Molecular Monitoring of Residual Disease in Chronic Myeloid Leukemia by Genomic DNA Compared with Conventional mRNA Analysis

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Translocation t(9;22), which produces the *BCR-ABL* gene, is pathognomonic of chronic myeloid leukemia. For clinical purposes, the amount of chimeric transcript is considered proportional to the leukemic clone; thus, mRNA is commonly used for molecular monitoring of patients. However, there is no consensus regarding the degree of increase in mRNA that should cause concern or whether the absence of transcript indicates a "cure." In this study, we analyzed 57 samples from 10 chronic myeloid leukemia patients undergoing imatinib treatment. For each sample, we compared *BCR-ABL* mRNA levels with the actual proportion of leukemic cells, which were measured through a novel genomic approach based on the quantitative amplification of DNA breakpoints. The two approaches gave similar patterns of residual disease, and the majority of patients were still positive after an average treatment period of 2 years. Nevertheless, in one of two patients with confirmed undetectable levels of chimeric transcript, DNA still revealed the persistence of leukemic cells at 42 months. These findings appear to justify the clinical practice of maintaining imatinib treatment indefinitely. However, the absence of leukemic DNA (observed in 1 of 10 patients) could be used to identify possible candidates for drug discontinuation. In conclusion, DNA analysis proved to be a reliable index of residual disease with potential applications in the field of clinical diagnostics and research. (*J Mol Diagn* 2009, 11:482–487; DOI: 10.2353/jmoldx.2009.080150)

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by the reciprocal translocation t(9;22)(q34;q11). Consequences of the translocation are the Philadelphia (Ph) chromosome and its molecular counterpart, the *BCR-ABL* fusion gene, which

encodes for a deregulated tyrosine kinase. In the absence of intervention, CML evolves from an initial chronic phase (CP) to an accelerated phase and ultimately to a blast crisis.¹ First-line therapy for patients in the CP is based on the tyrosine kinase inhibitor imatinib mesylate (IM) (Novartis Pharmaceuticals, Basel, Switzerland), which prolongs event-free and overall survival² but does not seem to eradicate the malignancy.³ Before the advent of IM, allogeneic stem cell transplantation was a common therapy and still remains an option. This procedure is associated with an appreciable level of morbidity and mortality but increases the probability of long-term leukemia-free survival.⁴ In contrast, most patients who stop taking IM quickly lose the response they achieved.^{5,6} As a consequence, IM therapy is indefinitely prolonged and patients are monitored regularly. Thus, decisions about the clinical management of CML should be informed by adverse effects⁷ and the costs⁸ of continuous treatment.

Response to IM can be assessed over time by observing the reduction of leukocytes, Ph-positive cells, and *BCR-ABL* transcripts.⁹ A detailed definition of hematological, cytogenetic, and molecular responses is closely connected to treatment failure and a suboptimal outcome.¹⁰ Complete cytogenetic response has been defined as the absence of Ph⁺ cells and can be achieved in 75% to 90% of patients treated in the early CP.¹¹ For these patients residual leukemia can be assessed only by reverse-transcription quantitative PCR (RQ-PCR). The detection of *BCR-ABL* mRNA reflects the presence of leukemic cells in a semiquantitative manner, and a rising level is considered an early indication of failing response. However, there is still no consensus regarding the degree of the increase in mRNA that should cause concern.^{12,13} This problem is hampered by the observation that apparent levels of chimeric transcript may be detected in healthy persons^{14,15} resulting in a background noise.

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In 2003, the Europe against Cancer (EAC) network established a standardized protocol for the quantitative detection of BCR-ABL transcripts.¹⁶ Currently there are various methods for reporting results, one of which is to normalize the amount BCR-ABL mRNA against an appropriate control transcript. More recently, expression levels were reported on a log₁₀ reduction scale from a standardized baseline for untreated patients.² The use of this scale has led to some degree of confusion, because it seems to imply that the value is a relative one. For this reason, a standardized international scale has been recently introduced,¹⁷ but its use is still limited, since the required reference materials are difficult to access. RNA technology is also affected by other limitations. Different RQ-PCR assays have been developed,¹⁷ and a number of different control genes (mainly *BCR*, *ABL*, and *GUSB*) are in use.¹⁸ Many of these protocols are far from being exhaustively tested, and expression levels are further influenced by the RNA extraction and the reverse transcription procedures.¹⁹

The inability to eradicate the disease through pharmacological treatment is probably due to the persistence of primitive hematopoietic progenitors³ that are highly resistant to IM.²⁰ In the last few years, a great effort was expended in an attempt to classify the leukemic “stem” cells in CML,^{21,22} but an accurate characterization is still lacking. In particular, it is not clear if these cells are transcriptionally silent,²³ or not.²⁴ This is of relevance, since molecular monitoring of CML patients is a circumstantial process based on the quantification of mRNA, rather than a direct observation of the leukemic clone. For these reasons, a definition of complete molecular response is still lacking, and whether the absence of chimeric transcript is consonant with “cure” remains an open question.

To better investigate the relationship between the proportion of leukemic cells and the expression of the *BCR-ABL* fusion gene, we developed a DNA-based assay to be used in parallel with conventional RQ-PCR to monitor patients with CML. A similar study has been previously undertaken only by Zhang et al.²⁵ in 1996. In that report mRNA from 10 patients after bone marrow transplantation was analyzed by competitive RT-PCR, while the presence of leukemic cells was assessed by nested PCR on genomic DNA. The majority of samples (79%) gave concordant results, thus discrepancies were attributed only to the different sensitivity between competitive RT-PCR and nested PCR. The authors concluded that patients do not generally have a pool of transcriptionally silent cells and mRNA analysis is a full satisfactory procedure for molecular monitoring of residual disease. However, recent improvements to quantitative PCR and the introduction of IM as front-line treatment revealed a new interest in these arguments. In this paper, we reported evidence of how the analysis of DNA from CML patients by real-time PCR could help the research and improve the practice of conventional molecular diagnostics.

Materials and Methods

Patients and BCR-ABL Breakpoint Sequencing

We monitored, for an average period of 25.5 months (range, 18–42), 10 patients with CML diagnosed in the early CP. All participants gave written informed consent and the research protocol was approved by the Ethic Committee of the Università dell’Insubria. Genomic junctions of *BCR-ABL* had been previously characterized in Mattarucci et al.²⁶ In the same paper, a detailed explanation of the sequencing protocol was also reported. Briefly, DNA from patients designated as 1, 2, 3, 4, 6, 8, 9, 10, PR1, and PR2 was extracted from blood or bone marrow, fragmented, ligated to adaptors, and amplified by a nested PCR using a *BCR* specific forward primer. Thus, genomic breakpoints were sequenced. The majority of patients were treated with IM monotherapy at a starting dose of 400 mg/day, except for patient 9 who was participating in an 800-mg/day trial. The dose for patients 6 and 10 was increased to 600 mg/day, as a consequence of suboptimal cytogenetic findings observed at 12 and 6 months, respectively.

Cytogenetic and mRNA Analysis

Conventional laboratory investigations (both cytogenetics and mRNA analysis) were performed at the reference laboratory of the CML network of the Italian Group of Hematological Malignancies in Adults at the Hospital of Bergamo, Italy. All of the analytical procedures were subject to the quality control process according to the ISO 9001:2000 accreditation of the laboratory and samples that did not conform were rejected. A cytogenetic analysis of bone marrow (approximately 20 metaphases for each patient) was performed before treatment using conventional QFQ staining. Cytogenetic tests were repeated every 6 months, until a complete response was achieved. Then bone marrow metaphases were analyzed less frequently. Levels of BCR-ABL mRNA were measured on diagnosis and approximately every 3 months thereafter using the M-bcr FusionQuant kit (Ipsogen, Marseille, France) according to the manufacturer’s protocol. This kit is approved for *in vitro* diagnostic and it contains the same primers and probes used by the EAC group to quantify the BCR-ABL¹⁶ and the ABL¹⁸ transcripts, plus a series of controls and calibrators. The performances of the RT-PCR assay were tested by the EAC network,^{16,18} briefly the sensitivity was at least of 10^{−4} dilutions of BCR-ABL-positive RNA in a negative RNA sample, the regression curve had an average slope of −3.45 with *R*² >0.95, and the precision of each measurement (ie, the error) was <6% of the corresponding average Ct value.

Results of mRNA analysis were reported as the ratio between the number of BCR-ABL and ABL transcripts, with this ratio expressed as a percentage (BCR-ABL/ABL). The sample preparation and the reverse transcription procedures were performed in compliance with the updated international recommendations.¹⁷ Total RNA was extracted with the RNA blood kit (Qiagen, Hilden,

Germany) from blood and bone marrow samples previously treated with the HetaSept gradient (Stem Cell Technologies, Vancouver, Canada) to eliminate red cells and erythroid precursors. As part of good laboratory practice, the integrity of RNA was tested by electrophoresis after each extraction; cDNA was synthesized from 1 μ g of RNA through the high-efficiency Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) following the EAC reverse transcription procedure,¹⁶ as recommended by the M-bcr FusionQuant kit user guide.

Patient-Specific Genomic Assays

For each patient, a genomic assay was developed on the basis of his/her *BCR-ABL* sequence. Each assay comprised two real-time PCR reactions: one directed against the breakpoint sequence (present in one copy, only in leukemic cells) and a second against the *BCR* sequence used as control (one copy in leukemic cells or two copies in normal cells) (see Supplemental Figure S1 at <http://jmd.amjpathol.org>). The percentage of leukemic cells (LCs) was calculated using the following formula: $LC = 100 \cdot (2/(2^{\Delta Ct} + 1))$, where ΔCt is the difference between the amplification cycles of the *BCR-ABL* and *BCR* reactions (see Supplemental Figure S2 at <http://jmd.amjpathol.org>). Common forward primers and probes (Table 1) were used to ensure that the efficiencies of the two reactions within each assay could be as similar as possible. Plasmids containing the *BCR-ABL* breakpoint and the correspondent *BCR* sequence were used as reference material to test the efficiency and sensitivity of each assay. According to the human C value, plasmids were diluted to simulate different concentrations of leukemic DNA starting from 400 ng. For a given assay, the amplification efficiency was considered acceptable when it was higher than 90% in the range from approximately 400 to 0.3 ng for both the *BCR-ABL* and *BCR* reactions. The acceptance criteria for the sensitivity was fixed at least 10^{-4} dilutions (ie, similar to the sensitivity of conventional RQ-PCR assays).¹⁶ Furthermore, genomic assays were tested for the absence of spurious amplifications after 45 cycles using *BCR-ABL* negative DNA from healthy subjects.

Genomic DNA was extracted with the DNA blood Kit (Qiagen) from the same samples used for molecular monitoring of mRNA. The reaction mixture of each real time PCR reaction contained 12.5 μ l of TaqMan Universal PCR MasterMix (Applied Biosystems, Foster City, CA); 900 nmol/L each primer; 200 nmol/L probe; DNA ranging from 100 to 300 ng depending on the sample availability; and nuclease-free water up to 25 μ l. The PCR thermal profile was 2 minutes at 50°C followed by 10 minutes at 95°C and 45 amplification cycles (95°C for 15 seconds and 60°C for 60 seconds). Reactions were prepared and run in triplicate on ABI Prism 7000 SDS (Applied Biosystems) and each experiment was repeated and confirmed a second time.

Table 1. Primer and Probe Sequences

Patient ID	
1	F 5'-CTGCTGCTGGGTGGTTGA-3' Ph-R 5'-GGATTTTAGTCCTTACTTGTTTCTATTTCAC-3' wt-R 5'-GCCAGATCCAAGGCACAGA-' Probe 6-FAM-AGATGCACGGCTTC-MGB
2	F 5'-CCCCCTTCCTGTTAGCACTTT-3' Ph-R 5'-GCTGCAACAGTACAAACAGTAACCC-3' wt-R 5'-CCCTAACAAAGCATAGCTCTTCCCT-3' Probe 6-FAM-ATGGGACTAGTGGACTTT-MGB
3	F 5'-GCCCTCCTCTCCTCCAGCTA-3' Ph-R 5'-AAGCCTCTGGCGTGTTC-3' wt-R 5'-TGAGCATATGTGCAACAGTGAATG-3' Probe 6-FAM-CACCTTTTGGTCAAGCTG-MGB
4	F 5'-TGGGACTAGTGGACTTTGGTTCA-3' Ph-R 5'-GTGCATGATCATCACTAGTTAAATGTAAA-3' wt-R 5'-CTAACCCACCTTGTCCTCCT-3' Probe 6-FAM-ACAAGAGGCCCTAACAA-MGB
6	F 5'-CACAGCATACGCTATGCACTGT-3' Ph-R 5'-GGGAAAAAAGTTTTCCTCCCTATATCG-3' wt-R 5'-ATAAGGTTCCAAGGACAGCAGAG-3' Probe 6-FAM-ACACACACCCACCC-MGB
8	F 5'-TGCTCTGTGCTTGGATCTG-3' Ph-R 5'-TTCGGTGTAAATCCTTCCATACCTT-3' wt-R 5'-TGCAAAACAGCTTGACCAAAA-3' Probe 6-FAM-CCCCACTCCCGTCCT-MGB
9	F 5'-TTGTACCTGCCTCCCTTTC-3' Ph-R 5'-TGAACCTCTGACCTCAAGTGATCT-3' wt-R 5'-GAGCCCCGAGACTCATCA-3' Probe 6-FAM-CGGGACACAGAGGC-MGB
10	F 5'-CACTGGTTTGCCTGTATTGTGAA-3' Ph-R 5'-GGACACACAGGGAACACTGTC-3' wt-R 5'-TGGGCCAAAAACATACTCATCA-3' Probe 6-FAM-TCCTGAGATCCCC-MGB
PR1	F 5'-CCGCTGACCATCAATAAGGAA-3' Ph-R 5'-TGCCACGCCTTCTCTCTG-3' wt-R 5'-CAAAGTCCACTAGTCCCATCAAAA-3' Probe 6-FAM-TTTCCTGTACAGGGCA-MGB
PR2	F 5'-TTTGTGTCAGCTGTTTGTGA-3' Ph-R 5'-GGCACCAGAAGCTGAGTGAAG-3' wt-R 5'-ACACATGTGCATAGCGTATGCTG-3' Probe 6-FAM-TGTTGCACATATGCTC-MGB

For each patient-specific assay, primers are designated as follow: F, common forward primer; Ph-R, reverse primer for the selective amplification of the *BCR-ABL* sequence; wt-R, reverse primer for the selective amplification of *BCR*.

Results

The presence of Ph chromosomes was evident in 100% of the bone marrow metaphases of each patient at diagnosis. The majority of patients (8 of 10) had a complete response within 6 months from the beginning of the treatment, and the results of subsequent cytogenetic investigations were negative. Only patients 6 and 10 still had evidence of the *BCR-ABL* translocation at 12 and 6 months, respectively; a stable cytogenetic remission was achieved and maintained after the dose of IM was increased to 600 mg/day. For each patient, a genomic assay was developed fulfilling the requirements for efficiency, sensitivity, and absence of spurious amplifications as established in *Materials and Methods* (see Supplemental Figures S3-S4 at <http://jmd.amjpathol.org>).

At the onset, the median baseline level of *BCR-ABL* mRNA reported as *BCR-ABL/ABL* was 164% (range, 60% to 280%, SD 77), whereas the average percentage of *BCR-ABL* positive cells quantified with the genomic approach was 81% (range, 35% to 95%, SD 19). Only patient 1 had a proportion of leukemic cells at the diagnosis (35%) that was significantly different from the average value of 81% (see Supplemental Figure S5 at <http://jmd.amjpathol.org>).

Table 2. Levels of Residual Disease (%) Measured by Simultaneous mRNA and DNA Analysis

Patient no.	Months of therapy from the diagnosis											
	0	3	6	12	15	18	21	24	27	30	33	42
1	228*	2.730 [†]	0.050*	UND [†]	—	—	UND*	—	—	—	—	—
	35*	0.077 [†]	0.004*	UND [†]	—	—	UND*	—	—	—	—	—
2	264*	1.630 [†]	0.180 [†]	—	—	—	—	UND*	—	—	—	—
	87*	1.064 [†]	0.391 [†]	—	—	—	—	UND*	—	—	—	—
3	144*	—	0.920*	—	0.200 [†]	0.180*	0.060*	—	—	—	—	—
	94*	—	0.801*	—	0.079 [†]	0.061*	0.027*	—	—	—	—	—
4	64*	7.070 [†]	1.450 [†]	0.580 [†]	1.660 [†]	0.450*	0.400 [†]	0.320*	—	—	—	—
	90*	4.575 [†]	0.672 [†]	0.418 [†]	0.032 [†]	0.045*	0.068 [†]	0.024*	—	—	—	—
6	92*	12.480 [†]	7.770 [†]	3.640 [†]	—	2.190*	7.030 [†]	1.260*	—	—	3.880 [†]	—
	90*	19.669 [†]	4.895 [†]	3.401 [†]	—	1.478*	3.567 [†]	0.855*	—	—	1.026 [†]	—
8	170*	28.220 [†]	1.820 [†]	0.090 [†]	2.080 [†]	0.040*	0.060 [†]	—	0.030 [†]	UND*	—	UND [†]
	72*	2.228 [†]	0.054 [†]	0.016 [†]	0.009 [†]	0.006*	0.005 [†]	—	0.003 [†]	0.002*	—	0.001 [†]
9	165*	—	0.350*	0.230 [†]	0.140 [†]	1.060*	0.020 [†]	UND*	—	0.260*	—	—
	90*	—	0.883*	0.204 [†]	0.128 [†]	0.533*	0.096 [†]	0.134*	—	0.678*	—	—
10	60*	—	—	0.040 [†]	—	0.050 [†]	—	—	—	—	—	—
	93*	—	—	0.007 [†]	—	0.012 [†]	—	—	—	—	—	—
PR1	280*	—	2.900*	—	—	0.400*	—	—	—	—	—	—
	94*	—	1.805*	—	—	0.212*	—	—	—	—	—	—
PR2	170*	—	—	—	0.070 [†]	—	—	0.020 [†]	—	—	—	—
	65*	—	—	—	0.012 [†]	—	—	0.010 [†]	—	—	—	—

For each patient's row, results are reported as follow: top, percentage of BCR-ABL/ABL mRNA; bottom, percentage of leukemic cells. Results at the onset are approximated to the unit. —, unavailable data; UND, undetectable levels.

*Bone marrow samples.

[†]Peripheral blood samples.

For the time points with both results available, the actual percentage of leukemic cells and the corresponding levels of BCR ABL mRNA measured during follow-up are reported in Table 2. For each of the 57 samples, the extent of sample degradation was evaluated by electrophoresis and PCR amplifications. The average Ct values of the reference sequences (ABL for the RNA samples and BCR for the DNA samples) were 24.63 (range, 22.85 to 25.82) and 22.42 (range, 21.71 to 23.54), respectively. These findings are consistent with the results of electrophoresis and confirm the integrity of the nucleic acids analyzed in this study.

Undetectable levels of chimeric mRNA were observed at least once in four patients (numbers 1, 2, 8, and 9), but only for patients 1 and 8 was it possible to confirm this finding during consecutive analyses. A correspondent DNA negativity was observed only in patients 1 and 2 but not in patient 8 and 9.

The pattern of residual disease of patient 8 is illustrated in detail in Figure 1A, because it represents a paradigm of how the genomic approach could be used to refine results of conventional mRNA analysis. Transcription levels of patient 8 were unstable until the 15th month of therapy and then gradually decreased. Absence of BCR-ABL mRNA was observed at the 30th month and then confirmed at the 42nd month. At the latter time point, our genomic assay still revealed the persistence of leukemic cells (Figure 1B). The same genomic assay was negative when applied to the DNA from a healthy subject used as negative control (Figure 1C), but it proved to have a sensitivity of at least 10^{-4} dilutions (Figure 1D).

Discussion

Molecular monitoring of CML patients is of particular relevance in the era of IM, because residual disease usually falls below the detection limit of cytogenetics. However, a definition of complete molecular response remains undetermined, and in practice, drug treatment is maintained indefinitely, even for patients who have undetectable levels of chimeric mRNA. To further investigate the correlation between the expression of BCR-ABL and the corresponding proportion of leukemic cells, we analyzed both the mRNA and DNA from a group of CML patients under IM therapy. For the mRNA we used a commercial kit approved for *in vitro* diagnostics, while the proportion of leukemic cells was determined by DNA analysis with the Δ Ct method. The use of the Δ Ct method in this setting was previously discussed by Mattarucchi et al,²⁷ who presented a protocol to monitor the residual disease of patients with myeloproliferative disorders.^{28,29} In this study, we adapted the method to the CML, following an accurate pre-validation procedure.

At the onset, levels of chimeric mRNA were extremely variable, and had little prognostic significance, as reported in other studies.³⁰ Conversely, 9 of 10 patients had a proportion of leukemic cells at diagnosis close to the average value of 81%. Only patient 1 had a considerably lower percentage (35%). The levels of BCR-ABL mRNA and DNA in patient 1 decreased suddenly and were undetectable only 12 months after the beginning of the therapy. The observation that the presence of a limited number of cells carrying the BCR-ABL sequence at the onset may be correlated with a rapid remission sug-

Figure 1. Molecular follow-up of patient 8. **A:** Comparison of DNA and mRNA results during the 42 months of IM therapy. UND, undetectable levels. **B:** The amplification of 300 ng of peripheral blood DNA extracted after 42 months of therapy still reveals disease eradication. **C:** The same amount of DNA from a healthy participant was positive for the control (*BCR*) but not for the *BCR-ABL* sequence. **D:** Amplification of 300 ng of DNA from patient 8, subsequently diluted 1:10 into the DNA from a healthy participant, demonstrates a sensitivity greater than the minimum value of 10^{-4} dilutions.

gests an as yet unexplored prognostic implication for the DNA technology.

During follow-up, results of DNA and mRNA investigations were more comparable than at diagnosis, although some considerable divergences were still present. The mRNA levels of patients 4 and 8 increased when measured at 15 months. However, this observation was not confirmed during subsequent mRNA follow-up and did not correspond to an expansion of the leukemic clone when assessed with the genomic approach. These findings, along with previous reports,^{12,13} point up the current lack of consensus regarding the degree of increase in mRNA production that should cause concern. A rising level should be confirmed at subsequent time points before it can be considered a clear sign of loss of response. In these situations, DNA analysis may help in making appropriate and timely decisions relating to patient care.

Further technical issues may affect the reliability of mRNA measurements. The reaction that amplifies ABL also amplifies BCR-ABL transcripts and therefore tends to underestimate the BCR-ABL/ABL ratio.¹⁷ Results may be further affected by the extent of mRNA degradation and by the efficiency of the reverse transcription procedure.

For clinical purposes, the amount of chimeric mRNA is considered proportional to the leukemic clone, but the absence of the chimeric transcript is a condition that is difficult to interpret. In our study, we observed that the *BCR-ABL* sequence was still detectable at trace levels in 100 ng of bone marrow DNA and in 300 ng of peripheral blood DNA from patient 8, respectively, at 30 and 42 months. At the same time points, the BCR-ABL transcripts were undetectable. Patient 9 also had undetect-

able levels of mRNA at 24 months, but this observation was not confirmed on the next follow-up. These findings support the inference that although BCR-ABL mRNA is a fairly good target for molecular monitoring in patients with CML, the absence of the chimeric transcript does not equate with eradication.

Most patients who stop taking IM quickly lose the response they achieved, but a minority have been reported to maintain lasting progression-free survival. In a large series published by Rousselot et al,³¹ six patients maintained undetectable levels of chimeric mRNA for a median of 15 months after stopping IM. In our report, both the DNA and mRNA analyses in patient 1 were negative at 12 and 21 months. A similar condition was achieved in patient 2, although the results could not be confirmed because of the absence of further follow-up samples. These observations raise the intriguing possibility that, in a prospective study, undetectable levels of *BCR-ABL* DNA could identify patients who are possible candidates for drug discontinuation. It is worth emphasizing, however, that the levels of DNA or mRNA below which assays are negative depend on the sensitivity of the method, which should be laboratory-defined. However, within the limits of the dynamic range, the extent of the normalization (ie, the BCR-ABL/BCR or the BCR-ABL/ABL ratio) guarantees that any moderate difference in the nucleic acids quality or quantity between different samples does not affect results.

In conclusion, although genomic technology is more labor-intensive than mRNA analysis, requiring an initial characterization of the breakpoint sequence, this approach has been proven reliable and intelligible, with potential applications in the fields of diagnostics and research.

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