RESEARCH ARTICLE

Diagnosis and Monitoring of Chronic Myeloid Leukemia: Chiang Mai University Experience

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Abstract

Background: A diagnosis of chronic myeloid leukemia (CML) is made on discovery of the presence of a Philadelphia (Ph) chromosome. The success of the treatment of this form of leukemia with tyrosine kinase inhibitor (TKI) is monitored by reduction of the Ph chromosome. Objective: To compare the role of conventional cytogenetic (CC) methods with a real time quantitative polymerase chain reaction (RQ-PCR) and fluorescence in situ hybridization (FISH) for diagnosis and treatment monitoring of CML patients. The secondary outcome was to analyze the treatment responses to TKI in CML patients. Materials and Methods: This was a retrospective study of CML patients who attended the Hematology clinic at Chiang Mai University Hospital from 2005-2010. Medical records were reviewed for demographic data, risk score, treatment response and the results of CC methods, FISH and RQ-PCR. Results: One hundred and twenty three cases were included in the study, 57.7% of whom were male with a mean age of 46.9 years. Most of the patients registered as intermediate to high risk on the Sokal score. At diagnosis, 121 patients were tested using the CC method and 118 (95.9%) were identified as positive. Five patients failed to be diagnosed by CC methods but were positive for BCR-ABL1 using the FISH method. Imatinib was the first-line treatment used in 120 patients (97.6%). In most patients (108 out of 122, 88.5%), a complete cytogenetic response (CCyR) was achieved after TKI therapy and in 86 patients (70.5%) CCyR was achieved long term by the CC method. Five out of the 35 analyzed patients in which CCyR was achieved by the CC method had a positive FISH result. Out of the 76 patients in which CCyR was achieved, RQ-PCR classified patients to only CCyR in 17 patients (22.4%) with a deeper major molecular response (MMR) in 4 patients (5.3%) and complete molecular response (CMR) in 55 patients (72.4%). In the case of initial therapy, CCyR was achieved in 95 patients (79.1%) who received imatinib and in both patients who received dasatinib (100%). For the second line treatment, nilotinib were used in 30 patients and in 19 of them (63.3%) CCyR was achieved. In half of the 6 patients (50%) who received dasatinib as second line or third line treatment CCyR was also achieved. Conclusions: CML patients had a good response to TKI treatment. FISH could be useful for diagnosis in cases where CC analysis failed to detect the Ph chromosome. RQ-PCR was helpful in detecting any residual disease and determining the depth of the treatment response at levels greater than the CC methods.

Keywords: Chronic myeloid leukemia - conventional cytogenetic method - FISH - Philadelphia chromosome

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm (MPN) which is characterized by the presence of leukocytosis with many stages of differentiation of neutrophils. It has a distinct pathogenesis due to the presence of the BCR-ABL1 fusion gene or a Philadelphia (Ph) chromosome resulting from reciprocal translocation of chromosome 9 and 22 [t (9;22) (q34;q11)] (Vardiman et al., 2008). CML is classified into the chronic phase (CP), accelerated phase (AP), and blastic phase (BP) according to the number of blast cells in the blood or bone marrow (BM), degree of basophilia, presence of persistent thrombocytopenia, response to therapy, clonal chromosomal abnormalities in Ph+ cells, and presence of extramedullary blast proliferation (Vardiman et al., 2008; Baccarani et al., 2013).

The diagnosis of CML can be obtained by conventional

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cytogenetic (CC) analysis to demonstrate the presence of the Ph chromosome. However, it has limitations in cases of variant translocation or cryptic translocation. In these situations, the presence of BCR-ABL1 fusion genes or transcripts by fluorescence in situ hybridization (FISH) analysis or reverse transcriptase polymerase chain reaction (RT-PCR) or real-time quantitative PCR (RQ-PCR) can be used for the diagnosis (Vardiman et al., 2008; Baccarani et al., 2013).

Before tyrosine kinase inhibitor (TKI) was available, allogeneic hematopoietic stem cell transplantation (allo-SCT) was widely used as a curative treatment but it had limitations including treatment-related mortality, availability of matched donors, age, and physical status of the patients. As a result, the therapy for CML was mainly oral chemotherapy such as hydroxyurea which could induce a hematologic response (HR) but not a cytogenetic response (CyR). Therefore, patients who received this treatment eventually died due to progression to BP at a median survival at about 4 years (Vardiman et al., 2008).

The first TKI that changed the paradigm of therapy for CML is imatinib. A phase 3 International Randomized Study of Interferon and STI571 (IRIS) showed that this targeted therapy can induce complete HR (CHR) and complete CyR (no Ph chromosome detected; CCyR) in 95.3% and 76.2% of CML-CP patients, respectively (O'Brien et al., 2003). For long-term outcomes, the best observed rate of CCyR was 82% and the 7-year event-free survival (EFS) and overall survival (OS) were 81% and 86%, respectively (O'Brien et al., 2008). These figures were higher than historical allo-SCT data (Gratwohl et al., 2006). As a result, TKI became the standard treatment for CML-CP. The second generation TKIs, nilotinib and dasatinib, were investigated rapidly and showed efficacy in both front-line therapy (Saglio et al., 2010 and Kantarjian et al., 2010) and second-line therapy in cases of imatinib intolerance or resistance (Giles et al., 2013; Shah et al., 2014).

Treatment with TKIs is initially monitored by physical examination and normalization of complete blood count (CBC) to determine HR. Apart from this, the reduction of the Ph chromosomes should be monitored by CC methods (CyR) or RQ-PCR for BCR-ABL1 transcript levels [molecular response (MR)] (Baccarani et al., 2013).

The primary objective of this study was to compare the role of the CC methods with RQ-PCR and FISH for diagnosis and treatment monitoring of CML patients. The secondary objective was to analyze the treatment responses of CML patients in Chiang Mai University Hospital.

Materials and Methods

Study overview

This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chiang Mai University, Thailand and performed at Maharaj Nakorn Chiang Mai Hospital. All CML-CP patients aged more than 15 years who attended the Hematology Clinic from 1st January 2005 to 31st December 2010 were retrospectively reviewed for demographic data, CML risk score including Sokal score (Sokal et al., 1984), Euro score (Hasford et al., 1998), EUTOS score (Hasford et al., 2011), and treatment response. The criteria of treatment responses including HR, CyR, and MR were defined according to European LeukemiaNet (ELN) criteria (Baccarani et al., 2009; Baccarani et al., 2013). The results of the CC methods, FISH and RQ-PCR in each patient at diagnosis and during follow-up after TKI therapy were also analyzed. SPSS statistical program version 16.0 was used for data analysis.

Conventional cytogenetic (CC) analysis

Metaphase chromosomes were prepared from 0.25-0.5 ml of bone morrow. The samples were cultured in 10 ml of RPMI 1640 medium containing 20% fetal calf serum, including 0.015 μg/ml colchicine, 6 mg/ml deoxythymidine, ampicillin and streptomycin. The cultures were incubated at 37^{O}C for 24 hours and after that 0.1 μg/ml colchicine were added. The cultures were incubated at 37^{O}C for a further 30 minutes. The chromosomes were harvested using conventional methods. Briefly, the bone marrow cultures were washed with phosphate buffer saline (PBS) and then exposed to 0.075 M KCl for hypotonic treatment (3 times), methanol: acetic acid (3:1) was used for fixation of cell membranes (3 times). Cells were dropped onto microscopic slides and kept overnight at room temperature. Chromosome spreads were stained with G- or Q-banding techniques. Ten metaphase chromosomes were analyzed for diagnosis and 20 metaphase chromosomes for follow up.

Fluorescence in situ hybridization (FISH) analysis

FISH analysis for the t(9;22)(q34;q11.2) were detected on both bone marrow interphase nuclei or metaphase chromosomes spreads prepared using CC methods. Slides were treated with 0.1 μg/μl RNase solution and 0.01% pepsin followed by 1% formaldehyde/1M MgCl in PBS and dehydrated in a series of ethanols. A LSI BCR/ABL1 dual color dual fusion probe (Vysis, Downers Grove, Illinois, USA) was used according to the manufacturer's instructions with slight modification. Briefly, the slide was incubated in denaturing solution (70% formamide/2XSSC) at 73^{O}C for 10 minutes, followed by dehydration in a series of ethanols. Probe mixture denaturation was carried out at 73^{O}C for 5 minutes. Ten microliters of the probe mixture were hybridized to a 22 × 22 mm area of slides for 16-20 hours at 37°C in a humidified chamber and the hybridized area was sealed with fixogum. Slides were washed in 0.4X SSC/0.3% NP-40 at 73°C for 2 minutes followed by 2X SSC/0.1% NP-40 at room temperature for 1 minute. Slides were then dehydrate in a series of ethanols and were then counterstained with 0.5 µg/ml of DAPI for 4 minutes.

Evaluation of the FISH signals, 200 interphase nuclei and metaphase chromosomes were performed under 1000x magnification using an Axioskop 2 fluorescence microscope (Carl Zeiss, Gottingen, Germany) and ISIS software (Meta Systems, GmBH, Altlussheim, Germany). Cells with normal chromosome 9 and 22 show two separate signals of each orange and green color. While cells containing t(9;22) show one red signal from normal chromosome 9 and one green signal from normal chromosome 22 and two orange/green fusion signals from

the derivative 9 and 22 chromosomes.

RT-PCR and RQ-PCR for BCR-ABL1 transcripts

Subtypes of the BCR-ABL1 transcripts (b2a2, b3a2) were determined by RT-PCR before quantitative analysis by RQ-PCR. Total RNA was extracted from 10 mL EDTA-blood using trizol method (Invitrogen, CA, USA). Two μg of total RNA was reverse-transcribed to cDNA with SuperScript® III First-Strand Synthesis System (Invitrogen, CA, USA). For RT-PCR, 2 μL of cDNA was amplified using sequence-specific primers (Invitrogen, CA, USA) for p210 BCR-ABL transcripts. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was used as an endogenous control gene was amplified in separate reaction. Primers used for RT-PCR were as follows: BCR-ABL (p210)-forward (F), 5'-GAT GCT GAC CAA CTC GTG TG-3'; BCR-ABL (p210)-reverse (R), 5'-AAC GAA AAG GTT GGG GTC AT-3'; GAPDH-F, 5'-ACC ACA GTC CAT GCC ATC AC-3' and GAPDH-R; 5'-TCC ACC ACC CTG TTG CTG TA-3'. PCR reactions were performed in 25 µl total volume with 5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.32 µM each primer and 1 unit of Taq DNA Polymerase (Invitrogen, CA, USA). The cycle conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec. PCR products were loaded onto 2% agarose gel for gel electrophoresis before being stained with ethidium bromide. The 194-bp, 265-bp and 400-bp PCR products represented b2a2, b3a2 and GAPDH, respectively.

For RQ-PCR using Applied Biosystems 7500 Fast Real Time PCR System, 2 μL of cDNA was amplified using the same sequence-specific primers (Invitrogen, CA, USA) as RT-PCR and TagManfluorogenic probe FAM-AGA CCC TGA GGC TCA AAG TCA GAT GCT ACT–TAMRA (AITbiotech, Singapore) for p210 BCR-ABL transcripts. ABL was used as an endogenous control to correct for the expression of p210 BCR-ABL genes. ABL primers (Invitrogen, CA, USA) and probe (AITbiotech, Singapore) were as follows: ABL-F, 5'-GCC TCA GGG TCT GAG TGA AG-3'; ABL-R, 5'-ACA CCA TTC CCC ATT GTG AT-3'; ABL-probe, FAM-AGA GTG TTA TCT CCA CTG GCC ACA AAA TCA–TAMRA. Plasmid cDNAs of p210 BCR-ABL and ABL were employed as a standard at concentrations of 10^2, 10^3, 10^4, 10^5 and 10^6 copies per µl. PCR reactions were performed in 20 µl total volume with 0.32 µM each primer, 0.1 µM probe and 1xPCR TaqMan Gene Expression Master Mix (Life Technologies, CA, USA). The cycle conditions were as follows: an initial denaturation at 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 30 sec.

Results

Demographic data

There were 190 CML patients during the study period but 67 patients were excluded due to being lost to follow-up or were referred to follow-up at other hospitals. Finally, 123 patients were included in the study. Table 1 shows the clinical characteristics of the CML patients included in this study. Seventy-one patients (57.7%) were male with a mean age of 46.9 years (range 17-78). Imatinib was the most prescribed TKI as the first-line therapy (120 patients, 97.6%). Two patients (1.6%) received dasatinib and one patient didn't take TKI due to economic problems.

Most patients were classified as high risk and intermediate risk according to their Sokal score (41.5% and 39.8%, respectively) and intermediate risk by Euro score (48.8%). However, when EUTOS score was used, most of the patients were categorized as low risk (78.9%).

Laboratory diagnosis of CML

From the CC analyses, 118 patients (95.9%) were correctly diagnosed with CML. The remaining 3 patients (2.4%) had no metaphase and 2 patients (1.6%) underwent bone marrow (BM) aspiration and CC analyses from other hospitals with negative results.

FISH analyses were performed on 20 patients at diagnosis and BCR-ABL1 fusion genes were detected in all cases including 5 patients who were not diagnosed by CC methods. RT-PCR and RQ-PCR were not used for the diagnosis of CML in this study on any patients.

Laboratory monitoring after TKI therapy

CC methods were used in 122 patients who received TKI in order to monitor response. CCyR was achieved in 108 patients (88.5%) with a median time to CCyR of 12.9 months. Eighty-six patients (70.5%) remained in continuous CCyR by CC method at the last follow up date.

There were 35 patients who had FISH analyses performed for monitoring and 10 patients (28.5%) remained positive for BCR-ABL1 fusion genes. Table 2 compares FISH analysis and CC analysis for monitoring of CML patients. FISH analyses led to the successful detection of BCR-ABL1 fusion genes in 1 patient who had no metaphase and 5 patients who did not have any Ph chromosomes detected by CC methods. On the contrary, 2 patients (5.7%) who had negative BCR-ABL1 fusion genes discovered by FISH were positive for Ph

Table 1. Clinical Characteristics of CML Patients in the Study

Clinical characteristics		N (Total=123)
Sex	Male	71 (57.7%)
	Female	52 (42.3%)
Age (Year)	Mean ± SD	46.9 ± 17.8
	Min/Max	17/78
Medication: First line	None	1 (0.81%)
	Imatinib	120 (97.6%)
	Dasatinib	2 (1.6%)
	Nilotinib	0 (0%)
Clinical risk score	Sokal (Mean ± SD)	2.9 ± 9.9
	High risk	51 (41.5%)
	Intermediate risk	49 (39.8%)
	Low risk	23 (18.7%)
	Euro (Mean ± SD)	1181.2 ± 662.1
	High risk	31 (25.20%)
	Intermediate risk	60 (48.78%)
	Low risk	32 (26.02%)
	EUTOS (Mean ± SD)	62.1 ± 44.0
	High risk	26 (21.14%)
	Low risk	97 (78.86%)

Table 2. Comparison Between Conventional Cytogenetics Analysis (CC) and Fluorescence in Situ Hybridization (FISH) Analysis for Monitoring of 35 CML Patients

Conventional cytogenetics (CC)	Fluorescence in situ hybridization (FISH)	N = 35	%
Positive	Positive	4	11.4
Positive	Negative	2	5.7
Negative	Positive	5	14.3
Negative	Negative	18	51.4
No interpretation	Positive	1	2.9
No interpretation	Negative	5	14.3

chromosomes when CC methods were used.

RQ-PCR was carried out for the monitoring of 103 patients. This method could determine the depth of response into BCR-ABL1 transcripts <10% which was comparable with major CyR (Ph chromosome <35%; MCyR) by CC methods, BCR-ABL1 transcripts <1% that correspond with CCyR, BCR-ABL1 transcript <0.1% or major MR (MMR), and undetected BCR-ABL1 transcript or complete MR (CMR). Analysis of 76 patients in whom CCyR was achieved who also had RQ-PCR monitoring showed CMR in most patients [55 patients (72.4%)] followed by CCyR [17 patients (22.3%)] and MMR [4 patients (5.3%)].

Response to TKI therapy

As describes previously, CCyR was achieved in 108 patients out of 122 TKI-treated patients (88.5%) after TKI therapy including 84 patients treated with imatinib, 19 patients with nilotinib, and 5 patients with dasatinib.

In the 120 patients who received imatinib as a first-line therapy, 95 (79.1%) of them showed initial achievement of CCyR. However, 11 patients (9.2%) eventually showed loss of CCyR in addition to another 25 (20.8%) patients who failed to have any CCyR. Overall 84 patients (70%) had continuous CCyR. In cases of failure, imatinib was switched to nilotinib and dasatinib in 30 and 3 patients, respectively. Nineteen patients (63.3%) who received nilotinib demonstrated CCyR both in patients who did not show any CCyR (15 patients) and patients that later showed a loss of CCyR (4 patients). The majority of them also showed CMR (10 patients) and MMR (5 patients).

Dasatinib as second-line therapy was prescribed for 2 patients who did not show any CCyR and 1 patient with a loss of CCyR after imatinib. All patients finally showed CCyR. Two imatinib and nilotinib failure patients subsequently received dasatinib but only one then achieved CCyR. Both patients who received dasatinib as first-line therapy showed CCyR and CMR.

Discussion

This study provides information about demographic data, laboratory diagnosis, monitoring and treatment responses of CML patients in Thailand. There was a slight male predominance of CML patients in this study with a mean age of 47 years. This age was equivalent to a study carried out in Iran (Payandeh et al., 2015) but higher than a Chinese ENEST study (39 years in imatinib group and 41 years in nilotinib group) (Wang et al., 2015). In a large prospective IRIS study (O'Brien et al, 2003), a greater number of patients were also male with a median age of 50 years, slightly higher than this study. The age range did not largely differ between the two studies (17-78 years in this study vs 17-70 years in IRIS study). However, the clinical risk scores of the patients in this study were mainly intermediate to high risk according to Sokal and Euro scores compared to low to intermediate risk in the IRIS study. This may be explained by a lack of knowledge, health check-up and awareness of CML in Thai people which consequently led to relatively late presentation.

This study confirmed that diagnosis of CML by CC methods had some limitations and may result in a critical delay of treatment. Three patients who had no metaphase and two patients who had negative results from this method could have had an established diagnosis by using the FISH analysis which has greater sensitivity (Kantarjian et al, 2008). Nevertheless, CC analysis remains the recommended method (Baccarani et al., 2013) since it is available in many hospitals and it can establish the diagnosis in more than 95% of patients as shown in this study. Moreover, it can demonstrate additional chromosomal abnormalities which influence the prognosis (Fabarius et al., 2011). More sensitive methods such as FISH or RT-PCR should be used in cases which have no metaphase or negative results from CC analysis but CML is still suspected (Vardiman et al., 2008).

CC analysis is also the recommended method for monitoring response after TKI therapy (Baccarani et al., 2013). The patients in whom CCyR was achieved had good clinical outcomes including progression-free survival and overall survival and CCyR is accepted as the minimal goal of CML therapy (Baccarani et al., 2013). Likewise, for purposes of diagnosis, the CC method has the problem concerning the presence of metaphases after treatment. Before the era of RQ-PCR monitoring, FISH analysis was investigated with the aim of solving this problem. The interphase FISH was shown to detect the BCR-ABL1 fusion gene in 17.3% of CCyR patients by CC method (Testoni et al, 2009) when a cut-off of less than 1% positive nuclei was used. In this study, the FISH analysis was not only helpful in a case of no metaphase but also enabled the detection of any residual disease in cases with CCyR. However, its use was limited by a lack of standardization and studies about the prognostic values of residual diseases determined by the FISH method. ELN recommended that FISH of blood interphase cell nuclei could be substituted for the CC method only for the assessment of CCyR, which is defined by <1% BCR-ABL1–positive nuclei out of at least 200 nuclei (Baccarani et al., 2013). Moreover, its sensitivity was questioned since about 5% of patients in this study who had negative BCR-ABL1 from FISH still had Ph chromosomes by CC methods as well as about 1.6% in the previous study (Testoni et al, 2009). FISH analysis was performed in the short period of time before RQ-PCR was available in our institution.

The RQ-PCR method for detection of BCR-ABL1

transcript levels or "molecular response" is widely used as a monitoring tool in CML because of its sensitivity and availability of international standardization method (Branford et al., 2008). It is also a recommended method for monitoring in addition to CC analysis depending on local facilities and on the degree of molecular standardization at the local laboratory (Baccarani et al., 2013). In this study, RQ-PCR could identify CMR and MMR in 72.4% and 5.3% of CCyR patients determined by CC methods, respectively. A deeper response, such as MMR, is also a recommended target of BCR-ABL1 transcripts for CML therapy according to current ELN guidelines (Baccarani et al., 2013). The patients who reach this milestone by 18 months have a very low possibility of suffering from disease progression to AP/BP as well as loss of CCyR (Hughes et al., 2010). In addition, a BCR-ABL1 transcript level <10% at 3 months which corresponds to MCyR was reported to be prognostically significant in several studies (Hughes et al., 2010 and Marin D et al., 2012) but the role of a change of treatment according to this milestone is still under investigation (Baccarani et al., 2013; Yeung et al., 2015).

Regarding a more extensive response, CMR [currently substituted by the term "molecularly undetectable leukemia" (Baccarani et al., 2013)] or $MR^{4.5}$ at 4 years was shown to be more predictive of long-term survival than CCyR (Hehlmann et al., 2013). In addition, about 40% of patients in whom CMR was achieved were found to have sustained remission even after TKI discontinuation trial (Mahon et al., 2010).

The role of RQ-PCR for BCR-ABL1 transcripts in the diagnosis were not evaluated in this study because this test was only recently available at Chiang Mai University. Therefore, it was used mainly for monitoring after TKI therapy especially in the patients who already were at the point of CCyR. However, there is some evidence that the baseline BCR-ABL1 transcripts may be useful in calculating the rate of decline or halving time of BCR-ABL1 transcript levels. Patients with a BCR-ABL1 halving time of less than 76 days also had good outcome even though they did not have early MR or BCR-ABL1 transcripts of less than 10% at 3 months (Branford et al., 2014).

Almost all patients in this study received imatinib as the front-line treatment although nilotinib and dasatinib were shown to induce more rapid and deeper response in the large randomized trials (Saglio G, et al., 2010 and Kantarjian H et al., 2010). This may be explained by national policy and economic status in Thailand as well as an emerging concern about long-term complications of both second-generation TKIs (Apperley, 2015). The patients who were given imatinib in whom 79.1% had CCyR were close to the successful outcomes in the IRIS study (76.2%) (O'Brien et al., 2003) in spite of patients included in this study having higher risk scores. Meanwhile, the long-term follow-up and monitoring are warranted since in the IRIS study, only 57% of patients had maintained CCyR (O'Brien et al., 2008).

Both nilotinib and dasatinib were effective TKIs for the patients in cases of imatinib resistance and intolerance. In the present study, nilotinib could induce CCyR in about 60% of cases as a second-line agent, a figure which was higher than the international phase II study of nilotinib after imatinib resistance or intolerance (Giles et al., 2013) (45%) and Expanding Nilotinib Access in Clinical Trail (ENACT) study (34%) (Nicolini et al., 2012). Only a few patients received dasatinib as first-, second-, or third-line in this study but in most of the patients who were given it there was a good response.

The limitation of this retrospective study was the limited numbers of CML patients who were analysed using FISH. The test was primarily performed in cases where CC methods showed no metaphase and its utility had declined after RT-PCR and RQ-PCR for the BCR-ABL1 method were available in the institution. No analysis of the correlation between response and time after TKI therapy as well as long-term outcomes such as treatment complications, progression-free survival and overall survival were carried out as they were out of the scope of this study.

In conclusions, FISH analysis is a sensitive method which can be used for the diagnosis of CML in cases where conventional cytogenetic analysis has failed to indicate a Ph chromosome or no metaphase. The RQ-PCR method provides greater sensitivity in detecting residual disease compared with CC methods. The treatment responses to TKI, the main one being imatinib, were not significantly different from studies carried out in Western countries.

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