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ARTICLE

Chronic myeloid leukemia with insertion-derived *BCR–ABL1* fusion: redefining complex chromosomal abnormalities by correlation of FISH and karyotype predicts prognosis

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Abstract

Chromosomal insertion-derived *BCR–ABL1* fusion is rare and mostly cryptic in chronic myeloid leukemia (CML). Most of these cases present a normal karyotype, and their risk and/or prognostic category are uncertain. We searched our database and identified 41 CML patients (20 M/21 F, median age: 47 years, range 12–78 years) with insertion-derived *BCR–ABL1* confirmed by various FISH techniques: 31 in chronic phase, 1 in accelerated phase, and 9 in blast phase at time of diagnosis. Conventional cytogenetics analysis showed a normal karyotype ($n = 19$); abnormal karyotype with morphologically normal chromosomes 9 and 22 ($n = 5$); apparent ins(9;22) ($n = 2$) and abnormal karyotype with apparent abnormal chromosomes 9, der(9) and/or 22, der(22) ($n = 15$). The locations of insertion-derived *BCR–ABL1* were identified on chromosome 22 (68.3%), 9 (29.3%), and 19 (2.4%). Complex chromosomal abnormalities were often overlooked by conventional cytogenetics but identified by FISH tests in many cases. After a median follow-up of 58 months (range 1–242 months), 11 patients died, and 3 lost contact, while the others achieved different cytogenetic/molecular responses. The locations of *BCR–ABL1* (der(22) vs. non-der(22)) and the karyotype results (complex karyotype vs. noncomplex karyotype) by conventional cytogenetics were not associated with overall survival in this cohort. However, redefining the complexity of chromosomal abnormality by correlating karyotype and FISH findings, CML cases with simple chromosomal abnormalities had a more favorable overall survival than that with complex chromosomal abnormalities. We conclude that insertion-derived *BCR–ABL1* fusions often involve complex chromosomal abnormalities which are overlooked by conventional cytogenetics, but can be identified by one or more FISH tests. We also suggest that the traditional cytogenetic response criteria may not apply in these patients, and the complexity of chromosomal abnormalities redefined by correlating karyotype and FISH findings can plays a role in stratifying patients into more suitable risk groups for predicting prognosis. (Word count: 292)

Introduction

The Philadelphia chromosome (Ph), a derivative chromosome 22 with a chimeric *BCR–ABL1* fusion derived mostly by a reciprocal t(9;22)(q34.1;q11.2), occurs in 95% of chronic myeloid leukemia (CML) [1], 2–10% of pediatric and 25% of adult acute lymphoblastic leukemia [2], and a small subset of acute myeloid leukemia [3]. *BCR–ABL1* fusion is a diagnostic hallmark of CML, however, 5–10% of CML patients at presentation lack a Ph by karyotype analysis, but are shown to carry *BCR–ABL1* as detected by either fluorescence in situ hybridization (FISH) or reverse transcription polymerase chain reaction (RT-PCR). Further studies have shown that most of these cases are caused by a three-way or even more complicated multi-way translocation involving chromosomes 9, 22, and one or more other chromosome(s). In these complex translocations, which are designated as "variant Ph" or "complex Ph" in the literature, *BCR–ABL1* is still located on the derivative chromosome 22 or der(22) [4–6]. Up to 50% of these cases with variant or

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complex Ph exhibit a del(9q) of the derivative chromosome 9 or der(9), compared with that of 12–15% of cases with a classic Ph [7–9]. The del(9q) has been considered to be a marker for a poor prognosis in CML patients [7–15], but this observation is not supported by recent observations [16–18].

A small subset of CML cases exhibit a normally appearing chromosome 22, but positive for *BCR–ABL1* fusion by FISH and/or RT-PCR, and these cases are often designated as "masked Ph" or "cryptic Ph" [4–6, 8]. Two underlying mechanisms have been previously proposed to explain this phenomenon: First, a cryptic insertion between chromosomes 9 and 22 may occur, manifested as either a partial *ABL1* from a chromosome 9 inserted into the *BCR* locus on a chromosome 22, e.g., ins(22;9)(q11.2; q34.1q34.1) referred to as ins(22;9), forming a *BCR–ABL1* located on the der(22) or vice versa, e.g., ins(9;22)(q34.1; q11.2q11.2, referred to as ins(9;22) with a *BCR–ABL1* located on the der(9). This mechanism is also considered as a main route for this type of *BCR–ABL1* in CML. Secondly, two or more sequential translocations between chromosomes 9 and 22 may take place that result in the affected chromosomes 9 and 22 having a normal appearance [8, 19]. Either a "masked Ph" or a "cryptic Ph" is apparently inadequate to describe those cases with *BCR–ABL1* located on a chromosome 9; the term of Ph negative, *BCR–ABL1* positive CML has been applied to more accurately describe these cases [20–22].

Fewer than 80 cases of CML with insertion-derived *BCR–ABL1* have been reported since the first case was described in 1981 [23], mainly in the form of case reports or limited case series (up to nine cases) [6, 19, 21–55]. Some early studies performed only karyotyping or chromosomal analysis plus interphase FISH (i-FISH). However, these methods may be insufficient for determination of an insertion-derived *BCR–ABL1* [23, 38, 40]. The frequent location of *BCR–ABL1* varies from der(9) or der(22) in previous reports [6, 19, 20, 22, 47, 55]. The prognostic significance of insertion-derived *BCR–ABL1* in CML patients remains unknown.

In this study, we present 41 new CML cases with insertion-derived *BCR–ABL1* confirmed by various types of FISH tests. The clinical, cytogenetic, and molecular features of these cases are investigated and discussed.

Materials and methods

Case selection

We searched the database of the Clinical Cytogenetics Laboratory at The University of Texas MD Anderson Cancer Center, from May 1, 2001 through October 31, 2019, for all cases with a positive *BCR–ABL1* by FISH that occurred as an insertion of either *BCR* into *ABL1* or *ABL1* into *BCR*. Within this time frame, the tyrosine kinase inhibitors such as imatinib, dasatinib, nilotinib, bosutinib, and ponatinib have been administrated to treat *BCR–ABL1* positive patients. Cases with positive *BCR–ABL1* FISH and/or RT-PCR result(s) but without further confirmation that the *BCR–ABL1* originated by an insertion were excluded from this study. Clinicopathologic and laboratory data were collected by electronic medical chart review. This study was approved by the Institutional Review Board, and was conducted in accordance with the Declaration of Helsinki.

Chromosomal analysis

As reported previously [56, 57], conventional G-banded chromosomal analysis was performed routinely on unstimulated 24 and 48 h bone marrow (BM) aspirate cultures using standard techniques. Usually, 20 metaphases are analyzed for each case, and the final results are reported following the International System for Human Cytogenomic Nomenclature guidelines [58]. A complex karyotype (CK) is defined as ≥3 chromosomal abnormalities with at least one being a structural abnormality. A balanced ins(22;9) or ins(9;22) is usually considered as one chromosomal abnormality, whereas unbalanced der(9) and der(22) are usually considered as two separate chromosomal abnormalities.

Fluorescence in situ hybridization (FISH) analysis

The Vysis LSI BCR–ABL1 ES Dual Color Fusion probe set (referred to as "ES probe") (Abbott Molecular, Des Plaines, IL) was employed as a routine *BCR–ABL1* fusion test in our laboratory. This assay is capable of distinguishing the major breakpoint (M, p210 *BCR–ABL1* transcript) and minor breakpoint (m, p190 *BCR–ABL1* transcript) when a typical signal pattern is observed: two-red, one-green, and one-fusion (2R1G1F) for p210 and one-red, one-green, and two-fusion (1R1G2F) for p190 [59]. For cases with atypical signal pattern(s), especially those cases with a suspicion of concomitant del(9q) in the der(9), the Vysis BCR/ABL1/ASS1 Tri-Color DF FISH probe set (referred to as "tricolor probe") (Abbott Molecular, Des Plaines, IL) was performed as a reflex test [59]. Both types of BCR–ABL1 FISH probe sets were fully validated before their utilization for clinical services.

Several types of FISH tests were performed in this study, encompassed as "intensive FISH studies". i-FISH: analysis of 200 or more (up to 500 if necessary) interphase cells with various FISH signal patterns, including both normal and abnormal signal patterns, which likely represent the heterogeneity of abnormal clones in a specimen. Metaphase

FISH (m-FISH): analysis of FISH signals on metaphase cells, which usually provides the chromosomal location of each signal. Map-back FISH (mb-FISH): performing FISH tests on previously G-banded/karyotyped slide(s) so that a correlation of m-FISH and karyotype results can be established. The latter test is extremely useful for identification/confirmation of FISH signal location in cases with unidentifiable chromosome(s) (marker chromosomes) and/or cryptic chromosomal abnormalities. Whole chromosome painting (wcp): a mixture of FISH probes targeting a whole chromosome is applied to confirm/exclude a subtle rearrangement between two or even among three or more chromosomes. All of these types of FISH tests were performed by following standard protocols as reported previously [59].

Quantitative *BCR–ABL1* real-time RT-PCR assay

A multiplex real-time RT-PCR assay was employed to quantitatively assess *BCR–ABL1* levels. This assay simultaneously detects the common *BCR–ABL1* transcripts e14a2, e13a2, and e1a2 [60]. Briefly, RNA was extracted from BM or peripheral blood (PB) specimens using Trizol reagent (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Reverse transcription (RT) was performed on total RNA (1 μg) using random hexamers and superscript II reverse transcriptase (Gibco-BRL). The resulting cDNA was then subjected to PCR to amplify *BCR–ABL1* transcripts on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) [60]. The quantitative *BCR–ABL1* mRNA levels were expressed as the percent ratio of *BCR–ABL1* to *ABL1* transcript levels. The sensitivity of this assay is between 1 in 10,000 and 1 in 100,000.

Morphological evaluation

Both diagnostic PB and BM samples for each case were reviewed. The white blood cell counts, hemoglobin (Hb) and platelet counts, percentages of blasts and neutrophils in the PB, and the percentages of blasts and granulocytes in the BM as well as the BM cellularity were collected.

Gene mutation analysis

Gene mutation analysis was performed using DNA extracted from BM aspirate samples in a subset of patients. Due to technologic advances and updating of assay equipment over time, various techniques were employed for mutation analysis: Sanger sequencing for *ABL1* and other (e.g., *JAK2* and *KIT*) mutation(s) and next-generation sequencing (NGS) using 28-gene, 53-gene, and 81-gene panels, where *ABL1*, *JAK2,* and *KIT* were constantly included in these panels. The NGS-based mutation analysis was performed using the Illumina MiSeq (Illumina, San Diego, CA) sequencer [61]. *FLT3* gene mutation analysis, including internal tandem duplication, ITD, and D835 point mutation, was assessed by PCR followed by capillary electrophoresis on a Genetic Analyzer (Applied Biosystems, Foster City, CA) [61].

Statistical analysis

Overall survival (OS) was defined as the time from the first diagnosis of CML to death or the last follow-up date in this study. The Kaplan–Meier curves were applied to estimate unadjusted OS durations. The Log-rank (Mantel–Cox) test and the Gehan–Breslow–Wilcoxon test were used to compare OS between groups. A student t test was applied to perform all univariate analyses. A chi-square (X^2) test was applied to compare the frequencies of different groups. A result was considered statistically significant if $p < 0.05$. All computations were conducted in GraphPad Prism 8.

Results

Clinical and laboratory findings, treatment response, and outcomes

In this study, 41 cases were identified as Ph negative, *BCR–ABL1* positive, in which the *BCR–ABL1* fusion was derived from an insertion confirmed by a combination of various FISH tests (i-FISH, m-FISH, mb-FISH, and/or wcp). The study group included 20 men and 21 women with a median age of 47 years (range 12–78 years) at time of initial diagnosis. Thirty-one patients had chronic phase (CML-CP), one accelerated phase (CML-AP), and nine blast phase (CML-BP), respectively. Among patients with CML-CP, one patient (case #2) had concurrent metastatic melanoma and another patient (case #34) had a history of essential thrombocythemia. All CML patients received therapy with at least one tyrosine kinase inhibitor (TKI). Fourteen (34.1%) patients received additional chemotherapy and/or interferon-alpha (IFN-α) prior to or in combination with TKIs and four patients (9.8%) also received allogeneic hematopoietic stem cell transplant.

The National Comprehensive Cancer Network criteria were applied to evaluate the outcomes of the CML patients in this study [62]. Three patients were lost for follow-up and their outcomes are unknown. Continuous follow-up was available for the remaining 38 patients. The median followed-up interval was 63 months (range, 11–242 months) after the initial CML diagnosis. Response to TKIs, chemotherapy, or other interventions led to complete molecular response (CMR, $n = 6$), major molecular response (MMR, n

Table 1 General information and outcomes of patients in this study.

General information							
Total 41 cases; 20 M/21 F							
Age: median 47 y (range: 12–78 y)							
Survival/follow-up: median 58 m (range: 1–242 m)							
Diseases	**Outcomes**						
	CMR ($n = 6$)	MMR ($n = 8$)	CCyR ($n = 4$)	PCrR ($n = 5$)	R ($n = 4$)	D ($n = 11$)	UN ($n = 3$)
CML ($n = 41$)							
CML-CP ($n = 31$)	6	8	4	5	3	2[a]	3
CML-AP ($n = 1$)	0	0	0	0	0	1	0
CML-BP ($n = 9$)	0	0	0	0	1	8	0

M male, *F* female, *y* years, *m* months, *CML* chronic myeloid leukemia, *CML-CP* CML chronic phase, *CML-AP* CML accelerated phase, *CML-BP* CML blast phase, *CMR* complete molecular response, *MMR* major molecular response, *CCyR* complete cytogenetic response, *PCyR* partial cytogenetic response, *R* relapse, *D* death, *UN* unknown (due to loss of follow-up), *CR* complete remission.

[a]One case with a metastatic melanoma.

Table 2 Comparison of the most recent hematological changes in 38 CML cases by disease phases (CP-CML vs. AP-CML + BP-CML).

	CP-CML ($n = 28$)	AP-CML + BP-CML ($n = 10$)	p values
General			
M/F	12/16	6/4	0.19
Age: mean (range) (y)	46 (12–78)	53 (40–72)	0.27
BM			
Blast: mean (range) (%)	1 (0–2)	39.7 (0–85)	<0.0001
Granulocytes: mean (range) (%)	29.6 (12–53)	17.8 (0–64)	0.026
PB			
Blast: mean (range) (%)	0	24.6 (0–91)	<0.0001
WBC: mean (range) (K/uL))	9.1 (2–77.9)	10 (0.1–31.4)	0.87
Neutrophils: mean (range) (%)	59 (18.3–83)	44.6 (0–76)	0.043
Hb: mean (range) (gm/dL)	13.4 (10.5–17)	9.3 (7.3–11.1)	<0.001
Platelet: mean (range) (K/uL)	223 (51–583)	102 (60–559)	0.032

M male, *F* female, *y* years, *CP-CML* chronic phase CML, *AP-CML* accelerated phase CML, *BP-CML* blast phase CML, *BM* bone marrow, *PB* peripheral blood, *WBC* white blood cells, *Hb* hemoglobin.

= 8), complete cytogenetic response (CCyR, $n = 4$), partial cytogenetic response (PCyR, $n = 5$), relapse ($n = 4$), and death ($n = 11$) respectively (Table 1). Among the 31 patients with CML-CP, 2 died; 1 patient with CML-AP, and 8 patients with CML-BP died of disease. One of the patients who died with CML-CP had concurrent metastatic melanoma.

The most recent hematologic laboratory findings in these groups of CML patients were compared (Table 2). Statistically significant differences in the percentages of blasts and granulocytes in the BM, and percentages of blasts and neutrophils, Hb levels and platelet counts in the PB were observed between these two groups consistent with their CML status and clinical presentation. No significant differences in male/female ratio and/or median age were observed in these two groups. The mean survival/follow-up was 96.6 months (range, 12–242 months) in the CML-CP group and 43.1 months (range, 11–201 months) in the CML-AP + CML-BP group. A significant difference in OS was observed between these two groups ($p < 0.0001$).

Chromosomal analysis

Chromosomal analyses of all 41 cases revealed a variety of karyotypes in this cohort: Group 1 ($n = 19$), normal diploid karyotype; Group 2 ($n = 5$), abnormal karyotype with morphologically normal chromosomes 9 and 22; Group 3 ($n = 2$), apparent ins(9;22) which could mimic the classic t (9;22) but presented with different i-FISH and m-FISH signal patterns (see below); and Group 4 ($n = 15$), abnormal karyotype with apparent abnormal chromosomes 9, der(9) and/or abnormal chromosomes 22, der(22). Even the

abnormalities in chromosomes 9 and/or 22 in group 4 cases might mimic a classic t(9;22), mostly depending on the resolution of available metaphases for analysis, but these results did provide a clue for further investigation to confirm or exclude the presence of *BCR–ABL1*. All cases in Group 4 had a CK whereas cases in the other three groups had a non-CK (except case# 36 in the Group 2) if determined by conventional cytogenetics only. Correlation between the karyotypes and *BCR–ABL1* positivity confirmed by FISH testing (see below) showed that 17 (41.5%) cases had apparent aberrations involving chromosome 9 and/or 22 or non-cryptic der(9)/der(22) (Group 3 + Group 4), whereas 24 (58.5%) cases carried cryptic der(9)/der(22) (Group 1 + Group 2) in this study.

Among the 38 CML cases with follow-up, 23 (60.5%) cases had a cryptic der(9)/der(22), and the remaining 15 (39.5%) cases had an apparent der(9)/der(22). Interestingly, patients with CML-AP and CML-BP seemed to be more prone to exhibit cryptic der(9)/der(22) than patients with CML-CP, although this difference was not statistically significant (8/10 vs. 15/28, $p = 0.28$) (Table 3). OS analysis also did not show significant differences between these two groups (Fig. 1a). Cases with a CK ($n = 14$, all cases in Group 4 and case #36) and a non-CK ($n = 24$, cases in Groups 1–3 except case #36) ($p = 0.97$) also did not show a significant difference in OS (Fig. 1b), suggesting that karyotype results alone are inadequate to predict prognosis.

Table 3 Aberrations involving chromosomes 9 and/or 22 in 38 CML cases.

	CP-CML ($n = 28$)	AP-CML + BP-CML ($n = 10$)
Cryptic		
Group 1	14	4
Group 2	1	4
Non-cryptic		
Group 3	2	0
Group 4	11	2
Total	28	10

Cryptic vs. non-cryptic der(9)/der(22) in CP-CML and AP-CML + BP-CML groups: x^2 test, $p = 0.28$.

Group 1: normal diploid karyotype ($n = 18$).

Group 2: abnormal karyotype with morphologically normal chromosomes 9 and 22 ($n = 5$).

Group 3: apparent ins(9;22) ($n = 2$).

Group 4: abnormal karyotype with apparent abnormal chromosomes 9 and/or 22 ($n = 13$).

FISH analysis

As mentioned above, the ES probe is used routinely for FISH testing for *BCR–ABL1* in our laboratory. All 41 cases were tested positive with the ES probe, which were initially considered to be discordant with the karyotypes which did not show a Ph. Therefore, m-FISH or ideally mb-FISH was performed, which allowed direct observation and documentation of apparent FISH signal(s) on the metaphases necessary to conclude, at least at the first-time FISH analysis, that a cryptic chromosomal insertion was responsible for *BCR–ABL1* in these cases [47, 59]. All cases had mb-FISH performed that also helped to identify the exact location of *BCR–ABL1* in each case. The *BCR–ABL1* was located on chromosome 22 in 28 (68.3%) cases, chromosome 9 in 12 (29.3%) cases, and chromosome 19 in 1 (2.4%) case. The latter case (case #22) had an ins(9;22) that formed *BCR–ABL1* which was likely sequentially relocated to chromosome 19 through a translocation.

Twenty-six (63.4%) cases in this study exhibited a signal pattern of 2R1G1F using the ES probe, the same as that of the p210 transcript derived from the classic t(9;22). In the remaining 15 cases, regardless of the karyotype results, their signal patterns were considered as atypical for either p210 or p190 (1R1G2F) transcripts; the results most likely suggested a more complex chromosomal rearrangement than a simple and balanced insertion, for which mb-FISH with the ES probe could not further confirm [59, 63]. Therefore, mb-FISH with a tricolor probe was performed in eight cases [55]. In another seven cases, wcp was performed due to the complexity of chromosomal abnormalities simultaneously involving chromosomes other than 9 and/or 22. A balanced three-way or multiple-way translocation involving chromosomes 9 and 22 was suspected initially, but an insertion was finally determined to be the cause of *BCR–ABL1* and complexity of chromosomal abnormalities, along with other simultaneous or sequential rearrangement in these cases. After correlating the chromosome analysis and all FISH test results in each case, the underlying causes of the atypical signal patterns mentioned above were attributed to more complicated chromosomal abnormalities undetected by conventional cytogenetics and interpreted as one the following: a cryptic deletion of 9q including *ASS1* gene; a cryptic der(9)ins(9;22) with two normal chromosomes 22; and gain of an extra der(9) or an extra der(22) with *BCR–ABL1* (see Supplementary Information, including the Supplementary Figure for detailed interpretation for each case).

Due to the additional chromosomal abnormalities detected by intensive FISH tests, we reclassified all cases into two new groups based on their complexity of chromosomal abnormalities defined by correlating both karyotype and FISH test results: 1. Cases harboring simple chromosomal abnormalities (SCAs, $n = 15$) that presented with a normal karyotype or a balanced ins(9;22) or ins(22;9) confirmed by FISH tests; 2. Cases harboring complex chromosomal abnormalities (CCAs, $n = 23$) that presented

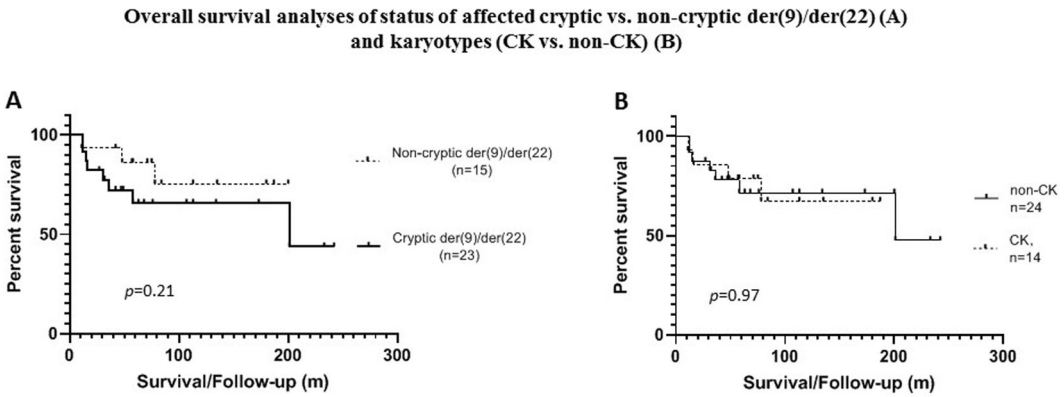

Fig. 1 Overall survival comparison of CML patients with cryptic vs. non-cryptic der(9)/der(22) and complex karyotype (CK) vs. non-CK. a Cryptic der(9)/der(22) ($n = 23$) vs. non-cryptic der(9)/der(22) ($n = 15$). The mean survival/follow-up lengths were 78.2 months (range 12–242 months) in the group with cryptic aberration and 96.8 months (range 11–200 months) in the group with non-cryptic aberration respectively. No statistically significant difference for overall survival was observed between these two groups ($p = 0.21$). **b** Complex karyotype (CK) ($n = 14$) vs. non-CK ($n = 24$). The mean survival/follow-up lengths were 87.6 months (range 11–187 months) in the group with complex karyotype (CK) and 84.4 months (range 12–242 months) in the group with non-CK respectively. No statistically significant difference for overall survival was observed between these two groups ($p = 0.97$).

with an obvious CK or a normal karyotype or even a non-CK but with unbalanced ins(9;22) or ins(22;9) and other abnormalities confirmed by FISH, such as gain of extra copy of der(9) or der(22). These cases possibly could be categorized as a CK, providing that these chromosomal abnormalities could have been detected by conventional cytogenetics. Many cases previously assigned to Groups 1 and 2 (Table 3) were now categorized in the CCAs group, e.g., cases #4, #7, #8, #18,#38 and #41 with a normal karyotype and cases #11, #24 and #36 with cryptic der(9)/der(22) and additional chromosomal aberration(s). Interestingly, cases #12 and #39 with an apparent ins(9;22) previously assigned in Group 3 were now in the SCAs group due to a balanced ins(9;22) confirmed by FISH tests. Statistical analysis showed a significant difference in the OS between these newly assigned groups (SCAs vs. CCAs) in 38 cases with clinical follow-up (Fig. 2). Therefore, we conclude that conventional karyotype results cannot reflect the entirety of chromosomal aberrations and thus should not be used, by themselves, to stratify patient risk and/or predict outcomes. Instead, the complexity of chromosomal aberrations (SCAs vs. CCAs) detected by FISH should be applied as a predictive marker. Our study also demonstrated the clinical relevance of FISH tests in the workup of CML patients.

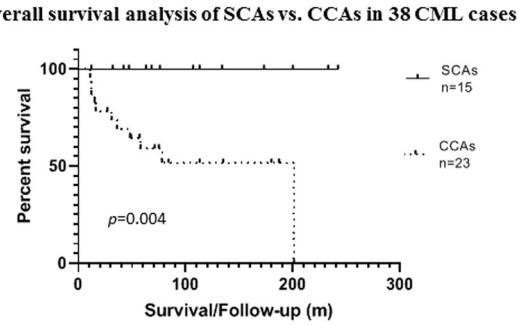

Fig. 2 Overall survival comparison of CML patients with simple chromosomal abnormalities (SCAs, $n = 15$) vs. complex chromosomal abnormalities (CCAs) ($n = 23$) according to a correlation of both chromosomal analysis and FISH test results. The mean survival/follow-up lengths were 102 months (range 12–242 months) in the SCAs group and 75 months (range 11–201 months) in the CCAs group respectively. A statistically significant difference for overall survival was observed between these two groups ($p = 0.004$).

RT-PCR analysis for *BCR–ABL1*

All cases tested were positive by RT-PCR for *BCR–ABL1* transcripts: e13a2 in 18 (43.9%) cases, e14a2 in 14 (34.1%) cases, e14a2 + e13a2 in 7 (17.1%) cases, e1a2 in 1 (2.4%) case, and undetermined in 1 (2.4%) case. Therefore, 95.1% of cases resulted in a p210 transcript, and 1 case (case #36) had a p190 (e1a2) transcript. The quantitative real-time RT-PCR Assay for *BCR–ABL1* played an essential role for determining treatment responses in this study, especially for those cases with a cryptic der(9) and/der(22), where cytogenetic response criteria are not applicable.

Gene mutation data

In this study, 23 cases were tested for *ABL1* mutations: 18 by Sanger sequencing and 5 by NGS. Five (12.2%) cases (cases #5, #8, #19, #28 and #37) had mutations: *p.E255K* (cases #8 and #19), *p.L298V* (case #5), *p.T315I* (case #28) and *p.P465S* (case #37). Among them, 2 patients (one was CML-AP, case #8; another was CML-BP, case #19) died,

whereas the remaining three (all CML-CP) achieved a MMR (case #5), CCyR (case #37), and PR (case #28), respectively. Due to the small size of cases with *ABL1* mutations and application of two methods with different coverage of *ABL1* gene in the mutation testing, the association of *ABL1* mutations and outcomes cannot be analyzed in this cohort. It also remains unknown whether cases with an insertion-derived *BCR–ABL1* are prone to *ABL1* mutation(s). Eight cases were tested for *FLT3* and all were negative for *FLT3 ITD* and *D835* mutations. Two cases (cases #23 and #25) were tested for *JAK2* and both were negative. Case #40 had an *ASXL1 p.G646fs*.

Discussion

To our knowledge, this study is the largest series of Ph negative, *BCR–ABL1* positive CML cases with the *BCR–ABL1* derived from an insertion. As shown in this study, an insertion-derived *BCR–ABL1* occurs in CML rarely, in <1% of CML cases at our institution. Chromosomal insertions that result in *BCR–ABL1* can take place in multiple ways as observed in this study. Approximately 70% of cases exhibited *BCR–ABL1* located on a chromosome 22, which was either a cryptic der(22), or "cryptic Ph" or non-cryptic der(22) or "masked Ph". The remaining cases had *BCR–ABL1* located on a cryptic or non-cryptic der(9) and in one patient, a der(19). Although this is different from some earlier reports in which the authors reported more frequent or even predominant *BCR–ABL1* located on der(9) [6, 22, 55], our results are similar to the sum of all cases reported previously (Table 4); *BCR–ABL1* locations reported in 78 patients in the literature have been identified on der(22) in 49 (62.8%), der(9) in 25 (32.1%), der(1)in 1 (1.3%) and simultaneously on both der(9) and der(22) in 3 (3.8%). Therefore, including the cases in this report, 77 of 119 (64.7%) cases with an insertion-derived *BCR–ABL1* possess a cryptic or a masked Ph. Our data also demonstrate that *BCR–ABL1* location does not correlate with OS in CML patients ($p = 0.41$, Fig. 3).

Sizes of the inserts can vary, which may be closely related to the morphological appearance of the chromosomes with the *BCR–ABL1* or those involved chromosomes without the *BCR–ABL1*. For examples, two cases (cases #12 and #39) with an ins(9;22) as well as a few cases reported previously with either ins(9;22) or ins(22;9) (Table 4) [36, 38, 53] exhibited morphologically abnormal der(22) and/or der(9) which might mimic the classic t(9;22), implying that a large insert changed the appearance of involved chromosome(s). By intensive FISH mapping using many probes targeting *ABL1*, *BCR,* and their flanking regions, Virgili et al. [47] identified inserts carrying 3′*ABL1* and flanking region or 5′ *BCR* and flanking region of sizes of 720 kb to 3.9 MB in 6 Ph negative, *BCR–ABL1* positive cases with a normal karyotype. Valle et al. [45] reported an insert of ~5.7 MB in a case also with a normal karyotype. A classic t(9;22) usually involves an exchange of approximately 10 MB of 9q (from the *ABL1* to the 9q telomere) and ~25 MB of 22q (from *BCR* to the 22q telomere).

Two mechanisms involved in the formation of a cryptic Ph were proposed in previous studies: an insertion of 3′*ABL* into 5′*BCR* or vice versa ("one-step") and two sequential translocations ("two-step) [8, 19]. However, more cases with different mechanism(s) have been identified subsequently ("multi-step") [6, 47, 55], e.g., simultaneous/sequential insertion + translocation involving chromosomes 9 and 22; additional insertion and/or translocation involving chromosome(s) other than 9 and/or 22; and gain or amplification of *BCR–ABL1* [64] (Table 4). In this study, no reciprocal translocation between chromosomes 9 and 22 has been observed and/or confirmed by various FISH tests. Therefore, we suggest that an insertion-derived *BCR–ABL1* fusion is likely (Supplementary Information). Although many cases in this study were intensively investigated by i-FISH, mb-FISH and/or wcp, the exact mechanism(s) (one-step vs. two-step vs. multi-step) cannot be completely determined due to the complexity of FISH results, even in some cases with a normal karyotype. Previous studies have suggested that concomitant deletion of 9q, del(9q) and/or del(22q) with ins(22;9) or ins(9;22) or even classic t(9;22) might be a predictive marker of a poorer prognosis in CML patients, in the era when interferon-alpha (INF-α) and hydrea were the mainstays of treatment [11, 12]. The role of these cytogenetic changes in the era of TKIs remains controversial [14, 15, 55], due to few patients reported previously treated with TKIs, as well as the uncertain status of del(9q) and/or del(22q) in many of those cases. In this study, eight cases were tested with a tricolor FISH test, which may be helpful to exclude/confirm a del9q including *ASS1* and its flanking region, but this test is not informative for cases with a potential del(9q) involving genes/loci beyond the target of ASS1/ABL1 probe.

A complete characterization of the mechanism(s) involved in *BCR–ABL1* fusion and the del(9q) and/or del (22q) status in each case usually requires intensively FISH mapping of *BCR*, *ABL1*, and their flanking regions [6, 47], or using other technologies with a genome-wide coverage, such as array-based comparative genomic hybridization [53, 65]. Genome-wide assays are often impractical in the clinical setting, especially for cases with extensive tumor heterogeneity. It is more important to explore the complexity of chromosomal aberrations involved/caused by the formation of *BCR–ABL1* n this cohort of cases. A FISH test usually analyzes 200–500 interphase cells at a resolution of approximately 100 kb, whereas conventional karyotyping routinely analyzes 20 selected metaphase cells at a much

Table 4 Summary of cases with insertion-derived *BCR–ABL1* fusion in the literature.

Ref#	Authors and Year	Case(s)	Karyotype(s)	*BCR–ABL1* location	Cryptic der(9) or der(22)
23	Lessard et al. 1981	1	ABN × 1[a]	chr. 22 × 1	No
[25]	Morris et al. 1990	1	NM × 1	chr. 22 × 1	Yes
[26]	Rassool et al. 1990	3	UN × 3	chr. 22 × 3	Yes
[20]	Lazaridou et al. 1994	9	NM × 9	chr. 22 × 9	Yes
[27]	Nishigaki et al. 1992	2	NM × 2	chr. 22 × 2	Yes
[28]	Macera et al. 1993	1	ABN × 1	chr. 22 × 1	Yes
[29]	Nacheva et al. 1994	2	NM × 2	chr. 22 × 1; chr. 9 × 1	Yes
[30]	Mohamed et al. 1995	1	ABN × 1	chr. 22 × 1	Yes
[21]	Hochhaus et al. 1996	1	NM × 1	chr. 22 × 1	Yes
[31]	Aurich et al. 1997	1	ABN × 1	chr. 9 × 1	No
[32]	Estop et al. 1997	1	NM × 1	chr. 22 × 1	Yes
[33]	Abruzzese et al. 1998	2	NM × 2	chr. 9 × 2	Yes
[34]	Seong et al. 1999	2	NM × 2	chr. 22 × 2	Yes
[35]	Vieira et al. 1999	1	ABN × 1	chr. 9 × 1	Yes
[36]	Martin-Subero et al. 2001	1	ABN × 1[a]	chr. 22 × 1	No
[37]	Mohr et al. 2001	2	NM × 2	chr. 22×1	Yes
[38]	Loncarevic et al. 2002	**2**	ABN × 2[a]	chr. 22 × 1; chr. 9 × 1	No
[39]	Morel et al. 2003	1	NM × 1	chr. 22 × 1	Yes
[40]	Aoun et al. 2004	1	NM × 1	chr. 22 × 1	Yes
[19]	Haigh et al. 2004	3	NM × 2, ABN × 1	chr. 22 × 3; chr. 9 × 1	Yes
[41]	Monma et al. 2004	1	NM × 1	chr. 22 × 1	Yes
[42]	Wan et al. 2004	1	NM × 1	chr. 9 × 1	Yes
[43]	Batista et al. 2005	2	NM × 2	chr. 22 × 1, chr. 9 × 1	Yes
[45]	Valle et al. 2006	1	NM × 1	chr. 22 × 1	Yes
[46]	Struski et al. 2007	1	ABN × 1	chr. 22 × 1	Yes
[47]	Virgili et al. 2008	8	NM × 8	chr. 22 × 5, chr. 9 × 3	Yes
[48]	Al-Achkar et al. 2010	1	NM × 1	chr. 1 × 1	Yes
[6]	Albano et al. 2010	6	UN × 6	chr. 22 × 3, chr. 9 × 3	Yes
[49]	Toydemir et al. 2010	1	ABN × 1	chr. 22 × 1	Yes
[50]	Boles et al. 2013	1	NM × 1	chr. 22 × 1	Yes
[51]	Brahmbhatt et al. 2014	2	NM × 2	chr. 22 × 1	Yes
[52]	Cattaneo et al. 2015	1	NM × 1	chr. 9 × 1	Yes
[53]	Shao et al. 2015	4	NM × 2, ABN × 3[a]	chr. 22 × 2; chr.9 × 1; chr. 22 + chr.9 × 1	Yes × 2
[54]	Wang et al. 2015	1	ABN × 1	chr. 22 × 1	No
[55]	Luatti et al. 2017	5	NM × 5	chr. 22 × 1; chr.9 × 4; chr. 22 + chr.9 × 1	Yes
[22]	Ratajczak et al. 2019	4	NM × 4	chr. 22 × 2; chr. 9 × 2	Yes
	Total	78	NM ($n = 54$, 69.2%), ABN ($n = 15$, 19.2%), UN ($n = 9$, 11.5%)	chr. 22 ($n = 49$, 62.8%), chr. 9 ($n = 25$, 32.1%), chr. 1 × ($n = 1$, 1.3%), chr. 22 + chr. 9 ($n = 3$, 3.8%)	Yes ($n = 72$; 92.3%), No ($n = 6$; 7.7%)

Ref reference, *NM* normal, *ABN* abnormal, *chr.* chromosome.

[a]ins(22;9) (q11;q22 to 34); ins(22;9)(q11;q34q21); ins(22;9)(q11;q34q34) and ins(9;22)(q34;q11q11); and ins (22;9) (q11;q21q34) respectively.

lower resolution of 5–10 Mb. Therefore, FISH testing is considered more sensitive and specific than conventional cytogenetics for detection of targeted chromosomal abnormalities, e.g., the *BCR–ABL1* and related chromosomal abnormalities in this cohort. As demonstrated in this study, cryptic chromosomal abnormalities or even some apparent chromosomal abnormalities presenting as minor clone(s) can be easily overlooked. Therefore, the results of conventional cytogenetics alone showing a normal karyotype, or even an abnormal karyotype but with cryptic der(22) and/or der(9), or misinterpretation of subtle changes in the karyotype can be misleading. FISH testing in these cases can clarify the findings and these results could have direct clinical impact, in terms of risk stratification or prognosis.

In this study, almost all CML cases presented a p210 *BCR–ABL1* transcript, except one with a p190 *BCR–ABL1* transcript and another case with a variant *BCR–ABL1*

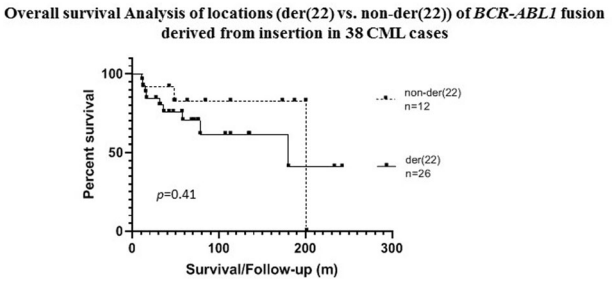

Fig. 3 Overall survival comparison of CML patients with *BCR–ABL1* fusion located on der(22) ($n = 26$) vs. non-der(22) ($n = 12$, including chr. 9, $n = 11$ and chr. 19). The mean survival/follow-up lengths were 75.6 months (range 11–242 months) in the der(22) group and 107 months (range 12–201 months) in the non-der(22) group respectively. No statistically significant difference for overall survival was observed between these two groups ($p = 0.41$).

transcript that was not characterized [21]. We have observed a high frequency of co-existing e14a2 and e13a2 *BCR–ABL1* transcripts in this study, ~16%, which was reported in a single case with an insertion-derived *BCR–ABL1* previously [35]. A co-existence of e14a2 and e13a2 *BCR–ABL1* transcripts is caused by a polymorphism within *BCR* gene [66], however, it is unknown whether the patients in this cohort are prone to carry *BCR* polymorphisms. Some hotspot *ABL1* mutations have been reported to be associated with TKI resistance in CML patients. It is necessary to point out that TKIs have dramatically evolved in the past two decades, and patients in this cohort have received various types of TKIs, either solely or subsequently according to the availability and specific indication (s) of each TKI. No statistical analysis for efficiencies of various TKIs has been performed for this cohort.

In summary, this study represents the largest cohort of CML cases with an insertion-derived *BCR–ABL1* fusion. A systemic investigation of clinical, laboratory and special testing information in this cohort reveals that approximately 40% of cases exhibited an apparent der(9) and/or der(22), with clues for a *BCR–ABL1* rearrangement, although these aberrations did not mimic a classic t(9;22). Therefore, chromosomes carrying an insertion-derived *BCR–ABL1* are not always cryptic. The locations of *BCR–ABL1* and sizes of the inserts were different among all cases in this cohort, indicating the complexity of this type of chromosomal rearrangement. In addition, the insert size is related to the morphology of affected chromosomes, and the FISH signal patterns may imply for a balanced or unbalanced insertion and/or a mixture of subclones. Our data further show that many CML cases with a normal karyotype actually possess complex chromosomal abnormalities that can be shown by various FISH tests, and that the presence of complex chromosomal abnormalities shown by FISH predict poorer OS. Therefore, any cases with a potential insertion-derived *BCR–ABL1* fusion warrant intensive FISH studies, at least at the time of initial diagnosis, and a normal or noncomplex karyotype obtained by conventional cytogenetics in these cases is misleading in terms of risk stratification, response categorization and clinical follow-up. We suggest that FISH testing is mandatory for the workup of cases of CML with *BCR–ABL1* fusion and that these results are relevant for patient management.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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