Original Article

Detection of *BCR-ABL1* Fusion Gene Transcripts in the Saliva of Nigerian Patients with Chronic Myeloid Leukemia

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ABSTRACT

Background: The presence of *BCR-ABL1* fusion gene resulting from a t(9; 22) reciprocal chromosome translocation is the molecular hallmark of chronic myeloid leukemia (CML). In the diagnosis and treatment of CML, peripheral blood or bone marrow samples are usually taken for analysis. However, both methods are invasive sample collection methods, thus a noninvasive saliva sample method for the detection of the fusion gene transcripts (BCR-ABL) was investigated in some Nigerians with CML. **Materials and Methods:** Real-time (RT)-polymerase chain reaction (PCR) analysis was used to detect *BCR-ABL1* fusion gene in the saliva and blood of 42 Nigerian CML patients. RNA was extracted using RNeasy kit and reverse transcribed by random hexamer priming using murine Moloney reverse transcriptase. *BCR-ABL1* transcript types were first detected by multiplex PCR and then quantified by a duplex RT-PCR-TaqMan chemistry with MGB probe and Black Hole Quencher. **Results:** Of the 42 subjects, transcript types were detected in 36 (85.7%) samples, e13a2 fusion transcript sub-type was detected in 9 (21.4%), whereas e14a2 subtype was found in 27 (67.3%); six (14.3%) of the samples did not reveal any of the fusion transcript subtypes. The median *BCR-ABL1* messenger RNA values were 9.38×10^2 in saliva and 10.29×10^4 in blood ($P < 0.05$). Similarly, the median *ABL1* value in saliva (3.11×10^3) was significantly lower ($P < 0.01$) than in blood (4.22×10^3). However, the median *BCR-ABL1* ratio in saliva (14.5%) was not significantly different ($P = 0.8$) from that of blood (12.0%). **Conclusion:** Saliva may offer an alternative easy-to-collect, readily available, and noninvasive sample for the diagnosis and treatment of CML.

KEYWORDS: *BCR-ABL1, chronic myeloid leukemia, Nigeria, Philadelphia chromosome, saliva*

INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disorder of hematopoietic stem cell.[1,2] CML is of special interest being one of the most extensively investigated and the first malignancy to be associated with a pathognomonic genetic abnormality – the Philadelphia (Ph) chromosome.[3,4] The Philadelphia (Ph) chromosome was first described by Nowell and Hungerford and was named after the city of its discovery.[5] Using G-banding technique, Rowley demonstrated the genetic basis of CML as a reciprocal translocation between chromosomes 9 and 22 [t(9:22) (q34:q11)].[6] The structural chromosomal aberration gives rise to a *BCR-ABL* fusion gene that is transcribed into hybrid messenger RNA (mRNA) molecules. The transcripts are translated into a *BCR-ABL* fusion protein, an oncoprotein with abnormal tyrosine kinase activity, which is responsible for the leukemic process.[7,8]

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Correct diagnosis is critical in CML for confirmation of suspected cases, choice of therapy, and prognosis.[2] Human body fluids like blood and saliva represent a common source of biological materials for diagnostic and forensic purposes.[9] However, despite the possible role of saliva as an easily obtainable sample for diagnosis of CML, peripheral blood and bone marrow have been samples of choice in testing for the *BCR-ABL1* gene mutation.[10] Reports from Foroni *et al.* considered total white blood cells from peripheral blood samples in CML as the most appropriate material for molecular investigation for diagnosis and monitoring in CML.[11]

It has been reported that *BCR-ABL1* mRNAs decay faster after 72 h of peripheral blood collection, and this affects the sensitivity of quantitative polymerase chain reaction (qPCR), which is the standard technique for *BCR-ABL1* transcript detection.[11,12] The result of this fast decay has been attributed to the high content of ribonucleases in blood plasma.[13] In addition, storage duration other than temperature has been shown to affect the rate of decline in RNA in blood samples.[14] This causes unnecessary delay due to repeated sampling of patients at extra costs. However, in saliva due to proprietary preservative used, mRNA decay is prevented. Moreover, blood samples require extra care in packaging and freight, a standard that might be difficult to meet in resource-poor countries. The alternative is that patients would have to travel longer distance for sampling since there are limited numbers of centers that currently perform the standard *BCR-ABL1* testing in Nigeria. The standard *BCR-ABL1* testing involves a combination of multiplex PCR to detect major, minor, and rare transcript variants and real-time (RT) PCR to quantify the detected transcript. This is superior to point of care testing devices such as Cepheid GeneXpert which can only quantify major transcript.[15] Saliva samples could also be a valuable, noninvasive source of genomic DNA for Tyrosine Kinase Domain mutation analysis by DNA sequencing.

Previous studies have shown that 74% of the total DNA in saliva is from leucocytes.[16] Saliva has been found to be an adequate source of DNA/RNA for genomic research, and it has the advantage of being noninvasive.[17] After online literature search on PubMed, only very scanty information on the use of saliva for detecting fusion transcripts in CML was available. The first report of using saliva sample for fusion transcript detection that we came across was by Chen *et al.*[17] The study had a small sample size of only seven leukemic patients.

This study was conducted on the detection of *BCR-ABL1* fusion gene transcripts in the saliva of CML patients at the University of Nigeria Teaching Hospital, Enugu, and patients were referred to the *BCR-ABL1* gene testing laboratory in Enugu from the Obafemi Awolowo University Teaching Hospital in Nigeria.

MATERIALS AND METHODS

Ethical approval for this study was obtained from the University of Nigeria Teaching Hospital, Ituku/Ozalla Health Research Ethics Committee with Reference No. NHREC/05/01/2008B. Written informed consent was obtained from all the participants whose samples were collected.

Subjects for the study

The subjects, 42 Nigerians (13 females and 29 males), were patients who had been diagnosed of CML and were referred to Safety Molecular Pathology Laboratory, University of Nigeria Enugu Campus, for *BCR-ABL1* testing as part of their clinical care. Those who were known to have Philadelphia chromosome (i.e., Ph$^+$) were also included in the study. Participating patients were sampled from their various locations through collaborations with their physicians.

Sample collection

The subjects were asked to rinse their mouths with water after which 2.0 mL of saliva was collected into Oragene RNA storage bottles (DNA Genotek, Canada). Venous blood samples were collected and stored in K3EDTA anticoagulant tubes. Saliva and blood samples were collected from each subject. The blood samples were analyzed immediately on their arrival at the testing laboratory, usually less than 72 h after sample collection. The saliva samples were analyzed after 72 h of storage at room temperature.

RNA extraction and reverse transcription

The blood samples were processed by red cell lysis to obtain white cell pellets which were further lysed in guanidiumisothiocyanate (GITC) buffer. About 350 μL of the GITC lysate was used for RNA extraction using RNeasy Mini Kit with DNAse I digestion (Qiagen, UK). Quality, quantity, and integrity of RNA were checked by spectrophotometric (A260/280) reading in Eppendorf Biophotometer and automated electrophoresis using BioRad automated electrophoresis system (BioRad, UK). Equal amount of RNA (0.5 μg) was reverse transcribed using SuperScript First-Strand DNA synthesis kit (Invitrogen, UK). In the same manner, saliva samples collected in the Oragene RNA kit were processed according to the manufacturer's instructions. Essentially, 350 μL of the stabilized saliva

from the Oragene kit was used in manual extraction of total RNA using RNeasy Mini Kit with DNase digestion (Qiagen). It was treated in the same way as GITC lysate. The amount of RNA used for cDNA synthesis was 0.5 μg. Equal volume of RNA elute was used in cDNA synthesis by random hexamer priming using murine Moloney reverse transcriptase (M-MuLV-RT) (Promega, UK). cDNA synthesis was done immediately after RNA extraction.

Multiplex and qRT-PCR analysis

Multiplex PCR was used for detecting transcript types. Multiplex PCR assay was designed to detect all transcript types. A duplex quantitative RT (qRT)-PCR based on TaqMan Chemistry using TaqMan MGB probes and primers of HPLC grade synthesized by Applied Biosystems (UK) was used to quantify *BCR-ABL1* gene. A control gene, *ABL1*, was amplified simultaneously with the target fusion gene, *BCR-ABL1*, in a 48-well StepOnePlus RT-PCR equipment (Applied Biosystems). The slope of the calibration curve was 3.32 corresponding to a PCR efficiency of 98%. The Y-intercept was equal to 40, and the correlation coefficient for log of starting quantity of target gene *BCR-ABL1* and *ABL1* control gene and their cycle of quantitation value was 0.98. An in-house plasmid calibrator containing *BCR-ABL1* prepared from TOPO vector cloning kit (Invitrogen) was used. For each sample, 3 μL of cDNA was used in a total reaction volume of 20 μL comprising 10 μL of TaqMan gene expression master mix with ROX as passive reference (Applied Biosystems) and 7 μL of primer and probe mix. The probe system consists of VIC and BHQ dyes for *BCR-ABL1* detection and FAM and BHQ dyes for *ABL* detection. The thermal profile used was as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 94°C for 15 s and 60°C for 15 s, and data were collected at the 60°C point. *BCR-ABL1* ratio for each sample was calculated by dividing transcript number of *BCR-ABL1* by *ABL1* and the quotient multiplied by 100.

Statistical analysis

Data analysis was by IBM SPSS Version 23, while graphical presentation was done by GraphPad Prism Version 7.04. We initially performed D'Agostino-Pearson Omnibus normality test to determine whether data approximately fit normal distribution. Levene's test for equality of variances and paired *t*-test for equality of means were used to determine whether there was a significant difference between the two normally distributed sets of data. Since transcript levels deviated significantly from normality and two samples (blood and saliva) were

obtained from each subject, Wilcoxon matched-pairs signed rank test, a nonparametric equivalent of paired *t*-test to assess differences between transcript levels, was used. Association between normally distributed continuous variable was by Spearman's correlation analysis; otherwise, Chi-square test for association for categorical variable was used. The level of significance was set at $P \leq 0.05$ as statistically significant.

RESULTS

Descriptive statistics

A total of 42 Nigerians (13 females and 29 males), 10–65 years of age, comprised three major ethnicities in Nigeria (Hausa, Igbo, and Yoruba). Demographically, 18 (42%) of the subjects were Igbos, 17 (40.5%) were Yoruba, and 5 (11.9%) were Hausa, while others constitute only 2 (4.8%). The majority [29 (69%)] of the subjects were male, whereas 13 (31%) were female.

A total of 36 (85.7%) samples tested positive for *BCR-ABL1*. Two subtypes of *BCR-ABL1* transcript, namely, e13a2 (9, i.e., 21.4%) and e14a2 (27, i.e., 67.3%), were detected in this study. Transcripts could not be detected in 6 (14.3%) of the paired samples. *BCR-ABL1* detection in both saliva and blood showed positive and negative concordance.

Comparison of *ABL1* level, *BCR-ABL* mRNA, and *BCR-ABL* ratios in saliva and blood

There was a significant difference in the comparison of *ABL1* levels in saliva and blood ($P = 0.005$). Figure 1 shows the median *ABL1* value in saliva (3.11×10^3; range: 1.28×10^1–1.02×10^4) which was also significantly different ($P = 0.005$) from that of blood which was 4.22×10^3 (range: 1.3×10^1–2.11×10^5). Figure 2 shows that the amount of *BCR-ABL1* mRNA in saliva with a median of 9.38×10^2 (range: 7.76×10^1–15.98×10^3) was significantly different ($P = 0.039$) from the median amount in blood (10.29×10^4) with a range of 8.3×10^1–23.21×10^4). In contrast, the *BCR-ABL1* ratio in blood [12.01% (0.00–76.33)] was not significantly different ($P = 0.808$) from that of saliva [14.50% (range: 0.00–75.03)] as depicted in Figure 3. Moreover, saliva gave comparable results for *BCR-ABL1* transcript levels and ratios when compared with blood judging from the more closely clustered data points for saliva when compared with those of blood in Figures 2 and 3. Six patients tested negative in both saliva and blood samples. They were patients on follow-up monitoring of treatment who had been on treatment for over 12 months.
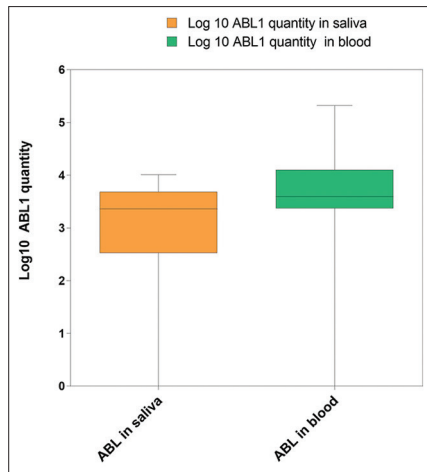
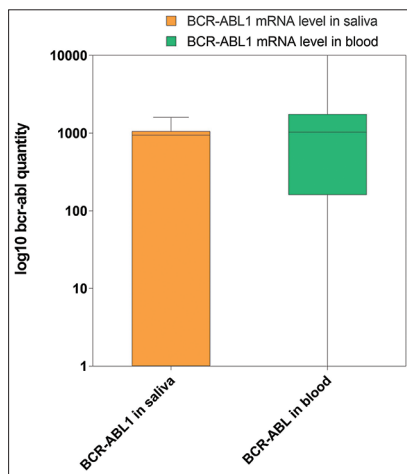**Figure 1:** ABL1 Level in saliva and blood

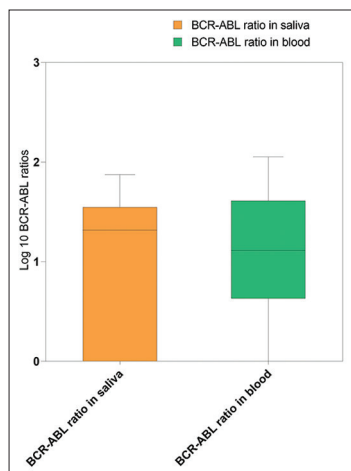**Figure 2:** BCR-ABL1 mRNA in saliva and blood

Figure 3: BCR-ABL1 ratio in saliva and blood

DISCUSSION

This study revealed that *BCR-ABL1* gene transcripts can be detected in the saliva of patients with CML, similar to reports by Chen *et al.*[17] who suggested that

human whole saliva could be a reliable and convenient sampling source for detection of *BCR-ABL1* fusion gene transcripts. In addition, our results suggested that measurement of *BCR-ABL1* fusion gene transcripts in saliva gives comparable results to measurements obtained using blood. The implication of this finding is not only of diagnostic importance but also of residual disease monitoring, which involves repeated sampling of patients to monitor response to therapy. It also provides genomic DNA for Tyrosine Kinase Domain mutation analysis for checking primary resistance in newly diagnosed patients. The possibility that Oragene RNA kit could preserve mRNA transcripts for more than 72 h is of interest in the light of situations where it might not be possible to analyze saliva sample immediately after collection. In many resource-poor countries, it may not be possible to analyze blood samples immediately after being collected in view of the need to send them to specialist laboratories or centers, where resources including well-trained personnel and modern equipment are available.

This study detected only the two major transcripts, namely, e13a2 and e14a2. Incidentally, these are also the most common ones reported in many other populations.[11] Those patients with undetected *BCR-ABL1* levels were found to have been on treatment for over a year and might have achieved complete molecular remission at the time of sampling.

Our findings support the use of saliva as an alternative to blood or bone marrow samples for molecular diagnosis of patients with CML. However, blood samples would still be required in morphological and cytochemical testing when necessary. Patients who test positive for *BCR-ABL1* are accepted for free imatinib treatment in Nigeria based on a nongovernmental organization support program. *BCR-ABL1* testing is also required for following up treatment and determining molecular remission. Saliva being an easily obtainable fluid will hopefully reduce burden and cost to the patients and the personnel. It will also increase accessibility to testing services especially from areas where there are poor electricity infrastructures and problems of prompt delivery of samples to the testing laboratory. Cost of sampling and transporting saliva was cheaper than that of sampling and transporting blood. Hence, this indicates that using this noninvasive means of sample collection could revolutionize the clinical management of patients with CML especially in resource-poor settings.

CONCLUSION

Saliva offers an alternative easy-to-collect, readily available, and noninvasive sample for diagnosis and treatment of CML.

Conflicts of interest

There are no conflicts of interest.

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