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Small percent of additional abnormal clones to Ph chromosome in the early chronic phase detected by large number of karyotype analysis and transformation to blastic crisis in chronic myelocytic leukemia

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Abstract

Extensive study on Chronic Myelocytic Leukemia (CML) was performed sequentially from the time of early chronic phase to the blastic crisis (BC) phase. A large number of 80-385 metaphases were karyotyped at the time of diagnosis in 16 patients and 13 in BC phase. Three patients were BC phase at diagnosis. All the patients had small percent of clones with additional chromosome aberration in 1.3-12.4% of the observed cells in the early stages of chronic phase. The additional abnormal clones which had existed at the time of diagnosis of early chronic phase remarkably increased in the BC phase of only 5 of 10 patients examined, because 3 of the 13 patients had no additional chromosome aberrations in BC phase. New abnormal clones which were not detected at the time of diagnosis emerged into the BC phase of 5 of the 10 patients. These additional clones were not always associated with the development of the BC. In all 3 patients diagnosed at 10.6 months before developing BC, small sized clone found in early chronic phase related to main clone in BC. Then, detection of additional clones in chronic phase for early detection of BC clone can be useful at only 10.6 months before BC. However, weak correlation between frequency of additional aberrations with Ph chromosome and duration of chronic phase was found. These findings also suggested that leukemic cells harboring Ph chromosome are high chromosome instability, in which are intrinsically labile for the acquisition of various additional chromosome aberrations at any stage, which might be related with resistance to chemotherapy, and therapies of interferon α and imatinib.

Keywords: chronic myelocytic leukemia; Ph chromosome; clonal evolution; large amount karyotype analysis.

Introduction

Cancer progression has been generally described as a stepwise clonal evolution driven by a series of gene or chromosome alteration (Nowell, 1976; Testa *et al.*, 1979). The clonal evolution of cells with Ph chromosome seen in chronic myelocytic leukemia (CML) has already been extensively studied from the cytogenetic point of views (Mitelman, 1993). CML is characterized by aberrant cells with Ph chromosome and *BCR-ABL* chimeric gene expression caused from reciprocal translocation between chromosomes 9 and 22 [t (9; 22) (q34; q11)] (Johansson *et al.*, 2002). After several or more years in chronic phase, it usually proceeds into the blast crisis (BC) phase (acute phase). Proliferation of single mutant cell with Ph chromosome takes place for a period of several years in the sub-clinical stage of CML, after which appearing of additional clone with Ph chromosome is well established as a marker for the BC phase diagnosis as confined clinically too (Kamada *et al.*, 1978). During the BC phase, additional chromosome aberrations such as +8,+22q- (additional Ph) and i(17q) and so on in addition to Ph chromosome appear in more than 70-80% of patients (Mitelman *et al.*, 1976; Kantarjian *et al.*, 1987; Mitelman, 1993; Johansson *et al.*, 2002; Tanaka *et al.*, 2004). In recently published article on sequentially analysis on chromosome aberrations, using an identical criteria and protocol for clinical therapies and observations, and using an identical protocol for chromosome analysis, in a large number of 114 CML patients in the same facility, development of some additional clones are closely related to chemotherapy (Tanaka *et al.*, 2004). Occurrence of major-route abnormalities [+8, i(17q), +22q-, +19] in BC phase was not associated with either duration of chronic phase or survival time after BC (Tanaka *et al.*, 2004).

However, these findings were reported by routine chromosome analysis in small cell numbers of about 20 metaphases. It was reported that low frequency of cell populations with additional aberrations with Ph chromosome have already found in chronic phase (Sonta & Sandberg, 1977; Sandberg, 1983; Ishihara *et al.*, 1983; Sadamori *et al.*, 1985). These might be some chance for the disappearance of small percent of clones during the course of treatment or appearance at BC phase. Furthermore, as additional clone seldom occurs in acute myelocytic leukema (AML) (Testa *et al.*, 1979; Hagemeijer *et al.*, 1981), so the development of additional aberrations has been considered to be closely related to the duration from the development of pathological clones until the time at which the diagnosis

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of CML can be established. However, no precise study on evolutional pattern of the small percent clones in individual patient has been performed. In an attempt to understand the biological meaning of these small percent of clones for the pathogenesis of CML, we examined precisely 80-385 metaphases in each patient sequentially from the early chronic phase to BC phases of the disease to detect all possible clonal evolution.

Materials and methods

Patients

Sixteen patients (10 males and 6 females, age ranging from 26 to 69) attending our hospital since 1980-1988, who diagnosed as CML formed subjects for the present study. All patients had been received no therapy at diagnosis. Clinical and hematological data at chronic phase in each patient are presented in Table 1. Subgroup of in BC of CML was classified according to clinical type and bone marrow smears observation by our criteria (Kamada & Uchino 1978; Kamada, 1979). All 16 patients were treated with busulfan (myleran) and followed with hydroxyurea at chronic phase. No patients were received bone marrow transplantation and interferon-α treatment. Chromosome analysis was performed at the time of diagnosis prior to the treatment, including 13 patients were serially analyzed their chromosome aberrations up to acquired BC phase. Three patients (cases 14, 15 and 16) had already BC phase at diagnosis, and following chromosome analyses were not available after that because they moved to other hospital. Ten of 16 patients were also confirmed molecular biologically by the presence of *BCR-ABL* chimeric DNA rearrangement by Southern blotting method at the time of diagnosis (Tanaka *et al*., 1989; Tanaka & Kamada 1990; Mansoor *et al*., 1992; Tanaka *et al*., 1993; Ge *et al*., 2001). *BCR/ABL* chimeric gene have three types of expression and produced chimeric proteins of p210, p190 and p230 (Ito *et al*., 2004), but gene expression and protein analyses did not performed in present study. Two patients (cases 8 and 13) were the atomic bomb survivors who had been exposed to radiation at 1.7 km and 4 km, respectively from the hypocenter of Hiroshima atomic bomb (Tanaka *et al*., 1989; Tanaka *et al.,* 2004). One (case 14) was a worker in a mustard gas factory.

Chromosome analysis

Chromosome analysis was performed according to our protocol (Kamada *et al*., 1981; Mansoor *et al*., 1992). Bone marrow cells were aspirated and cultured for 24 h in RPMI1640 containing 20% fetal calf serum. Cultures were arrested with 0.02 µg/l of colcemide at one hour before harvesting. Chromosome slides were treated with trypsin as standard Giemsa solution. All well-defined metaphases were photographed and karyotyped. Approximately 80-385 metaphases per a patient as a minimum target cell population were examined at the time of diagnosis for chromosome aberration. In our study, in which 20-131 cells/patient in the chronic and BC phases of 13 of the 16 patients analyzed. The abnormalities were scored as per ISCN (1995; 2005). In present chromosome analysis, more than two metaphases having identical karyotype were designated as clone, and only one metaphase having independent karyotype as a single aberration. Number of branches was scored as counting one in both clone and single abnormal cell.

Results

Clones with additional chromosome aberrations in diagnosis of early chronic phase

Cytogenetical data including the number of cells and frequencies of additional aberrations examined in each patient are presented in Table 1. In cases 14, 15 and 16 showed 25.7-62.6% of additional chromosome aberrations at the time of diagnosis, and they were diagnosed as BC, as confirmed by clinical findings and bone marrow smear examination. The percentage aberrations were 38.8% (36.1% showed missing Y chromosome positive clone) in case 14, 25.7% (out of which 11.7% showed missing 17 chromosome positive clone) in case 15 and 62.6% (out of which 53.3% showed trisomy of chromosome 8 positive clone) in case 16.

The 13 cases (from case 1 to 13) showed 1.3 to 12.4% of additional aberrations with Ph chromosome and also 0.3-1.3% had Ph negative cells at the time of diagnosis. Case 13 showed variant 9; 22 translocation with t(9;22)(q34;q11)t(9;22)(p13;q11) (Tanaka *et al*., 2000; 2001).

Fig. 1 shows the frequency of abnormal clones and single aberration with numerical and structural chromosome aberrations, and their distribution on each chromosome. The numerical aberrations were mostly trisomies of 9, 15, 18 and 21, and monosomy 8. The structural aberrations were involved in the changes of chromosomes 1, 2, 4, 5 and 8. The balanced type of aberrations such as translocation and inversion was rather rare. The unbalanced type such as deletion and duplication were common. Trisomies 8 and 19, and additional Ph chromosome(+22q-), which usually occur in blastic crisis phase, were found in low frequencies 2.8, 4, and 2%, respectively, at the diagnosis. In similar, no i(17q) was found in the total of 3,533 metaphase cells analyzed from 16 patients at the time of diagnosis.

Clonal evolution pattern in both chronic and BC phases

Of the 16 patients analyzed, 12 (cases 1-10, 12 and 13 in Table 1) had clinical and cytological information at BC phase. However, BC sample was not available in case 11, because he moved to another hospital outside city. The duration of chronic phase and karyotypes observed in the BC phase of these 13 patients are shown in Tables 1 and 2, respectively. Three patients (cases 5, 7 and 8) had no additional chromosome aberrations with t (9; 22) translocation. Cases 5, 7 and 8 showed additional chromosome aberrations in 1.6%, 1.3% and 2.8% of cells at diagnosis, but disappeared subsequently in the BC phase. These frequencies were lower than other cases

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examined in present study and the number of branches with additional aberrations was also lower. In case 5, additional aberrations had been observed in only 1.6% of the cells at the time of diagnosis. This frequency was quite low same as cases 7 and 9 during the BC phase. The patient acquired the BC phase 14 months later. The ordinary 46, XY, t (9;22)(q34;q11) karyotype remained unchanged and clones with additional aberrations did not occur with the progression into the acute phase(Table 1 & 2). In case 7, two cells with missing chromosome 15 and tetraploidy each was found at BC phase, but this abnormal cells was not detected in 154 metaphases

Table 1. Percentage of additional aberrations with Ph chromosome and clinical findings at chronic phase in 16 CML patients

Case No.	Age, Sex	At diagnosis or chronic (C) phase WBC (/mm^3)	NAP	BCR R+†	No. observed cells	No. cells with additional ab. with Ph (%)	Additional clone and aberration (%)	No. of branches¶	Percent of same clone as in BC	No. normal cells (%)	Duration of chronic phase (month)
1	56, M	236,500	82	R+	339	42 (12.4%)	**,+9 (7.5)** 4q- (1.5) ,+21 (1.5) ,+12 (0.6) ,+19 (0.3)	8	**7.5**	1 (0.3)	7
2	41, M	18,100	105	ND	305	18 (5.9)	,+22q- (2.3) 18q (1.3) ,+17 (1.0)	6	0	2 (0.7)	24
3	26, M	614,400	32	R+	309	21 (6.8)	4q- (1.2) 2q- (0.9) 8p- (0.6) **11p+ (0.3)** ,+22q- (0.3)	12	**0.3**	1 (0.3)	6
4	61, F	136,800	174	ND	80	7 (8.8)	92,XX (2.6) ,+8 (1.3) **,+4,+5 (1.3.)**	6	**1.3**	1 (1.3)	10.6
5	46, M	344,000	45	R+	385	6 (1.6)	1p+ (0.3)	5	ND	0	14
6	44, M	87,400	ND	R+	285	12 (4.2)	,+15 (0.7) ,+21 (0.7)	8	0	1 (0.4)	27
7	41, F	10,200	88	R+	154	2 (1.3)	1p- (0.6)	2	ND	1 (0.6)	65
8	68, F*	29,800	130	R+	107	3 (2.8)	5q- (1.9)	2	ND	0	78
9	69, F	88,300	ND	R+	240	3 (1.3)	17q+ (0.8)	2	0	1 (0.4)	27
10	34, F	294,000	12	ND	242	18 (7.4)	2p- (1.7) 17p- (1.2) **,+13 (0.4)** ,+19 (0.4)	11	**0.4**	0	27
11	59, M	25,100	75	ND	88	5 (5.7)	,+11 (4.5) ,+18 (1.1)	2	0	0	42
12	53, F	192,000	74	R+	275	12 (4.6)	**17p+ (0.4)** 10p- (0.4)	12	**0.4**	0	29
13	44, M*	161,800	ND	R+	185	13 (7.0)	,-Y (1.0)	10	0	1 (0.5)	31
14	67, M**	48,100	172	ND	255	99 (38.8)	,-Y (36.1)	8		1 (0.4)	0
15	61, M	9,600	ND	R+	137	28 (25.7)	,-17 (11.7)	2		0	0
16	33, M	24,800	128	ND	147	92 (62.6)	,+8 (53.3) ,+22q- (0.7)	6		6 (4.1)	0

* atomic bomb survivors, ** worker of Okuno island occupationally exposed to mustard gas, additional ab. : additional aberrations
Under lined chromosome is common aberrations found at blastic crisis phase in CML.. Bold letter shows same chromsome as found in blastic crisis phase of same patients. ND : not done, ¶ Number of subclones and aberrations , where single aberration was count one branch. † BCR gene rearrengement was observed by Southern blotting.

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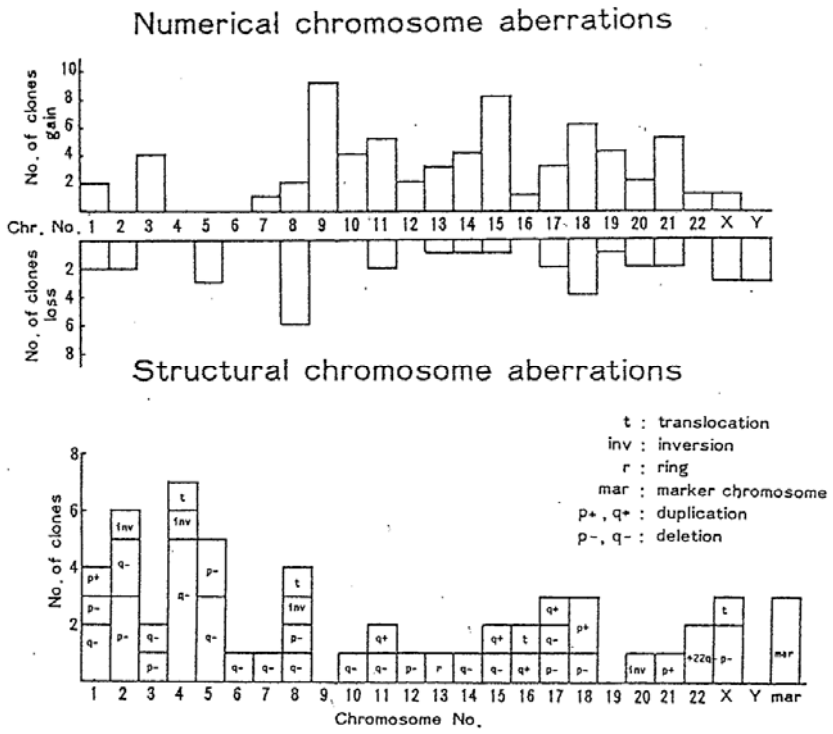
Fig.1 Distribution of numerical and structural additional aberrations to Ph chromosome on 23 pairs of chromosome

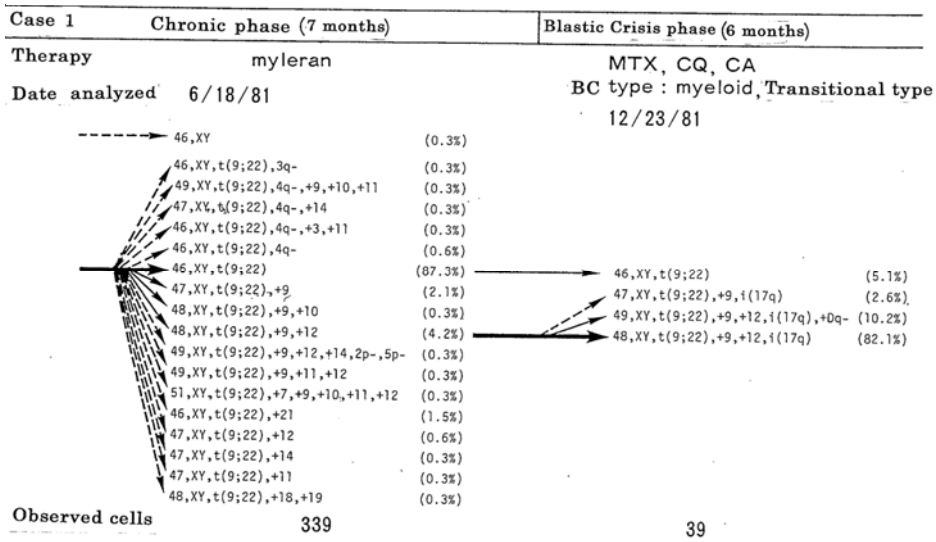


The upper row indicates the numerical aberrations and the lower the structural aberrations. In structural aberrations shown in the lower part of figure, the unbalanced type of aberrations at short or long arm of chromosome(p-, q-, p+, q+ etc.) is indicated downward and the balanced type (t, inv) upward. p: short arm of chromosome; q: long arm of chromosome.

analyzed in the chronic phase. Most metaphases (33 out of total cells) in BC phase showed only t (9; 22) translocation without additional chromosome aberrations. In case 8, ordinary 46, XY, t (9; 22)(q34;q11) karyotype remained unchanged at BC phase. Therefore these 3 cases were excluded from present analysis to find identical clones at both chronic and BC phases.

In 5 of 10 patients (cases 1, 3, 4, 10 & 12), clones found in BC phase were correlated to the clones detected in the chronic phase of the same patients. In case 1, clones of 48, XY, +9, t (9; 22) and *ibid.*, +19 was observed at the time of diagnosis with a proportion of 4.2% (5.1% if subclones were included) of number of cells analyzed (Fig. 2). Six months later in the BC phase, additional aberration of i(17q) appeared and clones of 48, XY, t (9;22), +9, +12, and *ibid.*, 1(17q) together were in 92.3% (82.1 & 10.2%, respectively) of the total cells (Fig. 2). In this case, clones with additional aberrations of 4q-(1.5%) and +21(1.5%) were also detected at the time of diagnosis but these clones disappeared in the BC phase. The results of the karyotype analysis in case 3, additional chromosome aberrations were found in 6.8% of the cells at the time of diagnosis (Table 1). In this case, cells with additional aberrations were considerably branched than other cases (8th column in Table 1). This case too had clones presently 4q- in 1.2%, 2q- in 0.9% and 8p- in 0.6%. Cell with +22q- was seen in one metaphase. The patient transformed into BC phase after 6 months. The karyotype of the major clone in the phase was 46, XY, t (9; 22), 11p+ (50.3%) (Table 2). The second analysis performed at the BC phase 3 months later reveled additional i(17q) in addition to the above-mentioned aberrations and clones of 46, XY, -17, t (9;22), 11p+, i(17q) were found in 16.3%. On the other hand, the clone with masked Ph chromosome that had appeared in the first analysis at the BC phase was no longer observed. The major clone having 11p+ was found only one cell (0.3%) during the chronic phase (Table 1). Also, an aberration suspected to be masked Ph chromosome was found first analysis of BC phase in 10.5%. Clones with masked Ph chromosome disappeared at this stage.

Fig. 2. Clonal evolution of Ph-positive cells from chronic phase to BC phase in case 1. Bold & broken lines show clone & single aberration, respectively.



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Only 80 metaphases could be analyzed at the time of diagnosis in case 4. Additional aberrations had been detected in 8.8% of these cells already during the chronic phase. These aberrations included hyperdipoid cells having 59 chromosomes in 1.3%. At the BC phase 11 months later, hyperdipoid clones having 57-58 chromosomes with +22q- (additional Ph chromosome) appeared as a major clone (22.7%). Although, the karyotype of the hyperdiplopid cell seen at the chronic phase were not entirely same as that seen at the BC phase, hyperdiploid clones with trisomies of 4 and 5 were seen in one cell (1.3%), which might became main hyperdiloid clone with +4 and +5 at BC phase (Table 1 & 2).

In case 10, BC cells had 53 chromosomes with trisomy 1, 5, 8 and 13, and additional Ph chromosome in 25%, but only one cells with trisomy 13 was detected in 242 analyzed cells (0.4%). In case 12, monosomies 17 and 19 were found in 73.8% of observed cells in BC phase, and 17p+ cell found in BC phase was also

Fig. 3. Clonal evolution of Ph-positive cells from chronic phase to BC phase in case 2. Bold & broken lines show clone & single aberration, respectively.

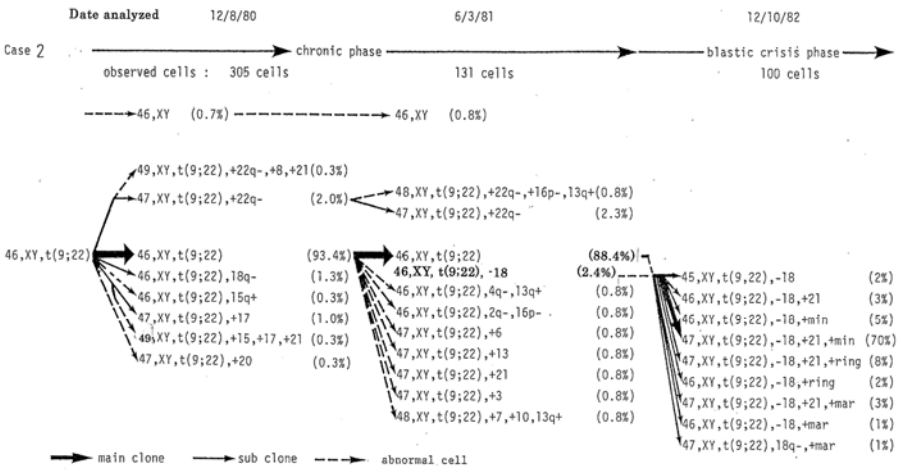

Table 2. Chromsome aberrations and clinical findings at BC phase in 16 CML patinets

Case No.	At blastic crisis (BC) phase		
	Main karyotype (%)	Clinical type of BC	Cell phenotype of BC
1	48, XY, t(9 22), +9, +11, i(17q), (94.9)	Trans	My
2	47, XY,t(9 22),−18, +22, +min, (99.0)	ND	ND
3	46, XY, t(9 22), 11p+, (95.9)	Trans	My
4	57, XX, t(9 22), +4,+5,+10,+10, ,+13,+17,+19,+20,+21,+22q− (22.7)	Acute	Ly
5	46, XY, t(9 22) (no change)	Acute	Mo
6	48, XY, t(9 22), +19, +22q−/46, XY,t(9 22),i(17q) (95.2)	Acute	My
7	46, XY, t(9 22) (82.5) (no change)	Trans	My
8	46,XY,t(9 22) (no change)	Acute	My
9	47, XX,t(9 22), +8, i(17q) (97.0)	Acute	My
10	53, XX, t(9 22),+1,+5, +8, +11,+12, ,+13, −17, 17p+, +22q− (25.0)	Tumor	My
11	46,XY, t(9 22) (91.2)/50,XY, t(9 22),6q− , +13, +14, ,+15, +20p− (3.5)	ND	ND
12	45, XX, t(9 22), −17, 17p+, −19, +19q+, (73.8)	Trans	My
13	45, XY,t(9 22),t(3;?;12),+15,−19,−22 (71.6)	Trans	Mo
14	ND	Acute	ND
15	ND	Trans	My
16	ND	Acute	ND

Trans:Transision type, Acute:Acute type;Tumor:Tumor formation type
My:Myebid crisis;Ly:Lymphoid crisis;Mo:Monocytic crisis

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detected in one cell at the chronic phase (0.4%) (Table 1 & 2).

On the other hand, five patients (cases 2, 6, 9, 11 & 13) did not show the same additional clonal abnormality in BC phase as observed in the chronic phase. For example, in case 2, at the time of diagnosis a clone with 22q-(additional Ph chromosome) was found in 2.3% of the total cells and also with other clones presently 18q- in 1.3% and +17 in 1.0% (Fig. 3). In our second in this patient, 7 months in the chronic phase, the +22q-clone remained at the same frequency (3.1%) as of our first analysis. However, the 18q- and +18 clones disappeared in this analysis. Instead, aberrant cells with 4q-, 2q- and +6 appeared newly in the second analysis. This patient progressed into BC phase 6 months later after the second analysis. The patient in this phase showed 47, XY, -18, +21, +min, t (9; 22) with minute and ring chromosomes. This clone seemed to have resulted from the abnormal clone having Ph chromosome with additional aberrations of -18. Cells having -18 were detected in 2.4% of the cells at the time of our second analysis at chronic phase, after 18 month which became main clone at the BC phase, although the possibility of random loss at the time of slide preparation could be dismissed.

Clones with additional aberrations having +15 and +21 had been detected in 0.7% respectively at the time of diagnosis in case 6 (Table 1). At the BC phase at 27 months later, two independent clones having 47, XY, t (9;22) and +19 and 22q-, and those 46, XY, t (9;22), +i(17q) and -17 were found in 95.2%. These two abnormal clones were interpreted to reflect different routes of evolution. In case 6, the clone having +22q- and i(17q) had been identified in none of the cells at the time of analysis of 285 metaphases performed at diagnosis. In the second analysis at the BC phase, clones having 48, XY, t (9; 22), +19, +22q- amounted to 45.6% of the metaphases examined. The clone having +22q- had an increased number of branches compared to the first examination at the BC phase. Any clones with +19 and +22q- were not found in chronic phase.

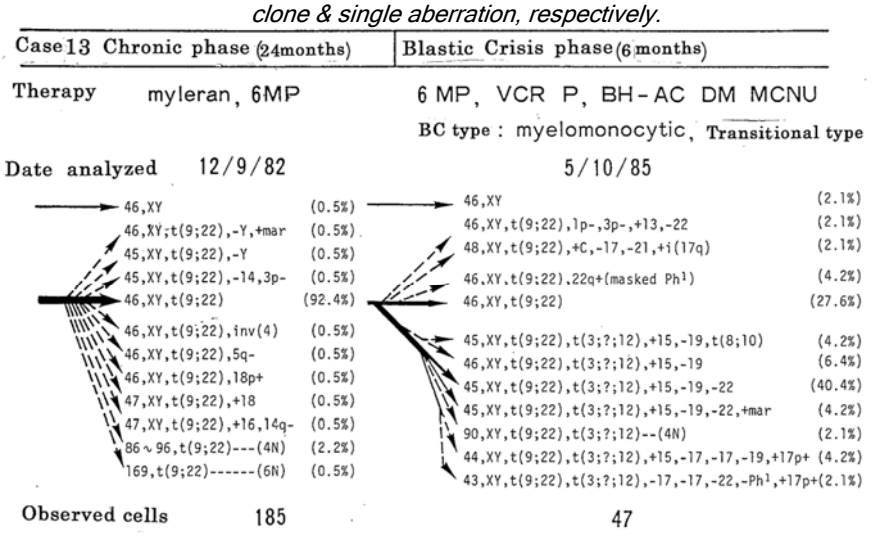
In case 9, the clones of +8 and i(17q) found at the BC phase were not detected in chronic phase. In case 11, two cells had hyperdiploid chromosomes and another two cells had tetraploid, but cells with these aberrations were not found in analyzed 88 cells at the chronic phase. In case 13, translocation of t (3;?;12) and trisomy 15 were found in 71.6% in BC phase, but those were not found in chronic phase(Fig. 4, Table 1 & 2).

Unlike the evolution pattern seen in cases 1, 3, 4, and 5 in which new aberrations were gradually added little by little, the clonal evolutions in cases 2 (Fig. 2) and 6 progressed simultaneously via two routes. The number of branches of clones with additional aberrations was lowest as only 2 in cases 7, 8 and 11, on the while other cases had 5-12 branches (Table 1). Patients (cases 8, 13 & 14) related with atomic bomb radiation exposure and mustard gas did not show any difference of clonal evolution with Ph chromosome in both chronic and BC phases.

Relationship between clonal evolution of Ph chromosome positive cells and prognosis rate

Further analysis was performed to know the correlation between clonal evolution in chronic phase with clinical type at BC, and with chemotherapy. The clinical findings of 16 patients are summarized in Table 1 and 2. Out of the 5 cases with detectable clone in BC phase, three cases (cases 1, 3 & 4) had less than 10.6 months duration of chronic phase. All of the 5 cases (cases 2, 6, 9, 11 & 13) without any detectable clone of BC in the chronic phase had more than 24 months of duration of chronic phase. Additional chromosome aberrations were detected at 7, 6 and 10.6 months in the cases 1, 3 and 4, respectively, before developing BC phase. The weak relationships between additional chromosome aberration frequencies and number of branches at diagnosis stage and duration of chronic phase were also observed (Fig. 5a & Fig. 5b). Generally, it was observed that a patient with higher number of additional chromosome aberrations at diagnosis was prone to develop BC faster. Cases 1, 3, 6-10, 12 and 15 developed myeloid crisis, cases 5 and 13 developed monocytic crisis and case 4 developed lymphoid crisis. There was a weak relationship frequency of additional chromosome aberrations at diagnosis stage and duration of chronic phase (Fig. 5a), but no correlation was found between number of branches (clonal evolution patterns) at diagnosis and duration of chronic phase (Fig. 5b). In these analyses, frequencies of additional chromosome aberrations of three patients (cases 14-16) were used as values

Fig. 4. Clonal evolution of Ph-positive cells from chronic phase to BC phase in case 13. Bold & broken lines show clone & single aberration, respectively.



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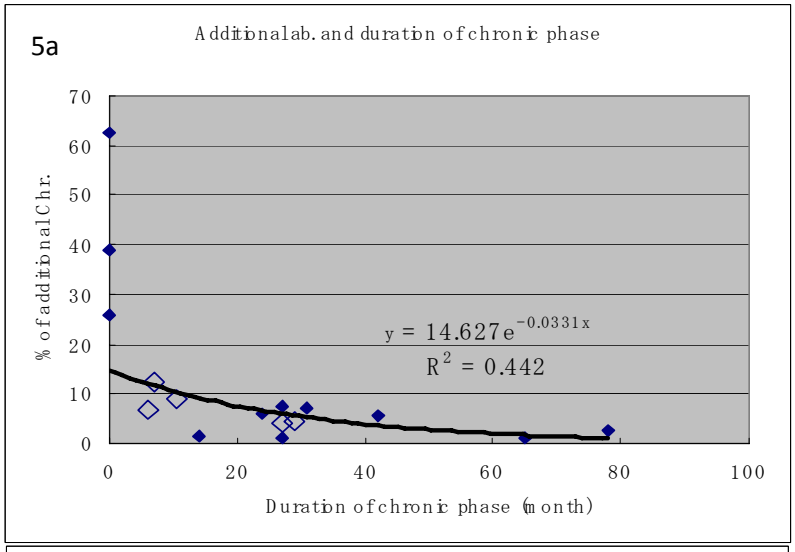
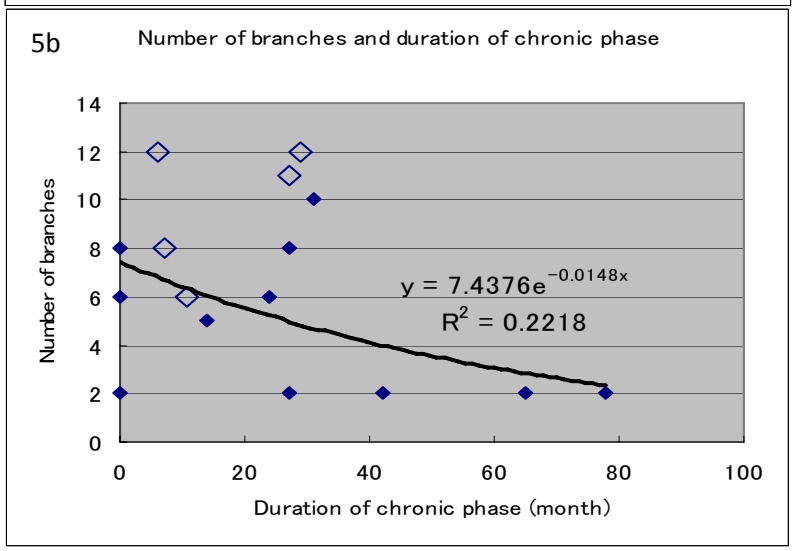
at 0 month of duration of chronic phase. However, the relationship between the frequency of additional chromosome aberrations and cell phenotype in BC cells was not found in a limited number of patients.

In summary overall of present analysis in 16 patients, diversity of clones were found at chronic phase, but they were not always related with duration of chronic phase before BC phase and cell type of BC such as myeloid cell, lymphoid or monocytic crisis. Within limited 10.6 months, additional clones found in chronic phase will be helpful to identify BC phase in CML. For example, minor clones found in BC phase could be detected in 3 of 3 patients by a large amount of karyotype analysis at 10.6 months before BC phase, but 5 of 10 patients could be detected before BC crisis phase at 31 months and only 5 of 11 patients 42 months (Fig. 5a).

Fig.5a, b. (a) Correlation between frequency of additional aberrations with Ph chromosome at diagnosis and duration of chronic phase (month), which shown weak correlation. (b) Correlation between number of branches at diagnosis and duration of chronic phase (month), which shown no correlation. ◇Cases 1,3,4,10 and 12, who had same aberrations as BC phase.





Discussion

Examination of 80-385 cells from patients with CML in the chronic phase prior to treatment revealed the 1.3-12.4% cells with additional aberrations only with Ph chromosome in all the 13 patients studied. Sonta *et al.* (1977) detected additional chromosome aberrations in 1.3-2.3% of cells by large number of 110-500 cells analysis. Our additional aberration frequency range was higher than those reported, but minimum range revealed the same. By contemplating a relationship between the low frequency clones with additional aberrations seen with detected in diagnosis and the clones observed in the BC phase, Sonta *et al.* (1977) reported that clones with abnormalities of 2p+, -Y, i(17q) and +22q-, which had existed in low frequency during the chronic phase and dominated as a major clone in the BC phase of 4 patients studied. Abnormal clones in 6 patients showed existing in diagnosis during the chronic phase, did not dominated as a major clone in BC phase. Additional chromosome aberration is significantly low (20%) or remained constant during most part of chronic phase, which rises suddenly during the last part of chronic phase to 80% (Smolin *et al.*, 1985). Krulik *et al.* (1987) also suggests that additional chromosome aberrations in chronic phase is a poor predictive indicator for the CML progression as only in 3 out of 28 patients showed additional chromosome aberrations in BC phase which had appeared only 6 months prior to the development of BC phase and these aberrations appear to be a hallmark for evaluating the progression of CML. It was also observed in present study that many of these existing clones disappeared during the course of the disease and were suddenly replaces with new type of clones before progressing into the BC phase. This study suggest that ph-positive clone with additional chromosome aberrations in chronic phase is not always undergone an evolution to BC phase.

Additional aberrations observed at the time of diagnosis in the chronic phase were found to consist mostly of the unbalanced type of aberrations such as deletion and duplication and rarely balanced translocation and inversion. Furthermore, additional aberrations such as +8, +22q- and i(17q) as commonly seen in more than 70-80% of CML patients in the BC phase (Mitelman *et al.*, 1976; Kantarjian *et al.*, 1987; Mitelman, 1993; Johansson *et al.*, 2002; Tanaka *et al.*, 2004), but those additional aberrations were less frequent in chronic phase, except i(17q), which was absent in diagnosis stage in present study. Our results suggests that i(17q) is closely

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associated with BC phase only. However, clone having i(17q) found in chronic phase was previously reported (Alimena *et al*., 1979), implying possibility of geographical difference. On the other hand, additional aberrations like 4q-, 5q- and 2p-, which are reported to be a low frequency in BC phase (Mitelman *et al*., 1976; First Workshop on Chromosomes in Leukemia 1977, 1978; Mitelman, 1993; Johansson *et al*., 2002; Tanaka *et al*., 2004), were detected more in chronic phase. It is interesting to note that the latter type of additional aberrations is more common in Ph-positive acute myelocytic leukemia (Sasaki *et al*., 1983). These findings suggest that cell differentiation stage development of leukemic stem cells in CML and Ph-positive acute myelocytic leukemia might be similar. Major or minor clones found in chronic phase of CML develop at which differentiation stage in hematopoietic cells is remained question to be resolved. Sato Y *et al.* (1988) revealed that main clones found in 2 CML BC patient were developed from pluripotent stem cells and that additional abnormalities were not restricted to occur in a specific cell line, using simultaneous assay for colony formation and chromosome analysis.

On studying the pattern of evolution of abnormal clones, the minor clones that had existed during the chronic phase continued to increase in their original forms during the course of diagnosis, and had increased in the BC phase of case 1. In case 2, the clones that had existed at the time of diagnosis were replaced by new clones. In cases 2 and 6, two new clones appeared simultaneously in the BC phase. Thus, the evolution of clones appeared to be erratic with no specific pattern. Although the cause for these varieties in the clonal evolution patterns is still unknown, it may be important to investigate the effects of exposure to chemical substances like by means of chemotherapy or radiation. Two (cases 8 & 13) out of 16 patients had been exposed to atomic bomb radiation in Hiroshima. Case 14 had an occupational history of working in mustard gas factory. Differences in the frequency of additional aberrations, the degree of branching of abnormal clones and aberrant karyotypes were similar between these three cases and other patients having no history of such exposure.

On examining the effects of chemotherapy in the evolution of abnormal clones, the influence of chemotherapy could not be ruled out, as in case 2 different kinds of additional aberrations appeared between initial analysis at the time of diagnosis and second analysis in the chronic phase, which may be related to the effects of chemotherapy after diagnosis. In cases 3 and 6, the branching of clones increased at the time of the second analysis when compared to the first analysis, and this may also be attributable to the chemotherapy effects. When analyzing the frequency of chromosome aberrations in busulfan-treated and multiple drug therapy CML group, Alimena *et al.* (1979) suggested a role of the chemotherapy effect in the evolution pattern of the clones. They revealed that no difference were found in the frequency and combination of +8, +22q- and i(17q) during the acute phase, the frequency of abnormal clones having chromosome 1 was higher in the multiple drug therapy group (Alimena *et al.,* 1979), however, no such association was confirmed by later studies (Fleischman *et al.,* 1981). In the present analysis, the abnormalities of +8, +22q- and +19 which occur often during the BC phase, had existed already at the time of diagnosis in the chronic phase prior to treatment. These abnormalities appeared with increased frequencies during the BC phase in 6 cases. Cases 1, 2, 3, 4, 10 and 16 had additional aberrations of +19 in 0.3%, +22q- in 2.3% and +22q- in 0.3%, +8 in 1.3%, +19 in 0.4% and both +22q- in 0.7% and +8 in 53.3%, respectively, in diagnosis at chronic phase (Table 1), and they had consistent abnormalities in their leukemic cells at BC phase. About 10% of CML patients who were diagnosed in the BC phase, also showed aberrations of +8, +22q- and i(17q) prior to treatment (Sadamori *et al.,* 1980; Oguma, 1980). These findings might suggest that the influence of chemotherapy was unlikely for the appearance of a common karyotype during the course of the disease. Several groups tried to know whether the cytogenetic evolution patterns in CML correlate with the type of therapy administrated in chronic phase, but discrepant results were obtained. The frequencies of trisomy 8 or other additional chromosome aberrations were higher in busulfan treated patients than in following hydroxyurea treatment (Nanjangud *et al.,* 1994). Our recent study showed that patients treated with busulfan showed higher frequency of major-route abnormalities and higher number of abonormal chromosomes than did patients treated with interferon-α, and that frequency of trisomy 8 was higher and monosomy 7 was lower in busulfan treated patients than in interferon-α treated patients(Tanaka *et al*., 2004). A more common (40% of the patients) feature in interferon-α treated patients was no change of additional chromosome aberrations. We concluded that interferon-α treatment is quite neutral, but not busulfan treatment (Tanaka *et al*., 2004). Thus, the clonal evolution pathway with Ph chromosome does not seem to be influenced by chemotherapy, rather than the pathway pattern. And, it can be concluded that they might be some minor role of the chemotherapy effect in the evolution of minor clones only rather than major clones presenting +8, +22q- and i(17q) either in combination or simple abnormality. These findings suggest that Ph-positive clone is intrinsically labile itself and readily acquire a variety of additional chromosome aberrations at any stage. Segmental jumping translocations of *BCR-ABL* chimeric region found in BC phase of CML also showed that Ph-positive clone prone to have genomic instability intrinsically (Tanaka *et al.,* 2000). Recent high-resolution array CGH analyses on CML also supported this conclusion (Hosoya *et al*., 2006; Brazma *et al*., 2007). On the other hand, 3 patients in present

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study, had original t (9; 22) translocation only at BC phase, and had clonal evolution with low number of branches at chronic phase. About a quarter patients (26.3%) did not have additional chromosome aberrations at BC phase (Mitelman *et al.*, 1993; Tanaka *et al.*, 2004), which suggests that these patients without clonal evolution might have multiple submicroscopic genomic changes, loss of heterozygosity and oncogne alterations such as *N*- or *K-RAS* are associated with transformation to BC in CML (Tanaka *et al.*, 1992a; 1992b; Khorashad *et al.*, 2008). BCR/ABL protein transforms hematopoietic cells and exerts a wide variety of biological effects including reduction in growth factor dependence, enhanced viability, and altered adhesion of CML cells (Sattler & Griffin, 2001). BCR/ABL also enhances DNA damage caused by endogenous reactive oxygen species and exogenous genotoxic treatment and also modulate the response to DNA damage, which can promote genomic and chromosome instabilities (Skorski, 2008; Koptyra *et al.*, 2008).

Chromosome analysis for large amount of cells is not only time consuming but also has its limitations in terms of cost and physical burden. To overcome these difficulties, the computer-assisted analysis system of large amount of metaphases had employed by the authors (Tanaka & Kamada 1986a; 1986b) may be immediately helpful. Further, application of more sensitive molecular biological method such as PCR and *in situ hybridization*, targeting genes such as methylated genes (Ge *et al.,* 2001) associating with BC, will be value for diagnosis of BC and also for further specific therapy regimen in CML patients.

Present study with large amount of karyotype analysis in 16 patients suggest that additional chromosome aberrations detected in the chronic phase of 10.6 month before development of BC phase appear to be a suitable indicator for assessing the progress of CML. These findings in present study will be helpful for promoting the chemotherapy and therapies of interferon-α and imatinib in CML patients.

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