.[24-26] In addition, the minimal region (1.8 Mbp) of amplifications/duplications/trisomy on chromosome 21 detected in 8% of unmatched and 9% matched MPN-blast phase samples harbored the oncogenic transcription regulator *ERG* (q22.2).

Table 2. Pretreatment in 47 MPN-blast phase cases and frequency of 17p and 7q deletions

Pretreatment				
Hydroxyurea	−	+	−	+
Alkylating agents	−	−	+	+
SNP-array alteration (17p vs 7q)				
No *del(17)(p)*, no *del(7(q)*	17	16	0	1
del(7)(q)	2	2	2	2
del(17)(p)	0	4*	0	1*
del(17)(p) and *del(7)(q)*	0	0	0	0

Numbers of blast-phase patients are presented.

MPN indicates myeloproliferative neoplasm; and SNP, single nucleotide polymorphism.

*A total of 5 cases with *del(17)(p)* pretreated with hydroxyurea ($P = .035$).

Complete or partial deletion (−7/7q−), as well as CNN-LOH of the long arm of chromosome 7, was one of the most common abnormalities detected by SNP-array analysis in up to 25% of unmatched and 27% matched samples evolved in the blast phase. SNP-array also revealed 3 unmatched cases (32, 87, and 116) with a heterozygous microdeletion encompassing the 7q22.1 locus, which was not detectable by cytogenetic analysis. Moreover, case 138 with 7qCNN-LOH had a homozygous deletion on 7q22.1 in both the matched MPN and MPN-blast phase samples (supplemental Figure 2A). The minimally deleted region spanned a small region of 0.88 Mbp at 7q22.1 covering only 2 target genes, *CUTL1* and *SH2B2*. The homozygous deletion of these genes in patient 138 was confirmed by QG RT-PCR (supplemental Figure 2B). Deletions of the long arm of chromosome 7 were found more often in MPN-blast phase patients pretreated with hydroxyurea and/or alkylating agents, but the findings were not statistically significant ($P = .2$; Table 2).

Also worth mentioning, 1 microdeletion encompassing the chromosome X–linked *PIG-A* gene occurred in male patient 121 at

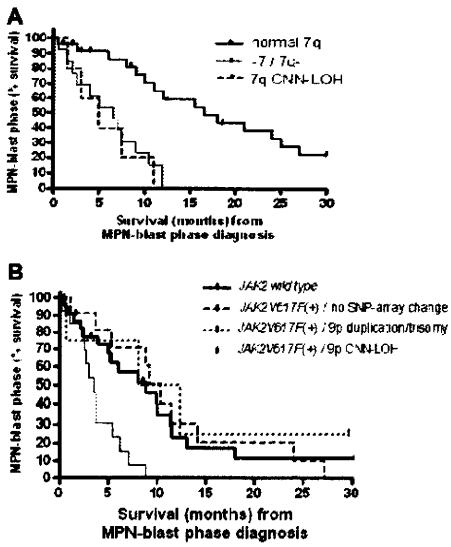


Figure 6. Survival analysis in MPN-blast phase. (A) Survival from the time of diagnosis of blast phase in transformed MPN patients with normal chromosome 7 (normal 7q) compared with either monosomy 7 (−7)/deletion of 7q (7q−) or 7qCNN-LOH. (B) Survival from the time of diagnosis of blast phase in transformed MPN patients with homozygous *JAK2V617F*+ associated with 9pCNN-LOH compared with either heterozygous *JAK2V617F*+ with 9p duplication/trisomy 9 or no abnormality, or patients without the mutation (*JAK2 wild type*). Median survival (months) and the case numbers for each group (transformed PV, ET, or PMF) are listed in supplemental Table 5.

leukemic transformation (supplemental Figure 2C). This patient had a normal chromosome X in his chronic phase of PMF.

CNN-LOH is a marker of poor survival in MPN patients after leukemic evolution

SNP-array technology provides efficient and effective detection of segmental CNN-LOH. In the present study, the most prominent regions for CNN-LOH besides chromosome 9p *(JAK2)* were on 7q and 17p *(TP53)* in patients with MPN-blast phase. In marked contrast to CNN-LOH on 9p, CNN-LOH on 7q or 17p almost never occurred in the chronic phase of the disorder in matched and unmatched samples. As mentioned previously, cases with CNN-LOH and/or deletion of 17p were associated with either complex karyotype or isochromosome 17 and decreased survival.

As also expected, survival in the MPN-blast phase was significantly decreased in patients with −7/7q− (median, 3.75 months) compared with those without chromosome 7 alterations (median, 9 months; $P = .008$). In addition, the unbalanced translocation, der(1;7)(q10;p10), a nonrandom chromosomal abnormality rarely found in AML, was detected by SNP-chip and FISH in 7% of unmatched samples after leukemic evolution and was also associated with an inferior outcome compared with patients without chromosome 7 imbalances ($P = .014$). Strikingly, survival continued to be significantly decreased in MPN-blast phase, when cases with only 7qCNN-LOH were compared with those with a normal 7q ($P = .01$; Figure 6A; supplemental Table 5).

The *JAK2V617F* mutational status in terms of heterozygosity or homozygosity appeared to have no influence on the duration to leukemic evolution. Regardless of the underlying MPN subgroup, no statistical difference in the time to leukemic transformation was found comparing *JAK2V617F*+ patients with normal chromosome 9 to mutant positive blast phase patients with either 9p duplication/trisomy 9 ($P = .28$) or 9pCNN-LOH ($P = .21$). Instead, we found that homozygous *JAK2V617F* had an impact on survival after MPN transformation. Blast phase patients with 9pCNN-LOH resulting in a homozygous *JAK2* mutation had a worse outcome (median, 4 months) compared with *JAK2V617F*+ MPN-blast phase patients with either 9p duplication/trisomy 9 (median, 7.5 months) or no abnormality on 9p (median, 9 months), as well as patients without *JAK2V617F* (median, 7 months, $P = .016$; Figure 6B; supplemental Table 5). Homozygous *JAK2V617F* in association with CNN-LOH diagnosed at leukemic transformation was independent of known risk factors such as 5q−, −7/7q−, or complex karyotype ($P > .05$).

Discussion

Oncogenic *JAK2* signaling is an important event in MPN.[1,2] Recently, we and others showed that homozygosity for *JAK2V617F* is closely related to chromosome 9pCNN-LOH in MPN patients.[1,6,8,9] However, the transformation process of MPN to MPN-blast phase is not well understood.

Recent findings suggested that transition from heterozygosity to homozygosity for *JAK2V617F* is associated with a hyperproliferative disease profile and may be important for disease progression, at least from PV to secondary myelofibrosis.[27] Moreover, Barosi et al showed in a longitudinal prospective study that the presence of a *JAK2V617F* hematopoietic clone was significantly associated with leukemic transformation in PMF.[28] This is in contrast to our present findings showing that not only the mutational status of *JAK2V617F*, but also 9pCNN-LOH with homozygous *JAK2V617F*, had no impact on the time to leukemic transformation in patients with MPN-blast phase. In addition, 2 of the 11 matched MPN samples, initially positive for *JAK2V617F* with either trisomy 9 or 9pCNN-LOH, became negative for these abnormalities after leukemic transformation. Although only tested in unpaired samples, PMF and PV samples also showed a significantly smaller number of both *JAK2V617F*+ and 9pCNN-LOH in the blast phase compared with the chronic phase. Interestingly, and also contrary to the previously cited studies, Tefferi et al noted a significant association between a low *JAK2V617F* allelic burden and evolution to blast phase in a large cohort of PMF patients.[29] Even though these data are not completely congruent with our findings, the results of Tefferi et al and our results point to the coexistence of a more dominant *JAK2V617F*-negative clone with a higher propensity to undergo clonal evolution. This is congruent with recent studies indicating that *JAK2V617F*+ MPN can result in *JAK2V617F*− MPN-blast phase.[30,31] But still, some of our matched cases with *JAK2V617F*+ had no change in abnormalities including *JAK2* mutational status as well as 9pCNN-LOH, allowing the existence of a common pre-*JAK2V617F* clone. Taken together, the presence of *JAK2V617F* appears not to be a prerequisite for leukemic transformation of MPN, suggesting that additional genetic events are required for full transformation.

SNP-array analysis was able to capture practically all cytogenetic abnormalities and to uncover additional lesions with potentially important clinical implications. The number of genomic alterations was more than 2 to 3 times greater in the blast phase as in the chronic phase of matched and unmatched cases with MPN. Noticeably, ET patients had fewer alterations in their chronic phase samples compared with the PV and PMF cases, whereas the number was comparable in all 3 MPN subgroups after their transformation. Being aware of the increased number of new

genomic changes enables investigators to focus on the identification of causative genes associated with the evolution of MPN to leukemia.

Commonly altered regions in blast crisis samples were detected on chromosomes 8, 12, 17, and 21 encompassing *MYC, ETV6, TP53,* and *RUNX1,* respectively, which are already known to be involved in the development of de novo and secondary AML.[18-22] Gain of chromosomal material at 8q24.21 was almost exclusively found in *JAK2V617F*$^-$ samples, suggesting that increased activity of *MYC* might allow selection of clones that do not require the *JAK2* gain-of-function mutation. Furthermore, deletion of 17p *(TP53)* was significantly associated with prior exposure to hydroxyurea as well as a complex karyotype in samples with MPN-blast crisis, which is in accordance with recent results.[32,33] Interestingly, not only deletion, but also 17pCNN-LOH, was associated with a complex karyotype, a poor prognostic marker in myeloid malignancies.

In addition, regions on chromosomes 1q, 7q, 16q, 19p, and 21q were frequently altered in the evolution to the leukemic phase and may harbor promising new candidate genes. Abnormalities involving chromosome 7 are frequently detectable in de novo and secondary AML,[34-37] and preceding studies have found a critical breakpoint region involving a locus at centromeric band 7q22, whereas the telomeric breakpoint varies from q32 to q36. Interestingly, the minimal deleted region in our cohort was located at 7q22.1 encompassing only 2 promising target genes, *SH2B2* (previously named *APS*) and *CUTL1*. *SH2B2* regulates and enhances *JAK2*-mediated cellular responses,[38] and the *CUTL1* gene encodes for a *CUT* family member of the homeodomain proteins that can repress the expression of developmentally regulated myeloid genes.[39] Moreover, genome-wide inspection for minimal regions of duplications/amplifications and CNN-LOH revealed several interesting genes, such as *PIN1, ICAM1,* and *CDC37* on 19p as well as *ERG* on 21q. Whereas the latter 3 targets have been shown to possess potential progrowth activity in de novo AML and/or MDS,[25,26,40] *PIN1* is known to be overexpressed in a variety of cancers and may act as an oncogene via promotion of cell cycle progression and proliferation.[24]

Mutations of the *c-CBL* gene are tightly associated with 11qCNN-LOH and are commonly diagnosed in patients with chronic myelomonocytic leukemia.[23,41,42] Although MPN shares clinical as well as hematologic features with chronic myelomonocytic leukemia, we detected 11qCNN-LOH only in a minority of our study population, suggesting that *c-CBL* mutations are rare events leading to transformation of chronic MPN to leukemic blast phase.

In contrast to recent findings showing frequent LOH on 4q associated with *TET2* mutations in patients diagnosed with MDS/MPN,[43] we detected CNN-LOH or deletions at 4q24 *(TET2)* only in a minority of our patients in the chronic as well as blast phase of MPN. Nevertheless, our study was not sufficient to explore these findings in more detail and make conclusions on tumor suppressor *TET2* and its potential role in leukemic transformation.

However, with regard to the variety of detected allelic imbalances, we suggest that no single candidate gene or molecular pathway is sufficient and necessary to cause transformation of chronic MPN to blast phase. Like de novo AML, MPN-blast phase appears to be a heterogeneous disease prone to have evolved multiple mechanisms to provide a proliferative advantage to the abnormal leukemic clone.

CNN-LOH involving chromosomal regions that are also frequently affected by deletions may have prognostic implications similar to the deletions visible by karyotyping. In our study, prognostic evaluation was based mainly on SNP-array data from blast phase samples without the incorporation of SNP-array results from the matched chronic phase. Moreover, we implied the survival and clinical outcome only of MPN patients who underwent leukemic transformation, without comparison with survival and outcome in untransformed chronic phase. However, as expected, blast phase patients with loss of chromosomal material on 7q showed poor survival, because this is known to be predictive for rapid progression and poor response in AML therapy.[35-37] MPN-blast phase patients with cytogenetically undetectable 7qCNN-LOH had comparable survival rates to those with −7/7q− in their leukemic cells, which is in accordance with previously published data.[44]

In addition, 9pCNN-LOH with homozygous *JAK2* mutation was also linked to an inferior outcome in MPN-blast crisis in comparison with patients with either heterozygous *JAK2V617F* or wild-type *JAK2*. In contrast to LOH on 17p, the prognostic impact of 9pCNN-LOH was independent of established risk factors such as −7/7q−, 5q−, or complex karyotype. Although *JAK2V617F* in association with 9pCNN-LOH appeared to have no impact on the time to MPN transformation, we suggest that the homozygous driver mutation in combination with additional newly acquired aberrations in terms of a second hit may have an implication on the clinical course of MPN-blast phase patients.

In conclusion, high-density SNP-array technology allowed precise identification of chromosomal aberrations, including CNN-LOH, and complemented conventional cytogenetic techniques in patients with chronic and transformed MPN. Our analysis provided prognostic details to further improve clinical prognosis, as well as novel interesting candidate genes potentially involved in the transformation of MPN.

Acknowledgments

We thank the members of all cooperating laboratories for helpful discussions.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG; TH 1438/1-1, N.H.T.), National Science Council (NSC; NSC96-2314-B-182-003, L.-Y.S.), Chang Gung Memorial Hospital (CMRP; CMRPG330303, L.-Y.S.), and the National Institutes of Health (NIH; grant 5R01CA026038-31, H.P.K.). H.P.K. is the holder of the Mark Goodson endowed Chair in Oncology Research at Cedars Sinai Medical Center and is a member of the Jonsson Cancer Center and the Molecular Biology Institute, UCLA, and is supported by the A*STaR award from the National University of Singapore.

Authorship

Contribution: N.H.T and U.O.K. performed the research, analyzed the data, and wrote the paper; D.H.T.L., N.K., G.B.I., T.L., T.W., D.N., M.K.-M., M.K., M.S., L.-Y.S., A.N., and S.D.R. assisted with the research; C.M.-T., R.M., T.H., D.G.G., and A.T. designed and performed the research; and S.O. and H.P.K. directed the overall study.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Nils H. Thoennissen, Division of Hematology and Oncology, Cedars Sinai Medical Center, UCLA School of Medicine, 8700 Beverly Blvd, Los Angeles, CA 90048; e-mail: nils.thoennissen@cshs.org.

References

1. Koppikar P, Levine RL. JAK2 and MPL mutations in myeloproliferative neoplasms. *Acta Haematol.* 2008;119(4):218-225.
2. Levine RL, Gilliland DG. Myeloproliferative disorders. *Blood.* 2008;112(6):2190-2198.
3. Grimwade D, Walker H, Harrison G, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. *Blood.* 2001;98(5):1312-1320.
4. Larson RA. Is secondary leukemia an independent poor prognostic factor in acute myeloid leukemia? *Best Pract Res Clin Haematol.* 2007;20(1):29-37.
5. Nannya Y, Sanada M, Nakazaki K, et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res.* 2005; 65(14):6071-6079.
6. Yamamoto G, Nannya Y, Kato M, et al. Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. *Am J Hum Genet.* 2007;81(1):114-126.
7. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood.* 2002;100(7): 2292-2302.
8. Kawamata N, Ogawa S, Yamamoto G, et al. Genetic profiling of myeloproliferative disorders by single-nucleotide polymorphism oligonucleotide microarray. *Exp Hematol.* 2008;36(11):1471-1479.
9. Scott LM, Scott MA, Campbell PJ, Green AR. Progenitors homozygous for the V617F mutation occur in most patients with polycythemia vera, but not essential thrombocythemia. *Blood.* 2006; 108(7):2435-2437.
10. University of California Santa Cruz. UCSC Genome Browser. http://genome.ucsc.edu. Accessed February, 2009.
11. Wellcome Trust Sanger. Ensembl. http://www.ensembl.org. Accessed September, 2009.
12. The Centre for Applied Genomics. Database of Genomic Variants. http://projects.tcag.ca/variation. Accessed March, 2009.
13. National Center for Biotechnology Information. Gene Expression Omnibus (GEO). http://www.ncbi.nlm.nih.gov/geo. Accessed December, 2009.
14. Schoch C, Schnittger S, Bursch S, et al. Comparison of chromosome banding analysis, interphase- and hypermetaphase-FISH, qualitative and quantitative PCR for diagnosis and for follow-up in chronic myeloid leukemia: a study on 350 cases. *Leukemia.* 2002;16(1):53-59.
15. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet.* 2005; 365(9464):1054-1061.
16. Weksberg R, Hughes S, Moldovan L, et al. A method for accurate detection of genomic microdeletions using real-time quantitative PCR. *BMC Genomics.* 2005;6:180.
17. Cervantes F, Passamonti F, Barosi G. Life expectancy and prognostic factors in the classic BCR/ABL-negative myeloproliferative disorders. *Leukemia.* 2008;22(5):905-914.
18. Hoffman B, Amanullah A, Shafarenko M, Liebermann DA. The proto-oncogene c-myc in hematopoietic development and leukemogenesis. *Oncogene.* 2002;21(21):3414-3421.
19. Bohlander SK. ETV6: a versatile player in leukemogenesis. *Semin Cancer Biol.* 2005;15(3):162-174.
20. Gaidano G, Guerrasio A, Serra A, et al. Mutations in the P53 and RAS family genes are associated with tumor progression of BCR/ABL negative chronic myeloproliferative disorders. *Leukemia.* 1993;7(7):946-953.
21. Merlat A, Lai JL, Sterkers Y, et al. Therapy-related myelodysplastic syndrome and acute myeloid leukemia with 17p deletion: a report on 25 cases. *Leukemia.* 1999;13(2):250-257.
22. Pabst T, Mueller BU. Transcriptional dysregulation during myeloid transformation in AML. *Oncogene.* 2007;26(47):6829-6837.
23. Dunbar AJ, Gondek LP, O'Keefe CL, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. *Cancer Res.* 2008;68(24):10349-10357.
24. Yeh ES, Means AR. PIN1, the cell cycle and cancer. *Nat Rev Cancer.* 2007;7(5):381-388.
25. Passam FH, Tsirakis G, Boula A, et al. Levels of soluble forms of ICAM and VCAM in patients with myelodysplastic syndromes and their prognostic significance. *Clin Lab Haematol.* 2004;26(6):391-395.
26. Pearl LH. Hsp90 and Cdc37: a chaperone cancer conspiracy. *Curr Opin Genet Dev.* 2005;15(1):55-61.
27. Passamonti F, Rumi E, Pietra D, et al. Relation between JAK2 (V617F) mutation status, granulocyte activation, and constitutive mobilization of CD34+ cells into peripheral blood in myeloproliferative disorders. *Blood.* 2006;107(9):3676-3682.
28. Barosi G, Bergamaschi G, Marchetti M, et al. JAK2 V617F mutational status predicts progression to large splenomegaly and leukemic transformation in primary myelofibrosis. *Blood.* 2007; 110(12):4030-4036.
29. Tefferi A, Lasho TL, Huang J, et al. Low JAK2V617F allele burden in primary myelofibrosis, compared to either higher allele burden or unmutated status, is associated with inferior overall and leukemia-free survival. *Leukemia.* 2008; 22(4):756-761.
30. Campbell PJ, Baxter EJ, Beer PA, et al. Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation. *Blood.* 2006; 108(10):3548-3555.
31. Theocharides A, Boissinot M, Girodon F, et al. Leukemic blasts in transformed JAK2-V617F-positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation. *Blood.* 2007;110(1):375-379.
32. Wattel E, Preudhomme C, Hecquet B, et al. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. *Blood.* 1994;84(9):3148-3157.
33. Haferlach C, Dicker F, Herholz H, Schnittger S, Kern W, Haferlach T. Mutations of the TP53 gene in acute myeloid leukemia are strongly associated with a complex aberrant karyotype. *Leukemia.* 2008;22(8):1539-1541.
34. Mitelman F, Kaneko Y, Trent JM. Report of the committee on chromosome changes in neoplasia. *Cytogenetic Cell Genet.* 1990;55(1–4):358–386.
35. Johnson E, Cotter FE. Monosomy 7 and 7q- associated with myeloid malignancy. *Blood Rev.* 1997;11(1):46-55.
36. van den Heuvel-Eibrink MM, Wiemer EA, de Boevere MJ, et al. MDR1 expression in poor-risk acute myeloid leukemia with partial or complete monosomy 7. *Leukemia.* 2001;15(3):398-405.
37. Erba HP. Prognostic factors in elderly patients with AML and the implications for treatment. *Hematology Am Soc Hematol Educ Program.* 2007; 2007:420-428.
38. Li M, Li Z, Morris DL, Rui L. Identification of SH2B2beta as an inhibitor for SH2B1- and SH2B2alpha-promoted Janus kinase-2 activation and insulin signaling. *Endocrinology.* 2007; 148(4):1615-1621.
39. Sinclair AM, Lee JA, Goldstein A, et al. Lymphoid apoptosis and myeloid hyperplasia in CCAAT displacement protein mutant mice. *Blood.* 2001; 98(13):3658-3667.
40. Warner JK, Wang JC, Takenaka K, et al. Direct evidence for cooperating genetic events in the leukemic transformation of normal human hematopoietic cells. *Leukemia.* 2005;19(10):1794-1805.
41. Sargin B, Choudhary C, Crosetto N, et al. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. *Blood.* 2007;110(3):1004-1012.
42. Sanada M, Suzuki T, Shih LY, et al. Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature.* 2009;460(7257): 904-908.
43. Jankowska AM, Szpurka H, Tiu RV, et al. Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. *Blood.* 2009;113(25):6403-6410.
44. Gondek LP, Tiu R, O'Keefe CL, Sekeres MA, Theil KS, Maciejewski JP. Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML. *Blood.* 2008;111(3):1534-1542.

Molecular Pathways

Clinical Cancer Research

Deregulated Intracellular Signaling by Mutated c-CBL in Myeloid Neoplasms

Seishi Ogawa[1,2], Lee-Yung Shih[3], Takahiro Suzuki[4], Makoto Otsu[5,6], Hiromitsu Nakauchi[5,6], H. Phillip Koeffler[7,8], and Masashi Sanada[1]

Abstract

c-CBL encodes a 120-kDa protein involved in intracellular signal transduction in a wide variety of cell types. Recently, frequent mutations of *c-CBL* have been reported in myeloid neoplasms showing both myelodysplastic and myeloproliferative features, in which most mutations are present in a homozygous state, as a result of allelic conversion in 11q. c-CBL has ubiquitin E3 ligase activity for a wide variety of tyrosine kinases, and thereby, negatively regulates tyrosine kinase signaling. Accordingly, c-CBL seems to have tumor suppressor functions, loss of which promotes tumorigenesis. On the other hand, once mutated, it is converted to an oncogenic protein and commits to myeloid leukemogenesis through a kind of gain of function causing aberrant signal transduction. The inhibition of mutant CBL protein or signaling pathways that it activates would have a role in therapeutics of myeloid neoplasms with *CBL* mutations. *Clin Cancer Res; 16(15); 3825–31. ©2010 AACR.*

Background

c-CBL proto-oncogene is a cellular counterpart of a viral oncogene, *v-CBL*, isolated from a transforming retrovirus that causes B-cell lymphoma and myeloid neoplasms in mice (1). c-CBL is recognized as a 120-kDa cytoplasmic protein rapidly phosphorylated after cytokine stimulation. Interacting with a broad spectrum of signaling and cytoskeletal molecules as a multi-adaptor protein as well as an E3 ubiquitin ligase, c-CBL is thought to be involved in intracellular signaling (2, 3). Although *c-CBL* was first identified through its oncogenic versions in mice, its role in human carcinogenesis has been elusive until recently, when frequent mutations of *c-CBL* have been reported in a subset of myeloid neoplasms (4–10). Mutations of *c-CBL* are found in a variety of myeloid neoplasms, including acute myeloid leukemia and myelodysplastic syndromes (4–7, 10, 11), but they are most frequent in those subtypes of myeloid neoplasms that are now grouped into myelodysplastic-myeloproliferative neoplasms (MDS-MPN) in the World Health Organization classification (12). MDS-MPN include chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML), and atypical chronic myeloid leukemia (aCML). c-CBL mutations are found in 5% of aCML (8) and up to 15% of JMML (9, 13, 14) and CMML (7, 10). They originate from immature hematopoietic progenitors and are commonly characterized by the production of dysplastic blood cells and myeloproliferative features (12, 15). Mutations seem to be somatic in most adult cases, but germline mutations were reported in some JMML cases in children (9). A conspicuous genetic feature of *c-CBL* mutations in these myeloid neoplasms is that mutations are homozygous in most cases, as a result of an allelic conversion of 11q arms that leads to duplication of the mutated parental copy of 11q and loss of the remaining wild-type allele, or "uniparental" disomy of the 11q arms. Mutations rarely accompany deletions of the wild-type allele (7–10), indicating the gain-of-function nature of the mutations rather than a simple loss-of-function (see below).

In mammals, three CBL homologs, c-CBL, CBL-b, and CBL-c, exist and are grouped into the CBL family of proteins (2, 3). All three homologs have a conserved *N*-terminal domain [tyrosine kinase-binding (TKB) domain], for their binding to phosphorylated tyrosine kinases, and a RING finger domain, as well as an intervening linker sequence. c-CBL and CBL-b, but not CBL-c, have extended C-terminal structures, including a proline-rich domain, a ubiquitin-associated–leucine zipper motif at the C terminus, and several tyrosine residues that are phosphorylated upon cytokine and/or growth factor stimulation (Fig. 1). The TKB domain consists of a four-helix bundle, a Ca^{2+}-binding EF hand, and a variant Src homology 2 (SH2) domain (16),

Authors' Affiliations: [1]Cancer Genomics Project, The University of Tokyo, Tokyo, Japan; [2]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan; [3]Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital, Chang Gung University, Taipei, Taiwan; [4]Division of Hematology, Department of Medicine, Jichi Medical University, Tochigi, Japan; [5]Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [6]Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Saitama, Japan; [7]Hematology/Oncology, Cedars-Sinai Medical Center, Los Angeles, California; and [8]National Cancer Institute of Singapore, National University of Singapore, Singapore

Corresponding Author: Seishi Ogawa, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan. Phone: 813-5800-9046; Fax: 813-5800-9047; E-mail: sogawa-tky@umin.ac.jp.

doi: 10.1158/1078-0432.CCR-09-2341

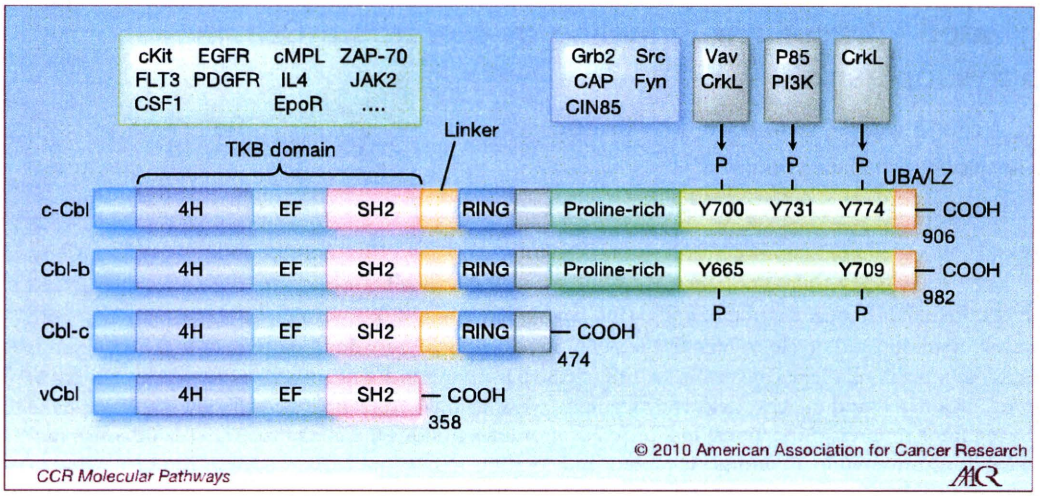


Fig. 1. Structure of CBL family proteins. Domain structures of CBL family proteins are depicted. Major tyrosine phosphorylation sites in c-CBL are indicated. Molecular interactions of c-CBL with cytokine receptors and other signaling molecules are also shown on top.

through which c-CBL binds to a phosphotyrosine-containing residue within a variety of activated tyrosine kinases. The spectrum of tyrosine kinases with which c-CBL can interact is thought to be determined by these N-terminal structures and includes receptor tyrosine kinases (RTK), such as epidermal growth factor receptor (EGFR; refs. 17–19), platelet-derived growth factor receptor (PDGFR; refs. 20–22), insulin-receptor (23, 24), c-Kit (25, 26), and FLT3 (5, 27), as well as non-RTKs (JAK2, ZAP70, and Syk; ref. 28). After being targeted to activated tyrosine kinases, c-CBL initiates a series of interactions with a variety of molecules as a multi-adaptor protein to transmit signals (Fig. 2A). First, c-CBL itself is phosphorylated at multiple tyrosine residues, to which a number of signaling molecules, including Vav (Y700; ref. 29), Crk/CrkL (Y700 and Y774; refs. 30–34), and the p85 subunit of PI3 kinase (Y731; refs. 35, 36) are recruited. The proline-rich domain provides binding sites for a variety of Src homology 3 (SH3)-containing proteins, including Grb2 (17, 18, 26, 32, 37–39) and NCK (40, 41), Src family tyrosine kinases (Fyn and Src; refs. 42–44), as well as CAP and CIN85 (45, 46). Grb2 constitutively binds to c-CBL in unstimulated cells, playing a role in recruitment of c-CBL to phosphorylated RTKs when cells are stimulated with their ligands. Src family kinases are responsible for phosphorylation of c-CBL on RTK stimulation. The long list of molecules making direct or indirect interactions with c-CBL is found in an excellent review (3), and the list is still growing. The complexity of molecular interactions of CBL comprises "the CBL interactome" and provides the basis for the diverse biological functions of c-CBL. Among these, the most extensively studied is its function as a negative regulator of tyrosine kinase signaling.

The negative regulation of tyrosine kinases by c-CBL was first implicated through genetic studies in *Caenorhabditis elegans*, in which the *c-CBL* ortholog, *sli-1*, was shown to be upstream of RAS (*let-60*) and Grb2 (*sem5*), and to suppress vulval induction that depends on *let23*, the ortholog of EGFR (47). Later, it was molecularly shown in mammalian cells that the negative regulation involves multi-ubiquitinylation of RTKs (21, 48, 49). c-CBL has E3 ubiquitin ligase activity, which is mediated by the linker-RING finger domains (50). c-CBL recruits E2 ubiquitin conjugating enzymes and ubiquitin monomers at the linker-RING finger interface and multi-ubiquitinylates the activated RTKs (Fig. 2A, upper panel). Depending on the multi-ubiquitinylation of the kinases, the kinase-c-CBL complexes are directed to endocytosis for subsequent degradation at lysosomes and/or proteasomes, or for recycling (21, 48, 49), which, in either case, limits kinase signals. Although multi-ubiquitinylation is critical for these reactions to occur, two c-CBL–bound adaptor molecules, CIN85 and CD2AP, mediate the endocytosis (45, 46). The negative regulatory roles of c-CBL in tyrosine kinase signaling suggest that the protein could have an antioncogenic function. In fact, *c-CBL* null mice have an enlarged thymus, expanded hematopoietic progenitor pools, splenomegaly with extramedullary hematopoiesis, as well as increased repopulating capacity of their bone marrow cells (10, 51–53). Blastic transformation of chronic myelogeneous leukemia in a bcr/abl-transgenic model is accelerated in the *c-CBL* null background (10). Finally, *c-CBL* null mice developed invasive cancers with complete penetrance.[9] Combined, these observations support that c-CBL can act as a tumor suppressor.

In contrast to the tumor suppressor function of the wild-type c-CBL, when transduced into NIH3T3 cells, *c-CBL* mutants isolated from human and murine neoplasms, as well as *v-CBL*, show clear transforming capacity in terms of

[9] Unpublished data.

anchorage-independent growth in soft agar and tumor generation in nude mice (10, 54). Bone marrow cells transduced with mutant *c-CBL* (R420Q and 70Z mutants) generate generalized mastocytosis, a myeloproliferative disease, and myeloid leukemia in lethally irradiated mice with long latency but high penetrance (55). The transforming activity of mutant c-CBL seems to be mediated by alteration of the E3 ubiquitin ligase activity. Except for rare mutations causing a premature truncation of the TKB domain, most *c-CBL* mutations in myeloid neoplasms are missense changes at highly conserved amino-acid positions within the linker and RING finger domains, or involve splice-site sequences, leading to amino acid deletions within these domains. Although the E3 ubiquitin ligase activity primarily depends on the RING finger domain, the intact linker sequence, which tightly packs with the TKB domain as well as with the E2 ligase, is also considered to be essential for efficient ubiquitinylation to occur (56). The crystal structure of the c-CBL/UBCH7 complex suggests that Y371 is important for the integrity of the linker-TKB interface (56). Thus, tumor-derived *c-CBL* mutations are expected to affect the E3 ubiquitin ligase activity. In fact, linker-RING finger mutations found in myeloid neoplasms, as well as other artificially introduced mutations within these domains, were shown to have compromised E3 ubiquitin ligase activity (5, 8, 10, 54). Moreover,

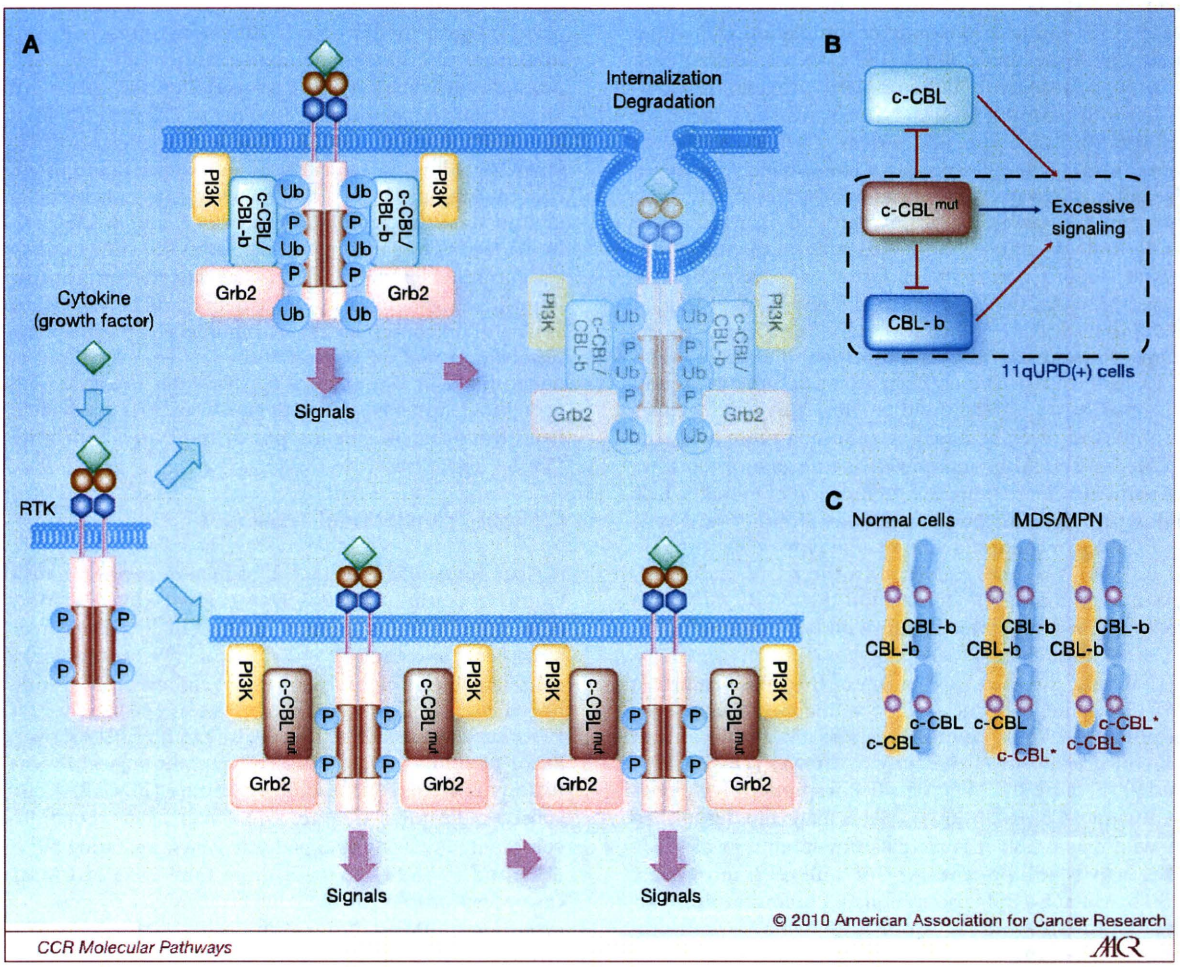


Fig. 2. Putative mechanism of gain of function of c-CBL mutants. A, after cytokine (growth factor) stimulation, RTKs are phosphorylated, to which c-CBL or CBL-b binds to ubiquitinate the receptors, while participating in signal transduction. Ubiquitinated RTKs are then subjected to degradation or recycling. On the other hand, when mutant c-CBL binds to the activated RTKs, downregulation of the RTKs is compromised, leading to prolonged signaling. B, putative mechanisms of the gain of function of c-CBL mutants; the CBL-b-inhibition model (red line) and the mechanism mediated by positive regulatory functions of c-CBL (blue line). C, in the CBL-b-inhibition model, a c-CBL mutant inhibits the E3 ubiquitin ligase activity of both c-CBL and CBL-b. In the heterozygous state, the inhibitory action of the c-CBL mutant is largely titrated out by three intact copies of c-CBL and CBL-b, leading to only modest increase in sensitivity to cytokines, as compared with the normal state (middle panel). When the mutant allele is duplicated by an allelic conversion in 11q, the mutant protein expressed from the two mutated alleles can effectively inhibit the remaining enzymatic activity from CBL-b (right panel).

these c-CBL mutants strongly inhibit the E3 ligase activity of wild-type c-CBL, indicating that linker-RING finger mutants act in a dominant negative manner against wild-type c-CBL (10). This finding is expected because a simple loss-of-function would not explain the dominant effect of c-CBL mutant on transforming activity in NIH3T3 cells expressing wild-type c-CBL. Interestingly, this inhibitory effect does not seem to depend on dimerization with the wild-type c-CBL, but on intact binding to phosphorylated tyrosine kinases, because a G306E mutation abolishes oncogenic capacity of these c-CBL mutants.[10] Thus, when overexpressed in EGFR-transduced NIH3T3 cells, mutant c-CBL inhibits ubiquitinylation of EGFR, leading to prolonged activation of the receptor after EGF stimulation. Similarly, transduction of c-CBL mutants into hematopoietic cell lines results in prolonged activation of c-Kit, FLT3, and Jak2 kinases after stimulation with either their ligands or interleukin 3 (IL-3; Fig. 2A, lower panel; refs. 10, 55). Murine hematopoietic progenitors transduced with tumor-derived *c-CBL* mutants show increased cell survival in the presence of stem cell factor, similar to those from *c-CBL* null mice (10). Unexpectedly, however, the effect of these c-CBL mutants becomes much more prominent in the *c-CBL* null background, in which these c-CBL mutants induce exaggerated survival or even proliferative responses to stem cell factor. Moreover, the augmented proliferative and/or survival responses of mutant c-CBL–transduced cells are also found for a broader spectrum of cytokines, including thrombopoietin, IL-3, and FLT3 ligand (10). These effects of *c-CBL* mutants found in the *c-CBL* null background are not explained by either a simple loss of c-CBL functions or inhibition of wild-type c-CBL, but should be interpreted as true gain of function. Of particular interest, the gain of function of mutant c-CBL is lost in large part by the presence of either wild-type *c-CBL* allele or cotransduction of wild-type *c-CBL*. The gain of function becomes apparent in the *c-CBL* null background, explaining the observation that *c-CBL* mutations are found in a homozygous state with loss of the wild-type c-CBL in most cases (7–10).

Currently, the exact mechanism of the gain of function of c-CBL mutants is unclear. A possible mechanism is inhibition of CBL homologs (Fig. 2B, red arrow) and/or CBL-intrinsic positive regulatory machinery (Fig. 2B, blue arrow). Because the hypersensitive response to cytokines in mutant *c-CBL*–transduced cells is markedly diminished by wild-type c-CBL, it is mediated by inhibition of "CBL-like" activity still present in *c-CBL* null cells, most likely CBL-b. Mutant c-CBL also inhibits E3 ubiquitin ligase activity of CBL-b, which is expressed in hematopoietic progenitor cells (10). *c-CBL/CBL-b* double knockout T cells show exaggerated proliferative response to anti-CD3 stimulation and prolonged T-cell receptor signaling, as compared with *c-CBL* or *CBL-b* single knockout T cells (57). According to this model, two mutant *c-CBL* alleles could functionally titrate out two wild-type *CBL-b* alleles, whereas one mutant *c-CBL* allele might not be sufficient to overcome one wild-type *c-CBL* plus two wild-type *CBL-b* alleles (Fig. 2C).

Another possible mechanism of the gain of function of mutated c-CBL is related to its function as a multi-adaptor, which is implicated in positive regulatory functions in signal transduction (Fig. 2B, blue arrow). As an adaptor protein, kinase-bound c-CBL recruits a number of molecules involved in signal transductions and cytoskeletal regulations. For examples, upon either IL-4 or granulocyte colony-stimulating factor stimulation, c-CBL is tyrosine-phosphorylated and binds to the p85 subunit of phosphoinositide 3 kinase (PI3K) to transmit mitogenic and/or survival signals (58, 59). Similarly, CBL was shown to regulate integrin-mediated cell adhesion, spreading, and migration in a PI3K-dependent manner (60, 61). CBL promotes activation of MAP kinases after stimulation of Met tyrosine kinase through binding to Crk (62). c-CBL is one of the downstream substrates and/or effectors of Src kinase signaling, necessary for bone resorption and osteoclast migration (63). It is also involved in cytoskeletal regulation via activation of Rac1 or Cdc42, and *R*-RAS (64). In the face of loss of negative regulatory functions due to compromised E3 ubiquitin ligase activity, the intrinsic role in positive signaling of c-CBL protein could be unmasked as gain of function (Fig. 2B). This model could explain the observation that c-CBL mutations were much more frequent than CBL-b mutations in MDS-MPN, because both proteins clearly have different functionalities, as evident from the different phenotypes of their knockout mice (51, 52, 65).

Clinical-Translational Advances

Gene mutations in signal transduction pathways are a common feature of MPN. Deregulated kinase activity caused by *bcr-abl* and mutated *JAK2* is a hallmark of chronic myelocytic leukemia and classical myeloproliferative disorders, including polycythemia vera, essential thrombocythemia, and primary myelofibrosis (66). Genes for RTKs, such as PDGFRs (PDGFRA/B) and fibroblast growth factor receptors (FGFR) are also recurrent targets of gene fusions in hypereosinophilic syndrome (PDGFRA) and subsets of CMML (FGFR; ref. 67). Finally, gene mutations commonly involving RAS pathway genes, including *NF-1*, *RAS*, and *PTPN11*, occur in more than 70% of CMML cases, responsible for their hypersensitivity to granulocyte-macrophage colony-stimulating factor (15, 67). The recent finding of frequent *c-CBL* mutations in the MDS-MPD subgroup revealed a novel mechanism for excessive cell signaling through deregulated kinase activity in MPN, especially MDS-MPN subtypes, and also provided an insight into the therapeutics of *c-CBL*–mutated myeloid neoplasms.

Because *c-CBL* mutations induce excessive tyrosine kinase signaling, use of tyrosine kinase inhibitors could be

[10] Unpublished data.

a logical approach to the control of *c-CBL*–mutated neoplasms. However, the broad spectrum of c-CBL–regulated tyrosine kinases may preclude the efficacy of selective kinase inhibitors, whereas the use of pan-kinase inhibitors would increase a risk of the development of unacceptable adverse effects. Otherwise, identification of functionally relevant kinases regulated by mutated c-CBL would enable efficient targeting of such inhibition. Alternatively, the downstream signaling pathways, including JAK/STAT, PI3K, as well as RAS/extracellular signal-regulated kinase (ERK) signalings, are also potential therapeutic targets for inhibition with low molecular-weight compounds.

Given the gain-of-function nature of c-CBL mutants, inhibition of these mutant proteins would be a more reasonable approach, regardless of the exact mechanism of the gain-of function. Because the oncogenic action of mutant c-CBL proteins depends on their intact binding to target kinases, inhibition of this binding would be a potential approach, especially when the inhibition could be specifically directed to mutant c-CBL, but be saved for CBL-b. Recently, piceatannol, a naturally occurring phenol stilbenenoid, was shown to induce loss of the CBL family of proteins including mutant CBL (70Z mutant; ref. 68). Piceatannol was initially isolated as an antileukemic agent from a domesticated oilseed and was shown to inhibit a broad spectrum of tyrosine kinases including Sky, Src, Lck, and FAK, as well as some serine-threonine kinases (69–72). It also induces selective loss of CBL-associated proteins; levels of PDGFRβ, c-Abl, and EGFR are reduced by piceatannol treatment, whereas those of c-Src, Lyn, Syk, and Grb2 are unaffected (68). The molecular mechanism that underlies piceatannol-induced CBL loss is still unclear. It does not depend on proteasome, lysosome, and caspase activation, but rather on reactive oxygen species, which seems to be distinct from the mechanism of inhibition of kinase activities (68). Although piceatannol shows a broad spectrum of biological activity as an anti-inflammatory, antihistamine, and general antitumor agent *in vitro* (73–75), because of its broad biochemical actions, it has not been determined if, or to what extent, the biological activities of piceatannol depend on piceatannol-induced loss of CBL proteins. Although loss of both c-CBL and CBL-b is likely to result in increased tyrosine kinase activity, it also induces CBL-associated molecules and inhibits activity of a number of kinases, actually showing general antitumor activity. Unfortunately, no information is currently available about the antitumor effect of piceatannol on c-CBL–mutated leukemia. In c-CBL–mutated leukemic cells, loss of mutant c-CBL may further augment antitumor activity of this agent.

Conclusion

c-CBL mutations are tightly associated with myeloproliferative myeloid neoplasms, especially the MDS-MPD subtype. c-CBL seems to act as a tumor suppressor, but when mutated, it is converted to an oncogenic protein. Although the oncogenic potential of c-CBL mutants is thought to be related to a type of gain of function, the molecular basis of this gain of function has not been fully understood. Undoubtedly, the effect of these mutations on the E3 ubiquitin ligase activity is essential for the gain of function. What complicates the mechanism is the fact that c-CBL has dual functionalities; it can behave as a multi-adaptor signal transducer, while also terminating signals by ubiquitinylating activated tyrosine kinases. Clearly, to understand the exact oncogenic mechanism of c-CBL mutants and to develop effective therapeutics, further *in vivo* and *in vitro* analyses are required.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Received 02/18/2010; revised 05/06/2010; accepted 05/10/2010; published OnlineFirst 06/14/2010.

References

1. Langdon WY, Hartley JW, Klinken SP, Ruscetti SK, Morse HC III. v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. Proc Natl Acad Sci U S A 1989;86:1168–72.
2. Thien CB, Langdon WY. Cbl: many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol 2001;2:294–307.
3. Schmidt MH, Dikic I. The Cbl interactome and its functions. Nat Rev Mol Cell Biol 2005;6:907–18.
4. Caligiuri MA, Briesewitz R, Yu J, et al. Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. Blood 2007;110:1022–4.
5. Sargin B, Choudhary C, Crosetto N, et al. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. Blood 2007;110:1004–12.
6. Abbas S, Rotmans G, Lowenberg B, Valk PJ. Exon 8 splice site mutations in the gene encoding the E3-ligase CBL are associated with core binding factor acute myeloid leukemias. Haematologica 2008;93:1595–7.
7. Dunbar AJ, Gondek LP, O'Keefe CL, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res 2008;68:10349–57.
8. Grand FH, Hidalgo-Curtis CE, Ernst T, et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood 2009;113:6182–92.
9. Loh ML, Sakai DS, Flotho C, et al. Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood 2009;114:1859–63.
10. Sanada M, Suzuki T, Shih LY, et al. Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. Nature 2009;460:904–8.
11. Reindl C, Quentmeier H, Petropoulos K, et al. CBL exon 8/9 mutants activate the FLT3 pathway and cluster in core binding factor/11q deletion acute myeloid leukemia/myelodysplastic syndrome subtypes. Clin Cancer Res 2009;15:2238–47.
12. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009;114:937–51.
13. Muramatsu H, Makishima H, Jankowska AM, et al. Mutations of E3

ubiquitin ligase Cbl family members but not TET2 mutations are pathogenic in juvenile myelomonocytic leukemia. Blood 2010;15: 1969–75.
14. Shiba N, Kato M, Myoung-ja P, et al. CBL mutations in juvenile myelomonocytic leukemia, but not in pediatric myelodysplastic syndrome. Leukemia. In press 2010.
15. Emanuel PD. Juvenile myelomonocytic leukemia and chronic myelomonocytic leukemia. Leukemia 2008;22:1335–42.
16. Meng W, Sawasdikosol S, Burakoff SJ, Eck MJ. Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase. Nature 1999;398:84–90.
17. Fukazawa T, Miyake S, Band V, Band H. Tyrosine phosphorylation of Cbl upon epidermal growth factor (EGF) stimulation and its association with EGF receptor and downstream signaling proteins. J Biol Chem 1996;271:14554–9.
18. Meisner H, Czech MP. Coupling of the proto-oncogene product c-Cbl to the epidermal growth factor receptor. J Biol Chem 1995;270: 25332–5.
19. Galisteo ML, Dikic I, Batzer AG, Langdon WY, Schlessinger J. Tyrosine phosphorylation of the c-cbl proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation. J Biol Chem 1995;270:20242–5.
20. Bonita DP, Miyake S, Lupher ML, Jr., Langdon WY, Band H. Phosphotyrosine binding domain-dependent upregulation of the platelet-derived growth factor receptor alpha signaling cascade by transforming mutants of Cbl: implications for Cbl's function and oncogenicity. Mol Cell Biol 1997;17:4597–610.
21. Miyake S, Lupher ML, Jr., Druker B, Band H. The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha. Proc Natl Acad Sci U S A 1998;95:7927–32.
22. Miyake S, Mullane-Robinson KP, Lill NL, Douillard P, Band H. Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain. J Biol Chem 1999;274:16619–28.
23. Ahmed Z, Smith BJ, Pillay TS. The APS adapter protein couples the insulin receptor to the phosphorylation of c-Cbl and facilitates ligand-stimulated ubiquitination of the insulin receptor. FEBS Lett 2000;475:31–4.
24. Ribon V, Printen JA, Hoffman NG, Kay BK, Saltiel AR. A novel, multifuntional c-Cbl binding protein in insulin receptor signaling in 3T3–1 adipocytes. Mol Cell Biol 1998;18:872–9.
25. Sattler M, Salgia R, Shrikhande G, et al. Steel factor induces tyrosine phosphorylation of CRKL and binding of CRKL to a complex containing c-kit, phosphatidylinositol 3-kinase, and p120(CBL). J Biol Chem 1997;272:10248–53.
26. Wisniewski D, Strife A, Clarkson B. c-kit ligand stimulates tyrosine phosphorylation of the c-Cbl protein in human hematopoietic cells. Leukemia 1996;10:1436–42.
27. Lavagna-Sevenier C, Marchetto S, Birnbaum D, Rosnet O. FLT3 signaling in hematopoietic cells involves CBL, SHC and an unknown P115 as prominent tyrosine-phosphorylated substrates. Leukemia 1998;12:301–10.
28. Lupher ML, Jr., Rao N, Lill NL, et al. Cbl-mediated negative regulation of the Syk tyrosine kinase. A critical role for Cbl phosphotyrosine-binding domain binding to Syk phosphotyrosine 323. J Biol Chem 1998;273:35273–81.
29. Marengere LE, Mirtsos C, Kozieradzki I, Veillette A, Mak TW, Penninger JM. Proto-oncoprotein Vav interacts with c-Cbl in activated thymocytes and peripheral T cells. J Immunol 1997;159:70–6.
30. de Jong R, ten Hoeve J, Heisterkamp N, Groffen J. Crkl is complexed with tyrosine-phosphorylated Cbl in Ph-positive leukemia. J Biol Chem 1995;270:21468–71.
31. Andoniou CE, Thien CB, Langdon WY. The two major sites of cbl tyrosine phosphorylation in abl-transformed cells select the crkL SH2 domain. Oncogene 1996;12:1981–9.
32. Buday L, Khwaja A, Sipeki S, Farago A, Downward J. Interactions of Cbl with two adapter proteins, Grb2 and Crk, upon T cell activation. J Biol Chem 1996;271:6159–63.
33. Ribon V, Hubbell S, Herrera R, Saltiel AR. The product of the cbl oncogene forms stable complexes *in vivo* with endogenous Crk in a tyrosine phosphorylation-dependent manner. Mol Cell Biol 1996; 16:45–52.
34. Sattler M, Salgia R, Okuda K, et al. The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway. Oncogene 1996;12:839–46.
35. Hartley D, Corvera S. Formation of c-Cbl.phosphatidylinositol 3-kinase complexes on lymphocyte membranes by a p56lck-independent mechanism. J Biol Chem 1996;271:21939–43.
36. Hunter S, Burton EA, Wu SC, Anderson SM. Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. J Biol Chem 1999;274:2097–106.
37. Brizzi MF, Dentelli P, Lanfrancone L, Rosso A, Pelicci PG, Pegoraro L. Discrete protein interactions with the Grb2/c-Cbl complex in SCF- and TPO-mediated myeloid cell proliferation. Oncogene 1996;13: 2067–76.
38. Wang Y, Yeung YG, Langdon WY, Stanley ER. c-Cbl is transiently tyrosine-phosphorylated, ubiquitinated, and membrane-targeted following CSF-1 stimulation of macrophages. J Biol Chem 1996; 271:17–20.
39. Husson H, Mograbi B, Schmid-Antomarchi H, Fischer S, Rossi B. CSF-1 stimulation induces the formation of a multiprotein complex including CSF-1 receptor, c-Cbl, PI 3-kinase, Crk-II and Grb2. Oncogene 1997;14:2331–8.
40. Miyoshi-Akiyama T, Aleman LM, Smith JM, Adler CE, Mayer BJ. Regulation of Cbl phosphorylation by the Abl tyrosine kinase and the Nck SH2/SH3 adaptor. Oncogene 2001;20:4058–69.
41. Adler CE, Miyoshi-Akiyama T, Aleman LM, Tanaka M, Smith JM, Mayer BJ. Abl family kinases and Cbl cooperate with the Nck adaptor to modulate Xenopus development. J Biol Chem 2000;275: 36472–8.
42. Tezuka T, Umemori H, Fusaki N, et al. Physical and functional association of the cbl protooncogen product with an src-family protein tyrosine kinase, p53/56lyn, in the B cell antigen receptor-mediated signaling. J Exp Med 1996;183:675–80.
43. Deckert M, Elly C, Altman A, Liu YC. Coordinated regulation of the tyrosine phosphorylation of Cbl by Fyn and Syk tyrosine kinases. J Biol Chem 1998;273:8867–74.
44. Feshchenko EA, Langdon WY, Tsygankov AY. Fyn, Yes, and Syk phosphorylation sites in c-Cbl map to the same tyrosine residues that become phosphorylated in activated T cells. J Biol Chem 1998;273:8323–31.
45. Bachmaier K, Krawczyk C, Kozieradzki I, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature 2000;403:211–6.
46. Baumann CA, Ribon V, Kanzaki M, et al. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. Nature 2000;407:202–7.
47. Yoon CH, Lee J, Jongeward GD, Sternberg PW. Similarity of sli-1, a regulator of vulval development in C. elegans, to the mammalian proto-oncogene c-cbl. Science 1995;269:1102–5.
48. Levkowitz G, Waterman H, Zamir E, et al. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev 1998;12:3663–74.
49. Lee PS, Wang Y, Dominguez MG, et al. The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. EMBO J 1999;18:3616–28.
50. Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science 1999;286:309–12.
51. Murphy MA, Schnall RG, Venter DJ, et al. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. Mol Cell Biol 1998;18:4872–82.
52. Naramura M, Kole HK, Hu RJ, Gu H. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. Proc Natl Acad Sci U S A 1998;95:15547–52.
53. Rathinam C, Thien CB, Langdon WY, Gu H, Flavell RA. The E3 ubiquitin ligase c-Cbl restricts development and functions of hematopoietic stem cells. Genes Dev 2008;22:992–7.

54. Thien CB, Walker F, Langdon WY. RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. Mol Cell 2001;7:355–65.
55. Bandi SR, Brandts C, Rensinghoff M, et al. E3 ligase-defective Cbl mutants lead to a generalized mastocytosis and myeloproliferative disease. Blood 2009;114:4197–208.
56. Zheng N, Wang P, Jeffrey PD, Pavletich NP. Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell 2000;102:533–9.
57. Naramura M, Jang IK, Kole H, Huang F, Haines D, Gu H. c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. Nat Immunol 2002;3:1192–9.
58. Ueno H, Sasaki K, Honda H, et al. c-Cbl is tyrosine-phosphorylated by interleukin-4 and enhances mitogenic and survival signals of interleukin-4 receptor by linking with the phosphatidylinositol 3′-kinase pathway. Blood 1998;91:46–53.
59. Grishin A, Sinha S, Roginskaya V, et al. Involvement of Shc and Cbl-PI 3-kinase in Lyn-dependent proliferative signaling pathways for G-CSF. Oncogene 2000;19:97–105.
60. Meng F, Lowell CA. A beta 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. EMBO J 1998;17:4391–403.
61. Zell T, Warden CS, Chan AS, et al. Regulation of beta 1-integrin-mediated cell adhesion by the Cbl adaptor protein. Curr Biol 1998; 8:814–22.
62. Garcia-Guzman M, Larsen E, Vuori K. The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. Oncogene 2000;19:4058–65.
63. Tanaka S, Amling M, Neff L, et al. c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. Nature 1996;383: 528–31.
64. Gotoh T, Niino Y, Tokuda M, et al. Activation of R-Ras by Ras-guanine nucleotide-releasing factor. J Biol Chem 1997;272:18602–7.
65. Chiang YJ, Kole HK, Brown K, et al. Cbl-b regulates the CD28 dependence of T-cell activation. Nature 2000;403:216–20.
66. Levine RL, Gilliland DG. Myeloproliferative disorders. Blood 2008; 112:2190–8.
67. Tefferi A, Gilliland DG. Oncogenes in myeloproliferative disorders. Cell Cycle 2007;6:550–66.
68. Klimowicz AC, Bisson SA, Hans K, Long EM, Hansen HC, Robbins SM. The phytochemical piceatannol induces the loss of CBL and CBL-associated proteins. Mol Cancer Ther 2009;8:602–14.
69. Thakkar K, Geahlen RL, Cushman M. Synthesis and protein-tyrosine kinase inhibitory activity of polyhydroxylated stilbene analogues of piceatannol. J Med Chem 1993;36:2950–5.
70. Geahlen RL, McLaughlin JL. Piceatannol (3,4,3′,5′-tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor. Biochem Biophys Res Commun 1989;165:241–5.
71. Law DA, Nannizzi-Alaimo L, Ministri K, et al. Genetic and pharmacological analyses of Syk function in alphaIIbbeta3 signaling in platelets. Blood 1999;93:2645–52.
72. Youn HS, Lee JY, Fitzgerald KA, Young HA, Akira S, Hwang DH. Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. J Immunol 2005;175:3339–46.
73. Larrosa M, Tomas-Barberan FA, Espin JC. The grape and wine polyphenol piceatannol is a potent inducer of apoptosis in human SK-Mel-28 melanoma cells. Eur J Nutr 2004;43:275–84.
74. Wieder T, Prokop A, Bagci B, et al. Piceatannol, a hydroxylated analog of the chemopreventive agent resveratrol, is a potent inducer of apoptosis in the lymphoma cell line BJAB and in primary, leukemic lymphoblasts. Leukemia 2001;15:1735–42.
75. Barton BE, Karras JG, Murphy TF, Barton A, Huang HF. Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines. Mol Cancer Ther 2004;3:11–20.