

## Analysis of Prevalence and Accumulation of Mutations Associated With Imatinib Based Therapy in Chronic Myeloid Leukemia Patients

Ratan J. Lihite, Nityanand Bolshette, Mangala Lahkar, Pabitra Kumar Gogoi, Pradeep Kumar Patnana

Ratan J. Lihite, Srimanta Sankaradeva University of Health Sciences, Gauhati Medical College, Guwahati-32, India

Nityanand Bolshette, Institutional level biotechub, National Institute of Pharmaceutical Education & Research, Guwahati-32, India

Mangala Lahkar, Department of Pharmacology, Gauhati Medical College, Guwahati-32, India

Pabitra Kumar Gogoi, Indian Society of Haematology and Blood Transfusion and Department of Haematology, Gauhati Medical College, Guwahati-32, India

Pradeep Kumar Patnana, Centre for DNA Fingerprinting and Diagnostics, Hyderabad-01, Telangana, India

Correspondence to: Mangala Lahkar, Head, Department of Pharmacology, Gauhati Medical College & Hospital and Chief Academic Coordinator, NIPER-Guwahati, Indrapur, Guwahati-781032, Assam, India.

Email: r.lihite@yahoo.com

Telephone: +91 9706143510

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### ABSTRACT

**AIM:** To identify accumulation of mutations in CML patients with chronic phase at ABL kinase codon 253 and 315 after the course of imatinib therapy for 12 months with respect to clinical outcome.

**METHODS:** In this cross sectional study, 103 CML patients with chronic phase attended between January 2013 and December 2014 at Gauhati Medical College & Hospital, Guwahati, Northeast India were enrolled. Peripheral blood samples were collected from CML patients who were on imatinib therapy for ABL kinase domain mutational analysis and again after 12 months, blood samples were obtained for mutational analysis to determine any accumulation of mutations in ABL kinase domain by RFLP method with mutation-specific restriction enzymes. Sokal scores were determined at the time of enrolment of CML patients and adverse effects to imatinib

therapy were also documented.

**RESULTS:** The median age at the time of enrolment of CML patients was 49.50 (45-56). Five patients (4.8%) were detected to have mutation at T315I after 12 months of imatinib therapy determined by mutation specific restriction digestion enzymes. Of these 5 patients, 2 patients were at high risk and other 3 patients were at intermediate risk as per sokal score. In our study, 425 adverse effects were reported to imatinib mesylate. Hypopigmentation was most commonly reported adverse effects to imatinib therapy. After one year of exposure to imatinib therapy, leukocytosis and thrombocytopenia were significantly found in CML patients with mutations. Hematologic and cytogenetic responses were significantly partial in CML patients with mutations.

**CONCLUSION:** In this study, 5 CML patients were reported to accumulate T315I mutations after 12 months of imatinib exposure and thus the prevalence of CML patients with mutation was 4.8%. Leukocytosis and thrombocytopenia were significantly reported in CML patients with mutations.

**Key words:** CML; T315I; Imatinib; Mutations; ABL kinase

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### INTRODUCTION

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of the primitive hematopoietic stem cell formed due to genetic rearrangement, caused by reciprocal translocation between chromosomes 9 and 22, expressed by fusion of breakpoints cluster (BCR) and human ABL1 gene, commonly referred as the

Philadelphia (Ph) chromosome t(9; 22)(q34; q11)<sup>[1]</sup>. In chronic phase of this disease gradual expansion of mature myeloid cells occurs in the bone marrow and peripheral blood. Without treatment, patients inevitably progress through an accelerated phase of disease to a terminal acute phase known as blast crisis, characterized by a massive increase in undifferentiated blast that can be either myeloid or lymphoid in nature. Selective inhibition of the BCR-ABL tyrosine kinase by imatinib is a promising therapeutic strategy in patients with CML. Imatinib mesylate was the first ATP-competitive inhibitor of the BCR-ABL oncoprotein and still widely used treatment for CML patients in India<sup>[2]</sup>. It has significant hematologic and cytogenetic responses in accelerated phase as well as in blast crisis of this disease. However in spite of these, resistance to imatinib therapy is still a hindrance in treatment of patients with CML<sup>[3,4]</sup> and their cross reactivity between host and cancer tissue often produce toxic effects in these patients<sup>[5]</sup>.

Mutations in BCR-ABL tyrosine kinase domain constitute a major cause of resistance to treatment with selective tyrosine kinase inhibitors. Most mutations gradually accumulate during the course of treatment in CML patients and produce significant impact on the treatment outcome<sup>[6-9]</sup>. Significant numbers of CML patients develop resistance during the course of imatinib therapy and move to advanced phase of this disease<sup>[10]</sup>. Several possible mechanisms of imatinib resistance are suggested such as clonal chromosomal evolution, BCR-ABL amplification, and ABL kinase domain mutation<sup>[11]</sup>. However, mutations in ABL kinase domain are recognized as one of the most common mechanism for imatinib failure.

CML is a commonest adult leukemia in India accounting for 30% to 60% of all adult leukemias<sup>[12]</sup>. The annual incidence range in male population was 0.8–2.2/100,000 while in female population it was 0.6–1.6/100,000<sup>[12]</sup>. As per our literature search it was observed that, very limited epidemiological data has been reported from Northeast India on this leukemia. Therefore, this hospital based study was conducted in one of the tertiary care hospitals of Northeast India with the aim to identify accumulation of mutations in CML patients with chronic phase after the course of imatinib therapy for 12 months with respect to clinical outcome. As per our available resources, finding of prognostic significance of all BCR-ABL kinase domain mutations were not achievable. Thus, this study was done to determine the clinical significance of mutations at ABL codon 253 and 315 with respect to cytogenetic and haematological response. The demographic data and laboratory investigations of these patients were also documented during study period.

## MATERIALS AND METHODS

### Patients

This cross sectional study was approved by institutional ethical committee for human research of Gauhati Medical College & Hospital, Guwahati, Northeast India (No. MC/108/2012/7 dated 29/11/2012). We have enrolled 103 CML patients with chronic phase attended between January 2013 and December 2014 at this tertiary care hospital. Informed consent was obtained from participants. CML progresses through distinct clinical stages, chronic phase characterized by expansion of terminally differentiated neutrophils followed by an accelerated phase and blast crisis with undifferentiated myeloid or lymphoid progenitor cells in maturation arrest. In this study, it was pretended that expression of the BCR-ABL gene is sufficient to cause chronic phase in CML, while disease progression to accelerated phase or blast crisis was thought to depend

upon additional genetic changes<sup>[13]</sup>. Therefore, chronic phase was solely considered in our study to avoid multiple additional genetic and molecular changes to achieve less mutational pre-existence in CML patients.

Initially, peripheral blood (PB) samples were collected from CML patients who were treated with 400 mg imatinib p.o. daily for mutational analysis in ABL kinase domain. Later on after 12 months, PB was again obtained for mutational analysis to determine any accumulation of mutations in ABL kinase domain after the exposure of imatinib therapy. Haematological and cytogenetic responses were documented as per the guidelines recommended by European Leukemia Net for the management of CML<sup>[14]</sup>. Sokal score of patients were also determined at the time of diagnosis according to Sokal *et al*<sup>[15]</sup>. Sokal index is a numerical value, calculated at the time of diagnosis from the percentage of blast cells in peripheral blood, the platelet count, the spleen size (measured in centimeters below the costal margin), and the age of the patient. Based on this index, patients were categorized into three risk groups as per their individual numerical value i.e., low, intermediate, and high. All the suspected adverse drug reactions occurred during study period were reported to the adverse drug reaction monitoring centre of this tertiary care hospital.

### RNA isolation

RNA isolations were performed twice for blood samples collected at the time of enrolment from CML patients and after 12 month of imatinib therapy as per manufacturer’s instructions (QIAGEN, Netherlands). Extracted total RNA was quantified on nanodrop, and confirmed on 1% agarose gel with ethidium bromide and subsequently observed on transilluminator.

### First strand cDNA synthesis

Two nanogram of total RNA was reverse transcribed into cDNA with oligo (dT) primer and RevertAid Reverse Transcriptase (Thermo Scientific, USA) in total volume of 20 µL in accordance with manufacturer’s recommendations.

### Genotyping PCR

The product of first strand cDNA synthesis was directly used for amplification of BCR-ABL genes. Set of forward and reverse primers used for the PCR reaction were: exon 4 primer F1: TCACCACGCTCCATTATCCA and ABL exon 4 R1: TCTTCCACACGCCCTCATAAACCT while for ABL exon 4/5 F2: AAGACCTTGAAGGAGGACACCATG and ABL exon 6 R2: GTTGCACTCCATCAAGTAGTCCA (Table 1). The thermal cycling conditions for PCR were followed as: 95°C for 5 minutes, 95°C for 30 seconds, 60.5°C for 30 seconds, and 72°C for 45 seconds (35 cycles), plus a 72°C extension for 5 minutes. Each PCR reaction employed 1µl of cDNA and 0.3 µM each of primer. PCR was performed with final volume of 20 µL and amplification was carried out using thermal cycler block (Thermo fisher scientific, Finland). Aliquots of 5 µL products were analysed and observed in 2% agarose gel containing ethidium bromide.

**Table 1** PCR primers and digestion products to confirm mutations by restriction digest analysis.

ABL codon	Primers	Restriction enzyme	Fragment size (bp)	
			wild type	mutant
253	F1: TCACCACGCTCCATTATCCA R1: TCTTCCACACGCCCTCATAAACCT F2: AAGACCTTGAAGGAGGACACCATG	Rsa1	138+30	168
315	R2: GTTGCACTCCATCAAGTAGTCCA	Dde1	134+49	183

**Restriction Fragment Length Polymorphism (RFLP) Analysis**

Mutation-specific restriction digestions were performed to confirm mutations of amino acids 253 and 315<sup>[16]</sup>. PCR products were digested separately with *RsaI* (codon 253), and *DdeI* (codon 315) restriction enzymes (Promega, Madison, USA). After digestion, products were subjected to electrophoresis on 3% agarose gel and photographed over UV light.

**Ethical approval**

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Statistical Analysis**

Descriptive statistics were used for data analysis. The non-normally distributed continuous data were presented in median (interquartile range). In our study, more than 20% of the cells have expected counts less than 5. Therefore, Fisher's Freeman-Halton test, extension of the Fisher exact probability test was applied for unordered row and column contingency table using IBM SPSS statistics software version 20. All statistical tests were two-sided and  $P < 0.05$  was considered statistically significant.

**RESULTS****Patient profile at the time of enrolment**

Responses of 110 CML patients comprised of 85 males and 25 females were recorded. The median age at the time of enrolment was 49.50 (45-56). Patient profiles at the time of enrolment were recorded as given in Table 2.

**Sokal risk at the time of enrolment in mutation induced patients**

Five patients were detected to have mutation at amino acid position T315I after 12 months of imatinib therapy. Out of these 5 patients, 2 patients were at high risk and other 3 patients were at intermediate risk as per sokal risk score (Table 3).

**Adverse events during monotherapy of imatinib mesylate**

During study period, 425 adverse effects were reported to imatinib mesylate. Among them, hypopigmentation was commonly reported adverse effect from CML patients with chronic phase (Table 4).

**Significance of variables in the CML patients with mutations**

After 1 year of exposure to imatinib therapy, leukocytosis and thrombocytopenia were significantly found in CML patients with mutations. Hematologic and cytogenetic responses were significantly partial in mutation induced patients. Frequency of adverse drug effect was more than two in CML patients with mutation (Table 5).

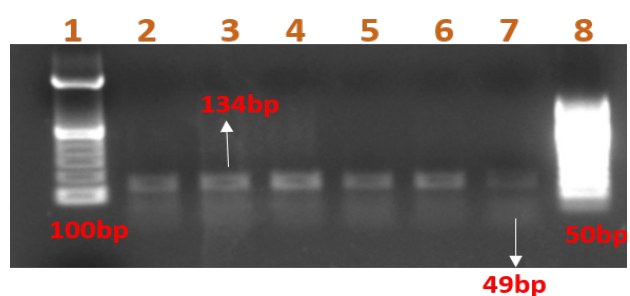
**Detection of mutations by RFLP analysis**

Five patients were detected to have mutation at amino acid position T315I after 12 months of imatinib therapy as determined by mutation specific restriction digestion enzymes (Figure 1, Figure 2, and Figure 3). Of these, 4 patients were male while 1 patient was female. All patients were of older adults ( $> 45$  years). As *DdeI* and *RsaI* are the mutation specific restriction enzymes. In our study, after 1 year of imatinib therapy *RsaI* enzyme haven't showed any mutant fragment by RFLP analysis, digested all PCR samples while *DdeI* enzyme have showed undigested fragment in 5 CML patients at 183 bp. Thus, after RFLP analysis, because of undigested PCR sample at 183bp

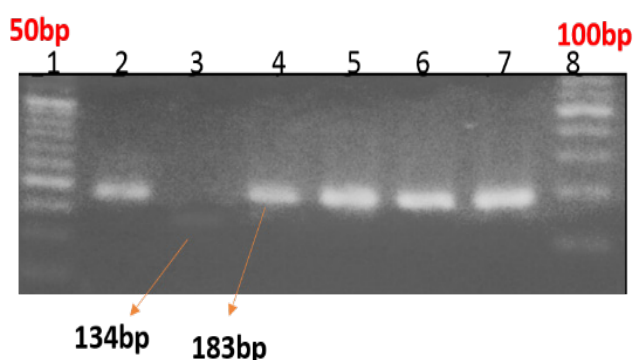
by *DdeI* enzyme, it was believed that out of 103 CML patients with chronic phase, 5 patients (4.8%) were accumulated mutation after 1 year of imatinib therapy.

**DISCUSSION**

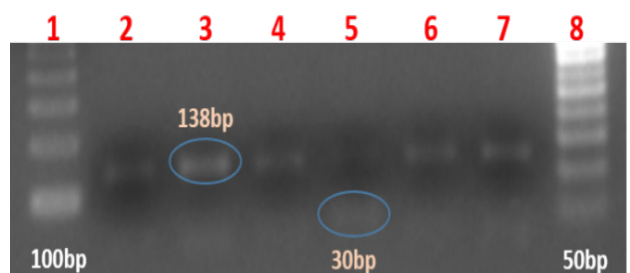
Male preponderance was observed in our study which was in agreement with other previous studies<sup>[12,17,18]</sup>. The male to female ratio was 3.4: 1. The median age in our study was 49.5 years. The median age of CML patients in India ranged from 33-42 years<sup>[12]</sup>. However, the median age in our study was above than this reported median age range.



**Figure 1** RFLP analysis of ABL codon 315 in CML before Imatinib therapy by *DdeI*. The amplicons of ABL exon 315 were digested with *DdeI* and observed on 3% agarose gel. Figure shows Lane 1: 1000bp ruler and Lane 2-7: digested sample with *DdeI* at 134 bp & 49 bp.



**Figure 2** RFLP analysis of ABL codon 315 in CML after 1 year of Imatinib therapy by *DdeI*. The amplicons of ABL exon 315 were digested with *DdeI* and observed on 3% agarose gel. Figure shows Lane 1: 50bp ruler; Lane 2, 4: undigested PCR sample at 183bp; Lane 3: digested sample with *DdeI* at 134 bp; Lane 5-7 are the mutated samples at 183bp and Lane 8: 100bp ruler.



**Figure 3** RFLP analysis of ABL codon 253 in CML before Imatinib therapy by *RsaI*. The amplicons of ABL exon 253 were digested with *RsaI* and observed on 3% agarose gel. Figure shows Lane 8: 50bp ruler and Lane 1-7: digested amplicons of exon 253 with *RsaI* at 138 bp and 30 bp.

**Table 2 Patient profile at the time of enrolment.**

Variables (Median, Interquartile range)	Response of CML patients (%)
<b>Age (49.50, 45 - 56)</b>	
≤ 40	8 (7.27)
41-50	48 (43.63)
51-60	40 (36.36)
≥ 61	14 (12.72)
<b>Gender</b>	
Male	85 (77.27)
Female	25 (22.72)
<b>Phase</b>	
Chronic	103 (93.63)
Accelerated	7 (6.36)
Blast Crisis	0 (0)
<b>Splenomegaly</b>	
Present	98 (89.09)
Absent	12 (10.90)
<b>Hepatomegaly</b>	
Present	76 (69.09)
Absent	34 (30.90)
<b>Anaemic</b>	
Yes	52 (47.27)
No	58 (52.72)
<b>Platelet Count (218023.50, 251642.50 - 63479)</b>	
Normal (150,000-450,000/μL)	70 (63.63)
Thrombocytosis (> 450,000/μL)	2 (1.81)
Thrombocytopenia (< 150,000/μL)	38 (34.54)
<b>WBC count (5834, 23507 - 4587)</b>	
Normal (4,500-10,000/ μL)	55 (50)
Leukocytosis (> 10,000/ μL)	32 (29.09)
Leukopenia(< 4,500/ μL)	23 (20.90)
<b>Sokal risk</b>	
Low risk	19 (17.27)
Intermediate risk	83 (75.45)
High risk	8 (7.27)

**Table 4 Reported adverse events (425) during monotherapy of imatinib mesylate.**

Adverse effects	Frequency of event (%)
Hypopigmentation	54 (12.7)
Anaemia	42(9.88)
Musculoskeletal pain	37(8.7)
Skin rash	36(8.47)
Abdominal pain	33(7.76)
Nausea	31(7.29)
Weight gain	28(6.58)
Muscle cramps	25(5.88)
Fatigue	23(5.41)
Thrombocytopenia	22(5.17)
Abnormal liver enzymes	14(3.29)
Flatulence	13(3.05)
Face oedema	10(2.35)
Pancytopenia	9(2.11)
Vomiting	9(2.11)
Neutropenia	7(1.64)
Leukopenia	5(1.17)
Diarrhea	5(1.17)
Gastritis	3(0.7)
Headache	3(0.7)
Marrow depression	3(0.7)
Arthralgia	2(0.47)
Oedema genital	2(0.47)
Oedema peripheral	2(0.47)
Hypotension	2(0.47)
Gingival bleeding	1(0.23)
Insomnia	1(0.23)
Pruritus	1(0.23)
Rash maculopapular	1(0.23)
Urticaria	1(0.23)

**Table 3 Sokal risk at the time of enrolment in mutation induced patients.**

Sl. No.	Age	Sex	Hb (g/dL)	WBC count (μL)	Platelet count(μL)	Basophils (%)	Eosinophils (%)	Sokal risk
1	49	male	10.1	12458	21458	2	0	Intermediate risk
2	47	female	8	23458	41236	0	0	high risk
3	49	male	12.5	65489	45879	1	0	high risk
4	47	male	10.5	23654	57896	2	0	Intermediate risk
5	45	male	11.5	23675	57796	2	0	Intermediate risk

**Table 5 Significance of variables in the CML patients with mutations.**

Variables	Patients without mutation	Patients with mutation	P-value
<b>Gender</b>			
Male	81	4	1.000
Female	24	1	
<b>Age</b>			
≤40	8	0	0.157
41-50	43	5	
51-60	40	0	
≥61	14	0	
<b>WBC count</b>			
Normal (4,500-10,000/ μL)	55	0	0.002*
Leukocytosis (>10,000/ μL)	27	5	
Leukopenia (<4,500/ μL)	23	0	
Normal (150-450,000/μL)	70	0	
<b>Platelet</b>			
Thrombocytosis (>450,000/μL)	2	0	0.007*
Thrombocytopenia (<150,000/μL)	33	5	
<b>Hematologic response</b>			
Complete	78	0	0.001*
Partial	20	5	
Minimal	7	0	
<b>Cytogenetic response</b>			
Complete	59	0	0.026*
Partial	39	5	
Minimal	7	0	
<b>Frequency of adverse effects</b>			
None	20	0	0.000*
One	73	0	
Equal to or more than two	12	5	

\*statistically significant.

Splenomegaly & palpable liver are most common abnormalities could be noticed in CML patients<sup>[17,18]</sup>. In our study, at the time of enrolment splenomegaly was present in majority of the patients. Palpable liver was also observed in more than half of the patients. Platelet count was within normal range in most of the patients while some had thrombocytopenia and thrombocytosis. WBC count was within normal range in half of patients while other had experienced leucopenia and leukocytosis. The scoring system developed by Sokal *et al* is most widely accepted system for assessing prognosis of disease in patients with CML<sup>[15]</sup>. In our study, at the time of enrolment most patients were under intermediate risk category followed by low risk and high risk category. In the study of Ono *et al.*, 2011, CML patients in chronic phase with high Sokal score showed significantly higher incidence of mutations<sup>[25]</sup>. However, our study does not showed any significant changes in mutation induced patients after 12 months of imatinib therapy.

In our study, imatinib has shown remarkable haematological response in patients with CML. Most patients have achieved complete haematological response within 12 months of imatinib therapy. However, hypopigmentation, anaemia, musculoskeletal pain, skin rash, abdominal pain, nausea, weight gain, muscle cramps, fatigue, and thrombocytopenia were most commonly reported adverse effects during imatinib therapy. In our study, therefore, weekly blood counts for the first 4 weeks, twice weekly during the second month, then at 2-4 week intervals were recommended to the patients in identifying non-responders as well as those who develop major toxicities requiring dose adjustment and/or granulocyte colony stimulating factor (G-CSF) support<sup>[19]</sup>.

In India, the overall survival of patients with CML on imatinib is 93%<sup>[12]</sup> and most of them have received imatinib through Glivec International Patient Assistance Program<sup>[20]</sup>. Imatinib was also established choice of treatment for newly diagnosed CML patients from IRIS study because most of the patients had complete hematologic and major cytogenetic response when treated with imatinib in early chronic phase<sup>[21]</sup>. However, these patients were reported to have one or more ABL kinase domain mutations on imatinib based therapy. Higher frequency of mutations is usually seen at amino acids T315, F359 and G250 as compared to other residues<sup>[22]</sup>. Ethnic difference among populations and low socio-economic status may also predispose to advanced disease phase in CML patients with high mutational accumulations<sup>[23]</sup>. Thus, these accumulated mutations imply failure to imatinib therapy and induce resistance in CML patients.

In this study, out of 103 CML patients with chronic phase, 5 patients (4.8%) were identified to accumulate T315I mutation after 12 months of imatinib exposure while none of the patients were with amino acid mutation at 253 positions. In the study of Branford *et al.*, 2002, T315I mutations was not detected before the initiation of imatinib therapy in CML patients with chronic phase but this mutation was predominant at the time of sequence analysis after imatinib exposure<sup>[24]</sup>. In another study of Branford *et al.*, 2003, it was also observed that patients who took imatinib therapy for more than 4 years from the diagnosis had significantly higher incidence of mutations as compared to those who treated with less than 4 years exposure<sup>[18]</sup>. In the study of Wongboonma *et al.*, 2012, T315I resistant mutation was completely undetectable in naive cases but its incidence increased with drug exposure<sup>[9]</sup>. Thus, among other mechanisms for mutational accumulation, our finding emphasizes accumulation of T315I mutations after the exposure of imatinib therapy in CML patient with chronic phase.

## CONCLUSIONS

Our study has some potential limitations. Due to lack of funding and low resource setting we were unable to detect BCR-ABL kinase domain mutations by appropriate screening tool. Various types of sensitive and resistant mutations may pre-exist in BCR-ABL kinase domain with no previous drug exposure and their frequency may increase after treatment with tyrosine kinase inhibitors. We have not entertained alternative mechanisms such as mutations outside the kinase domain in the neighbouring regions, clonal chromosomal evolution, BCR-ABL amplification, and pharmacogenomic variations for accumulation of mutations. Variations in the kinase domain mutation frequency and types from various studies may also reflect geographic heterogeneity in various ethnic populations. However, in our study after imatinib therapy T315I mutation was accumulated in 5 CML patients with chronic phases and thus the prevalence of CML patients with mutation was 4.8%. Leukocytosis and thrombocytopenia were significantly observed in these CML patients with chronic phase after imatinib exposure. Hypopigmentation was commonly reported adverse effect to imatinib therapy in CML patients.

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## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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- Peer reviewer:** Akihiro Yokoyama, MD, PhD, Division of Hematology, Department of Internal Medicine, National Hospital Organization Tokyo Medical Center, 2-5-1, Higasigaoka, Meguro-ku, Tokyo, 152-8902 Japan.