Biomarkers for BCR-ABL1 as a Potential Therapeutic Option Among Adult Chronic Myeloid Leukaemia Treated With Tyrosine Kinase Inhibitors at Molecular Response in Multi-racial Malaysia: Hsa-mir-181a-5p for Decrement; hsa-mir-182-5p and hsa-mir-26a-5p for Increment.

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Abstract

Background

Tyrosine kinase inhibitors (TKIs) as first-line therapy for Chronic Myeloid Leukaemia (CML) show a high success rate. However, low number of patients with long-term treatment-free remission (TFR) is observed. Molecular relapse after imatinib discontinuation occurred at 50% at 24 months with 80% occurrence within the first 6 months. One of the reasons for relapse is untimely TKIs discontinuation caused by large errors from estimates at very low-level or undetectable disease, thus warranted new biomarkers for CML.

Methods

Next Generation Sequencing (NGS) was used to identify microRNAs (miRNAs) at molecular response in CML adult patients receiving TKIs treatment. A total of 86 samples were collected, 30 from CML patients responsive and 28 from non-responsive to imatinib therapy, and 28 from blood donors. NGS was conducted whereby 18 miRNAs were selected and validated by real time RT-qPCR in triplicate.

Results

Hsa-miR-181a-5p was expressed significantly (p-value<0.05) with 2.14 and 2.3 fold down-regulation in both patient groups respectively while hsa-miR-182-5p and hsa-miR-26a-5p were significant only in the non-responsive group with 2.08 and 2.39 fold up-regulation respectively. The down-regulation was consistent with decreased amount of BCR-ABL1 in patients taking TKIs regardless of molecular responses. The up-regulation was consistent with substantial presence of BCR-ABL1 in CML patients treated with TKIs at molecular response.

Conclusion

Therefore, these miRNAs have potential as new therapeutic biomarkers for BCR-ABL1 status in adult CML patients treated with TKIs at molecular responses. These could improve current approaches and require further analysis to look for targets of these miRNAs in CML.

Background

CML occurs mainly in adults with Philadelphia chromosome (Ph) presents in most patients [1] as a result of a reciprocal translocation between chromosome 9 and 22 that gives rise to BCR-ABL1 transcripts, t(9;22)(q34.1;q11.2) [2-3]. There are 3 phases of CML, namely chronic, accelerated and blast crisis and is treated using Tyrosine Kinase Inhibitors (TKIs). Imatinib (Glivec®, Novartis) a first generation TKI, has been widely used since the Food and Drug Administration approval in 2001 and given orally (400mg) once daily [4]. This is a targeted therapy whereby imatinib competes for Bcr-Abl tyrosine kinase site thus prevent phosphorylation and inhibits proliferation. Patients treated with imatinib are usually experiencing mild to moderate adverse effects [5]. Second generation TKIs, nilotinib and dasatinib are also choices of
front-line treatment and patients in chronic phase usually respond well to treatment [4]. Progress is measured based on haematologic, cytogenetic and molecular responses. Molecular response is the major treatment endpoint with optimal molecular response at BCR-ABL1 transcript level $\leq 10\%$ by 3 months, $< 1\%$ by 6 months and $\leq 0.1\%$ by 12 months [4].

BCR-ABL1 expression less or equal to 0.1% known as major molecular response (MMR) corresponds to a 3-log reduction from the International Randomized Study of Interferon and STI571 (IRIS) baseline. BCR-ABL1 expression less or equal to 0.01%, 0.0032% or 0.001% are known as deep molecular response (DMR) and corresponds to 4, 4.5 or 5-log reduction respectively [6]. DMR of 4.5-log reduction or lower is preferable for discontinuation of imatinib by controlled studies due to fewer events observed and better clinical outcome [7, 8, 9]. These low-level estimates were measured using RT-qPCR and have large errors especially from Poisson variation, which is substantial with limit of detection (LoD) of $10^{-4}$, $10^{-4.5}$ and below [10, 11, 12]. These led to disagreements in reporting as recommended by EUTOS especially in assigning targets to 3 for less than 3 copies [10-11, 13]. Reporting were suggested to include confidence limit [11], average targets or as individual results for undetectable replicate [10] and LoD was proposed [14]. In most centres MR$^{4.5}$ is regarded as the routine LoD for RT-qPCR [15]. All these have compromised consistency of DMR in which could hinder timely TKI discontinuation; therefore, a new therapeutic biomarker is necessary.

MicroRNAs (miRNAs) are small non-protein coding, endogenous RNAs around 22 nucleotides long and function as regulators in gene expression. Expression could be downregulated or upregulated. Mature miRNAs are incorporated in RNA-induced silencing complex and annealed to mRNA. Regulation of gene expression is by mRNA cleavage or translational repression [16]. miRNAs have been extensively studied in cancer and studies showed miRNAs have potential as tumour suppressor or oncogene with potential use in diagnosis, prognosis and treatment of cancers [17, 18, 19, 20, 21, 22, 23, 24].

NGS allows deep sequencing with high sensitivity, wide coverage and suitable for detection of low level variants. There are several platforms available with unique specifications [25]. Miseq system (illumina®), an easy to use NGS instrument that offers short sequencing run times, long read lengths and high data quality is suitable for targeted resequencing. The sequencing workflow involves library preparation, cluster generation, sequencing by synthesis and data analysis. Except for library preparation, the rest are carried out by the instrument making it a reliable platform.

In this study, adult CML patients responsive and non-responsive to imatinib therapy at molecular response were looked at. miRNAs from whole blood of these patients were profiled using NGS and compared with normal control. Suitable miRNAs were selected and verified by real-time RT-qPCR, the known gold standard for quantifying gene expression. Therefore, this study aimed to identify miRNAs that have therapeutic potential in relation to TKIs at molecular response in CML. These miRNAs could lead to discovering conditions that favour TKI discontinuation accurately thus making long term TFR achievable.
Methods

Patients and samples

A cross sectional study was conducted among 86 respondents in Ampang Hospital and National Blood Centre, Malaysia between 2013 to 2014. Of these respondents, 30 were CML patients responsive to imatinib therapy, 28 were CML patients non-responsive to imatinib therapy and 28 were normal controls. Only adults aged 18 and above from both sexes were included in this study. Peripheral blood samples were collected from all the respondents. CML cases were taken from haematology clinic at Ampang Hospital. Imatinib is the front-line treatment used to treat CML and in the event of intolerance or failure, other approved TKIs will be used as appropriate. Current treatment success is measured by minimal presence or undetectable of BCR-ABL1 transcripts by RT-QPCR. Responsive is defined as CML patient who has been assessed and confirmed by clinician through clinical diagnostics and evaluations, has been receiving imatinib not less than 18 months, the latest BCR-ABL1<sup>IS</sup> ratio was 0.1% or less and ABL1 control gene was 10 000 copies or more. Non-responsive is defined as any of the above criteria are not met and is treated with imatinib, other TKIs and/or other drugs as necessary. Control is defined as normal people taken from healthy donors mostly from the National Blood Centre who came to donate blood. The conversion factor of 0.81 was used to convert BCR-ABL1 ratio to the International Scale (IS) ratio. Clinical data was captured using a standardized data collection form. The form was divided into three domains namely sociodemographic information, treatment history with imatinib and treatment status responsive or non-responsive. Blood samples and clinical data were collected from the clinic and sent to research laboratory.

Preparation of samples

Peripheral blood (2.5 ml) was collected from each participant using BD® Vacutainer Safety-Lok™ Blood Collection Set and drawn into PAXgene® Blood RNA tube (PreAnalytiX). Purification of total RNA (tRNA) including miRNA was conducted using PAXgene® Blood miRNA Kit (PreAnalytiX) with silica-membrane technology. Quantity of tRNA was measured by NanoDrop® ND-1000 spectrophotometer while quality of tRNA and small RNA were analysed by Agilent 2100 Bioanalyzer using Eukaryote Total RNA Nano assay and Small RNA assay (Agilent) for RNA Integrity Number (RIN) and amount (%) of miRNA respectively.

Next Generation Sequencing

tRNA of a CML patient responsive to imatinib therapy with RIN 7.7, a CML patient non-responsive to imatinib therapy with RIN 7.4 and a normal control with RIN 8.1 were selected for NGS. Libraries were prepared according to Illumina® TruSeq Small RNA kit (illumina®) which involved adapter ligation, reverse transcription, PCR amplification, and pooled gel purification. The kit contained adapters that are designed to directly and specifically ligate to a 5’-phosphate and a 3’-hydroxyl group of mature miRNAs. The ligated small RNA was reverse transcribed and amplified to create cDNA constructs. PCR amplification was performed with cycling conditions of 30 seconds at 98°C, 15 cycles of 10 seconds at
98°C, 30 seconds at 60°C and 15 seconds at 72°C; followed by 10 minutes at 72°C and hold at 4°C. Each sample was analysed using High Sensitivity DNA assay (Agilent) to view bands and peaks.

Purification of the amplified cDNA construct was conducted using gel. The amplified cDNA constructs with unique indices were pooled and Novex DNA Loading Dye (Invitrogen) was added. The mixture was loaded into 2 wells of 6% Novex TBE PAGE Gel, 1.0 mm (Invitrogen). The gel was run for 60 minutes at 145 V in XCell SureLock™ Mini-Cell electrophoresis unit (Invitrogen™) containing 1X TBE buffer. Immediately after electrophoresis, gel was removed from the cassette and stained with 0.5 mg/ml ethidium bromide in water for 2 to 3 minutes. Stained gel was placed on a UV transilluminator and under UV light; bands on the gel were cut out using a razor blade (in between 160 and 145 bp Ladder bands). The excised gel fragment was placed and passed through 0.5 ml Gel Breaker tube, added Ultra-Pure water, filtered to recover purified construct and concentrated by ethanol precipitation. The pellet was then resuspended in 10 mM Tris-HCl, pH 8.5 and kept at -15°C to -25°C. Library was validated using DNA 1000 assay and High Sensitivity DNA assay (Agilent) by Agilent Technologies 2100 Bioanalyzer.

The sample library was normalized to 4 nM library using 10 mM Tris-Cl pH 8.5 with 0.1% tween 20. The 4nM library was denatured using 0.2N NaOH and added pre-chilled HT1 giving a 20 pM denatured library in 1 mM NaOH; then diluted to 13 pM using pre-chilled HT1. These were prepared according to Preparing Libraries for Sequencing on the MiSeq® guide, Part # 15039740 Rev.D October 2013 (illumina®). PhiX control was later spiked-in at least 5% to the sample library. The supplied 10 nM PhiX library was diluted to 4nM with 10 mM Tris-Cl pH 8.5 with 0.1% tween 20, denatured to 20 pM then further diluted to 12.5 pM. The denatured and diluted PhiX control and sample library were combined and the libraries were dispensed into sample reservoir of reagent cartridge according to MiSeq® Reagent Kit v2 (50 cycles) Reagent Preparation Guide (illumina®) and ran on MiSeq (illumina, USA) using Standard Flow Cell with 14 tiles imaged top and bottom for 15 M reads (approximately 5 M reads per sample). Clusters were generated and sequenced by synthesis with single read. Imaging was conducted using LED and filter combinations meant for each fluorescently-labeled nucleotide. Alignments were performed using BaseSpace (illumina®). miRNAs of responsive sample and non-responsive sample were compared to miRNAs of normal control, and 18 miRNAs were selected mainly with regards to substantial expression level and clinical significance.

**Real-time reverse transcriptase quantitative PCR (RT-qPCR)**

The selected miRNAs were ordered as ready-to-use 96-well plate Custom miScript™ miRNA PCR Array (Qiagen). The array plate consisted of the 18 miRNAs, normalisation controls (SNORD61, SNORD95, SNORD96A, RNU6-2), reverse transcription control (miRTC) and positive PCR control (PPC) in the 24 x 4 format. Preparation of cDNA was conducted using miScript® II RT kit (Qiagen) and tRNA samples for a 20 μl reaction. Starting tRNA (containing miRNA) input of 250 ng was used for all samples as recommended by the kit which will result in 0.5 to 1 ng cDNA per array well. The 5x miScript HiSpec buffer facilitated conversion of mature miRNAs into cDNA while suppressed long RNAs. The reaction was incubated for 60 min at 37°C followed by 5 min at 95°C and placed on ice.
Reaction mix, consisted of miScript® SYBR® Green PCR kit (Qiagen) and template cDNA was added to the verified array plate in a 25 μl reaction mix per well. The miScript PCR system allowed sensitive and specific detection and quantification of the mature miRNA in triplicate in all case and control samples by real-time RT-qPCR using Light Cycler 480 (Roche). The cycling conditions used were initial activation for 15 min at 95°C, 45 cycles of denaturation for 15 s at 94°C followed by annealing for 30 s at 55°C and extension for 30 s at 70°C. PCR amplification was followed by dissociation curve analysis performed for 10 s at 95°C, 1 min at 65°C and a continuous 95°C.

Similar threshold settings were used to enable data comparison between sample runs whereby threshold cycle (C_T) of PPC (C_T^{PPC}) values between 19 ± 2 and C_T^{miRTC}-C_T^{PPC} values less than 7 were obtained. These indicated high quality RNA samples, correct cycling program run and correct thresholds were used; and no apparent inhibition of the reverse-transcription reaction occurred respectively. In addition, single melting peak was exhibited in all samples representing specific PCR product. The Light Cycler uses crossing point with Fit Points method while Gene Globe Data Analysis centre (Qiagen) uses C_T in which are similar. Data was formatted in Excel Spreadsheet using template provided and uploaded into the Gene Globe Data Analysis Center in a web browser (Qiagen). Normalisation was conducted using Global Threshold Cycle Mean of expressed miRNAs. Relative quantification was performed using ΔΔC_T method whereby ΔC_T = C_T^{miRNA} - C_T normalisation factor, while ΔΔC_T = ΔC_T (experimental sample) − ΔC_T (control sample). Fold change was calculated as 2^{-ΔΔC_T} whereby greater than 1 was reported as fold upregulation while less than 1 as fold downregulation. P-values were calculated based on Student’s t-test of the replicate 2^{(-ΔC_T)} values for each miRNA. Any significant miRNA observed will be linked to TKIs received and clinical outcomes of patients.

Results

Demographic characteristics of participants were generally representative of local population. Majority of the respondents were Malays, followed by Chinese and Indians. There were more males than females in this study and male outnumbered female in the control group. All patients were diagnosed with CML whereby BCR-ABL1 transcripts, the hallmark of CML were present. In responsive patients, BCR-ABL1 ratios were observed declining with imatinib therapy. Common chromosomal abnormalities usually seen with CML were observed such as trisomy 8 which was seen in both responsive and non-responsive patients. Loss of chromosome Y, an age related chromosomal abnormality was seen in few older male patients with Ph negative (Ph-ve). In the Malays, the number of responsive and non-responsive patients was similar. The numbers of Chinese and Indians participants were small for comparison (Table 1).

Table 1 Demographic characteristic of CML patients responsive and non-responsive to imatinib therapy and blood donors
In responsive patients, MMR was observed in 53% of patients comprising of 7 Malays, 6 Chinese and 3 Indians. DMR was observed in the remaining 47% of 6 Malays, 6 Chinese and 2 Indians in which 40% were with 4-log reduction (MR$^4$) and 7% with 4.5-log reduction (MR$^{4.5}$) from the IRIS baseline. Longer duration on imatinib of around 6 years (mean 71.64 ± 34.50 months) was observed in DMR patients compared to around 5.5 years (mean 66 ± 30.43 months) in MMR patients. Overall, mean duration on imatinib was 5.7 years with 53% were on imatinib for more than 5 years (Table 2). In non-responsive patients, imatinib was changed to other TKIs (82%) or other treatment options (11%) with regards to mutations in the kinase domain and according to patients’ conditions and tolerability. BCR-ABL1$^{IS}$ ratio was < 0.1% in 29% of patients and the average of latest available BCR-ABL1$^{IS}$ ratio was 13.7%.

**Table 2** Clinical findings of CML patients responsive to imatinib at molecular response
| No | Age (year) | Duration on imatinib (month) | IRIS molecular response (BCR-ABL1 IS*) |
|----|------------|------------------------------|---------------------------------------|
| 1-9| 41-65      | 32-131                       | MR4                                   |
| 10-12| 42-71    | 20-94                        | (undetectable with 10,000-31,999 ABL1 transcripts) |
| 13-14| 32-54     | 24-50                        | MR4                                   |
| 15-30| 25-75      | 26-122                       | (<0.01% BCR-ABL1 IS) MR 4.5           |
|     | 25-75**    | 20-131**                     | (undetectable with 32,000-99,999 ABL1 transcripts) MMR |
|     | (47.87 ± 13.23)*** | 68.63 ± 31.95)** | (<0.1% or >3-log reduction) |

*International Scale, **Range, *** mean ± sd

tRNA samples of normal control with RIN 8.1 and 13% small RNA, responsive patient with RIN 7.7 and 14% small RNA, and non-responsive patient with RIN 7.4 and 21% small RNA, produced high yield of amplified cDNA constructs. The amplified cDNA constructs were pooled as a small RNA library, gel purified and showed the 147 nt band (approximately 22 nt small RNA fragments) and 157 nt band (approximately 30 nt RNA fragments). The purified small RNA library showed peaks at around 150 bp with DNA 1000 chip and 152 bp including molarity of 1495 with High Sensitivity DNA chip which were suitable for cluster generation.

Profiles of miRNA expression by NGS showed similar amount of mature miRNA was present in all samples with 40.8% in normal control, 40.9% in responsive sample and 40.3% in non-responsive sample. A list of miRNAs with various expression levels was generated from each sample and compared to of normal control. Among these, hsa-miR-181-5p was shown expressed 0.45 and 0.51 times in responsive and non-responsive samples respectively.

These were consistent with real-time RT-qPCR data whereby significant down-regulations of hsa-miR-181a-5p were observed in both responsive (Group 1) and non-responsive (Group 2) groups when compared to control group (Figure 1 and 2 respectively). Fold change of hsa-miR-181a-5p was 0.47 (95% CI= 0.36, 0.58, p-value < 0.05) in responsive group (Figure 3) and 0.43 (95% CI= 0.31, 0.55, p-value < 0.05) in non-responsive group (Figure 4) which corresponded to 2.14 and 2.33 fold down-regulation respectively compared to control group.
In addition, another 2 miRNAs were also significant but only in the non-responsive group. These were hsa-miR-182-5p with fold change of 2.08 (95% CI= 1.60, 2.57, p-value < 0.05) and hsa-miR-26a-5p with fold change of 2.39 (95% CI= 1.82, 2.96, p-value < 0.05) as shown in Figure 4 which corresponded to 2.08 and 2.39 fold up-regulation respectively when compared to the control group.

In the responsive group, a reference gene, RNU6-2 is a small non-coding RNA was also significant with fold change of 0.48 (95% CI= 0.37, 0.58, p-value < 0.05) which corresponded to 2.10 fold down-regulation when compared to the control group (Figure 3).

**Discussion**

We looked at our CML patients with regards to imatinib therapy, a front-line treatment for CML in our government hospitals in view of treatment discontinuation for optimal CML responders as suggested by the European LeukemiaNet 2013. Data showed that half of our responsive patients received imatinib therapy for more than 5 years with mean age of 48 years old, average of 5.5 years for patients with MMR and 6 years for DMR. These indicated on average, MMR achieved by 18 months extended survival for at least 4 years and DMR is achievable with longer treatment in our CML patients. DMR was observed in almost half of the responsive patients and if consistent these patients can be considered for imatinib discontinuation studies, as minimum requirements are at least 24 months of DMR with MR\(^4\) or deeper [26]. DMR was reported suitable for imatinib discontinuation as it has survival benefits and lesser events [7]. However, determining the right time for imatinib discontinuation is critical. Long term TFR is still low, with relapse after discontinuation is rather high in remission patients. Weighted mean molecular relapse rate of CML was 42% with mean molecular remission was more than 37 months before discontinuation of imatinib. Shorter duration on imatinib and shorter duration with undetectable level of BCR-ABL transcripts were pointed out as reasons for relapse [27]. Therefore sufficient duration on imatinib and consistency in DMR is crucial before imatinib discontinuation.

Management of CML has remarkably improved over the years with high success rate for treatment using TKIs in chronic patients. This is assisted by new analysis techniques complementing existing ones for a comprehensive detection and monitoring of CML. RT-qPCR, a sensitive technique has become a gold standard at molecular response and is used to analyse treatment response especially from TKIs as first line. Since RT-qPCR has limitations at very low-levels thus new biomarkers are needed not only to assist analysis but also to understand better molecular and cellular interactions and associations in order to prolong TFR.

Numerous novel findings were reported from CML studies using NGS, a new technology with relatively high sensitivity and wide coverage. In CML patients treated with imatinib, NGS has enabled identification of novel BCR-ABL1 fusions gene comprising BCR intron 14 and ABL1 intron 2 breakpoints, giving partial deletion of SH3 domain [28]. Meanwhile with whole genome sequencing, NGS has enabled identification of e13a2-like BCR-ABL1 fusion comprising broken BCR exon 13 and 9 ABL1 intron 1 nucleotides forming a novel chimeric exon [29]. In our study, NGS has assisted in identifying miRNAs at molecular response
in local CML patients responsive and non-responsive to imatinib therapy. Validation by custom array real-time RT-qPCR has identified hsa-miR 181-5p, hsa-miR-182-5p and hsa-miR-26a-5p as potential new therapeutic biomarkers in response to treatment using TKIs in CML. Hsa-miR 181-5p could be a tumour suppressor miRNA and could be involved in major or partial suppression of CML. Meanwhile hsa-miR-182-5p and hsa-miR-26a-5p could be oncomiRs in which their expressions may indicate non-responsiveness to TKIs treatment or relapse.

Hsa-miR-181a-5p was significantly down-regulated in both the responsive and non-responsive to imatinib groups in comparison with the control group. The down-regulation indicated significant interactions between hsa-miR-181a-5p and TKI therapies. These showed that imatinib, nilotinib, bosutinib and dasatinib taken by CML patients over the time had caused inhibition to CML cell proliferation, led to apoptosis thus reduced the amount of BCR-ABL1 in CML patients. Fold down-regulation of 2.14 observed in the responsive to imatinib group represented 3 to 4.5 log reduction of BCR-ABL1 levels from IRIS standardized baseline with average 5.7 years of intake. Similarly 2.3 fold down-regulation observed in the non-responsive to imatinib group represented less than 1 to 5 log reductions except in 6 (21%) patients in which were either on TKI break or imatinib. The log reduction to MMR and DMR in the imatinib responsive group was consistent with down-regulation of hsa-miR-181a-5p. Various log reduction seen in the non-responsive to imatinib group was also consistent with down-regulation of hsa-miR-181a-5p. Thus indicated significant down-regulation of hsa-miR-181a-5p has positive correlation to log reduction or reduced levels of BCR-ABL1 in CML patients treated with TKIs. Therefore hsa-miR-181-5p has potential to be used as a therapeutic biomarker for positive performance of TKIs shown by reduced levels of BCR-ABL1 indicating patients were responding to TKIs treatment in CML. Hsa-miR-181-5p could be a tumor-suppressive miRNA which prevents mRNA from coding specific protein directly or associated to CML in patients treated with TKIs.

On the other hand, hsa-miR-182-5p and hsa-miR-26a-5p were observed significantly up-regulated and only in the non-responsive to imatinib group with 2.08 and 2.39 fold up-regulation respectively in comparison with healthy donors. These were patients whereby their latest available BCR-ABL1 IS ratios were mostly more than 0.1 % that were neither in MMR nor DMR, with substantial average and only 7% (2) were treated with imatinib. Fold regulation of these miRNAs in the imatinib responsive group, whereby BCR-ABL1 in these patients were minimal or absent (MMR or DMR), were not significant in comparison with healthy donors. Thus indicated imatinib had reduced the amount of BCR-ABL1 in these responsive patients to the extent not significant in comparison with healthy donors. Thus the significant up-regulation could indicate that hsa-miR-182-5p and hsa-miR-26a-5p have positive correlation to substantial presence of BCR-ABL1 in CML patients treated with TKIs. Therefore, these miRNAs could be used as new therapeutic biomarkers for substantial presence of BCR-ABL1 in CML patients treated with TKIs. Hsa-miR-182-5p and hsa-miR-26a-5p could be oncogenic miRNAs (oncomiRNAs) and could be associated to CML in patients treated with TKIs.

Studies have shown hsa-miR-181-5p as a therapeutic biomarker, has potential use for clinical improvement in cancers. This is demonstrated in gastric cancer cells whereby hsa-miR-181-5p has been
extensively studied and was reported as a potential regulator of MEG2, a tumor suppressor gene [30], regulated RASSF6 and in combination predicts poor prognosis in gastric cancer [31]. It also inhibited MTMR3 expression in AGS gastric cancer cells and was identified as a novel autophagy [32]. In non-small-cell-lung cancer tissues and cell lines, hsa-miR-181a-5p was reported significantly reduced and has potential role in tumor suppression by partially targeting Kras [33], a protein that regulates cell growth. In breast (BC) and colon cancers (CC), hsa-miR-181a-5p was reportedly down-regulated and inversely related to matrix metalloproteinase-14, which is elevated in tumors, in which, in order to prevent cancer metastasis in BC and CC, is by elevating hsa-miR-181a-5p [34]. In hepatocellular carcinoma, miR-181 was reported significantly turned on the MAPK/JNK pathway, the regulator of cell proliferation and by limiting it, would suppress the pathway [35]. Hsa-miR-181a-5p, a tumor suppressor is considered as prognostic marker in Acute Myeloid Leukaemia patients treated with intensive induction chemotherapy and autologous stem cell transplant [36].

Higher expression of hsa-miR-182-5p as observed in our study was also seen in other studies as in prostate cancer (PCa), lung squamous cell carcinoma tissues analysed from The Cancer Genome Atlas database, the Gene Expression Omnibus database, and real-time qPCR [37] and also in colorectal cancer cells using tumorigenic variant cell line MICOL-14\textsuperscript{tum} compared to MICOL-14\textsuperscript{h-tert} cells [38]. In PCa, biphasic role of hsa-miR-182-5p was observed which was higher expression in localized PCa and contrarily lower expression in aggressive tumor [39]. Lower expression was also observed in renal cancer whereby hsa-miR-182-5p was downregulated in tumor tissue compared with adjacent normal tissues and overexpression decreased tumor growth in mice, demonstrating antitumor effect [40].

Hsa-miR-26a-5p expression in our study is consistent with a larger study on CML patients indicating miR-26a expressions are influenced by response to TKIs used [41,42]. The study showed miR-26a was differentially expressed in HL-60.BCR-ABL cells treated with tyrosine kinase inhibitors when compared to HL-60 cells. miR-26a expression levels increased gradually with both dasatinib and nilotinib treatments. The increased expression levels with dasatinib and nolitinib were consistent with our findings of hsa-miR-26a expression was upregulated in CML patients molecularly non responsive to imatinib in comparison with controls whereby most patients were treated with nilotinib or dasatinib. Thus with dasatinib and nilotinib treatments, upregulation of hsa-miR-26a was observed in CML patients non-responsiveness to imatinib. However, HL-60.BCR-ABL cells treated with imatinib mesylate showed increased miR-26a expression at 4 hour but decreased expression at 8 hour. This correlates with miR-26a expression was lower in chronic phase patients (62.5% Complete Cytogenetic Response (CCyR) and 37.5% imatinib resistant) in comparison to healthy individuals. Moreover, miR-26a expression was observed lower in imatinib resistant patients compared to patients who achieved CCyR (<1% BCR-ABL1). In our study hsa-miR-26a-5p expression was insignificant in CML patients molecularly responsive to imatinib when compared to controls as these were patients either in MMR or DMR (\leq 0.1% to undetectable BCR-ABL1). These showed that with imatinib, decreasing expressions of hsa-miR-26a were observed from responsiveness towards resistance. Thus these indicated imatinib, nilotinib and dasatinib have altered BCR-ABL kinase activity differently.
These studies showed hsa-miR-181-5p, hsa-miR-26a-5p and hsa-miR-26a-5p are promising therapeutic biomarkers and can be manipulated to improve cancer treatment. In this study, only 18 miRNAs from NGS profiles were validated using real time RT-qPCR thus the significant miRNAs observed could be solely or partially involved in the decreasing and/or increasing of BCR-ABL1 in CML patients. Therefore further studies are needed to determine their targets and effect of involvements in CML. Knowing their interactions and targets in CML will give their net effects that allow manipulation by inhibit oncomiRs or stimulate tumor suppressor miRNAs. These will give new values for TKIs responses in which could assist in determining accurate time for TKIs discontinuation and better prediction of relapse in CML. Timely discontinuation of TKIs in CML remission patients is crucial for long-term TFR, and prolong TFR would subsequently cure CML.

**Conclusions**

In our CML adult patients, hsa-miR-181-5p has shown potential as a therapeutic biomarker for positive performance of TKIs shown by reduced levels of BCR-ABL1 indicating adult CML patients were responding to TKIs treatment at molecular response and could be a tumor-suppressive miRNA. On the other hand, hsa-miR-182-5p and hsa-miR-26a-5p have potential as therapeutic biomarkers for substantial presence of BCR-ABL1 in CML adult patients treated with TKIs at molecular response and could be oncomiRNAs.

**Abbreviations**

TKI: Tyrosine Kinase Inhibitor; CML: Chronic Myeloid Leukaemia; TFR: Treatment free remission; NGS: Next Generation Sequencing; RT-qPCR: Real-time reverse transcriptase quantitative PCR; miRNA: MicroRNA; Ph: Philadelphia chromosome; IRIS: International Randomized Study of Interferon and STI571; IS: International Scale; DMR: Deep Molecular Response; CCyR: Complete Cytogenetic Response; LoD: Limit of Detection; tRNA: total RNA; RIN: RNA Integrity Number; PPC: Positive PCR Control; miRTC: Reverse Transcription Control; C_T: Threshold Cycle; BC: Breast Cancer; CC: Colon Cancer; PCa: Prostate Cancer.

**Declaration**

**Ethics approval and consent to participate**

All procedures performed involving human participants were approved by the Medical Research and Ethics Committee (Ministry of Health Malaysia, P12-837), 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. Written informed consent was obtained from all the respondents in the study prior to blood collection.

**Consent for publication**
Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study will be available upon request with the approval by the Ministry of Health, Malaysia.

Competing Interests

The authors declare that they have no competing interests.

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Authors’ contributions

AMY actively involved in all aspects of the study except for clinical selection of patients, epidemiology and biostatistics analysis and was a major contributor in writing the manuscript, NAM involved in epidemiology and biostatistics research designed and critically reviewed the manuscript, CKM selected suitable CML patients for the study, HAH contributed in NGS preparation and reviewed the manuscript, YMY reviewed hematopathology analysis of the manuscript and LI reviewed molecular analysis of the manuscript. All authors read and approved the final manuscript.

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