RESEARCH ARTICLE

Low Level of TERC Gene Amplification between Chronic Myeloid Leukaemia Patients Resistant and Respond to Imatinib Mesylate Treatment

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Abstract

The amplification of telomerase component (TERC) gene could play an important role in generation and treatment of haematological malignancies. This present study was aimed to investigate copy number amplification status of TERC gene in chronic myeloid leukaemia (CML) patients who were being treated with imatinib mesylate (IM). Genomic DNA was extracted from peripheral blood of CML-IM Resistant (n=63), CML-IM Respond (n=63) and healthy individuals (n=30). TERC gene copy number predicted (CNP) and copy number calculated (CNC) were determined based on Taqman® Copy Number Assay. Fluorescence in situ hybridization (FISH) analysis was performed to confirm the normal signal pattern in C4 (calibrator) for TERC gene. Nine of CML patients showed TERC gene amplification (CNP=3), others had 2 CNP. A total of 17 CML patients expressed CNC>2.31 and the rest had 2.31>CNC>1.5. TERC gene CNC value in healthy individuals was 2 and their CNC value showed in range 1.59-2.31. The average CNC TERC gene copy number was 2.07, 1.99 and 1.94 in CML-IM Resistant patients, CML-IM Respond and healthy groups, respectively. No significant difference of TERC gene amplification observed between CML-IM Resistant and CML-IM Respond patients. Low levels of TERC gene amplification might not have a huge impact in haematological disorders especially in terms of resistance towards IM treatment.

Keywords: Telomerase RNA component - gene amplification - CML - imatinib mesylate resistance

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Introduction

Chronic myeloid leukaemia (CML) is a myeloproliferative disorder characterized by increased growth of myeloid cells in bone marrow and accumulation of these cells in blood. Genetic abnormality in CML is the presence of Philadelphia (Ph) chromosome, the event of reciprocal translocation between chromosomes 9 and 22 that result in formation of BCR-ABL fusion gene (Goldman, 2008). Imatinib mesylate (IM: Novartis formerly CGP57148B, STI- 571, now Imatinib mesylate; Gleevec® or Glivec®, Novartis, Basel, Switzerland) was the first tyrosine kinase inhibitors getting an approval from US Food and Drug Administration (FDA) for CML treatment. It is an ideal drug for first-line therapy to treat CML diseases (Gugliotta et al., 2011). However, there was an observation of drug failure or resistant to IM during treatment and at least 1/3 of patients did not achieved good outcome (Cortes et al., 2011). Although it was reported that half of the CML patients who were resistant towards IM treatment are due to the BCR-ABL point mutations or BCR-ABL gene amplification (through BCR-ABL dependence mechanisms), the other half of the CML resistant patients involved in BCR-ABL independence mechanisms. Examples of BCR-ABL independence mechanisms of IM resistant includes clonal evolution, steroid receptor co-activator (SRC) overexpression, intracellular uptake of IM, pharmacokinetic consideration, CML stem cell quiescence and other pathway which is still not being elaborate yet (Quintás-Cardama et al., 2009).

Telomerase is a ribonucleoprotein enzyme aids in synthesizing telomere DNA by adding repetition DNA sequence (TTAGGG) onto the 3’ end of telomere DNA chromosome. Telomerase is an enzyme that responsible for maintaining telomere length and provides a part of contribution to the telomerases stability (Masutomi et al., 2003). Human telomerase relies on its two core components - functional telomerase RNA component (encoded by TERC gene), telomerase reverse transcriptase (encoded by TERT gene), and its protein subunit together

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with other twenty telomerase associated proteins (Podlevsky et al., 2008).

Telomerase activity is well known to express more than 85-90% in immortal cell lines and human cancer cells (Kim et al., 1994; Shay and Bacchetti, 1997), yet in most human tissues it is either totally absent or undetectable (Wright et al., 1996). Activation and up regulation of telomerase activity play important roles in the development of many cancers, strengthen the facts that telomerase is a relevant and possible target that might involves in the BCR-ABL independence mechanisms. The correlation of shorter survival of CML patients and increased activity of telomerase speculated that telomerase has a potential effect.

IM treatment required for BCR-ABL reactivation (Ohyashiki et al., 2002). A study done by Bakalova et al., (2003) found that the decreased amount of BCR-ABL in K562 leukemic cell line after IM treatment evoked telomerase activation as BCR-ABL interfered and altered the telomeric association proteins. Other study conducted by Yamada et al., (2008) found that the K562 with hTERT cells demonstrated a resistance to IM through telomerase activation. They suggest that the K562-hTERT cells may partially escape from IM which induced apoptosis of leukemic cells via increased its telomerase activity.

Amplification of the TERC gene may cause high activity in telomerase activity and enhance the progression of human cancers. TERC gene is in the band 3q26.3 of human chromosomes 3 and present ubiquitously in cells as it serves as template for telomeres synthesis (Cong et al., 2002). The chromosomal region of TERC gene is the frequent region being amplified in human cancers and it changed in term of copy number (gain or amplify) (Cao et al., 2008). Recent studies also reported that TERC gene amplification were found in many cases of the human cancers such as in cervical cancer, esophageal cancer and laryngeal squamous cell carcinoma (Kokalj et al., 2009; Chen et al., 2011; Liu et al., 2012). Inhibition may lead to enhanced sensitivity to treatment (Chen and Xing, 2012).

Until now, increased of telomerase activity in clinical samples from CML via amplification of TERC gene has not been well documented. There also has been limited report on gene abnormality or gene aberration to associate the correlation of telomerase essential genes especially amplification of TERC and involvement of TERC and its interaction with IM treatment in CML. Thus, this present study focuses on the TERC gene amplification status in CML patients and healthy individuals as normal controls were obtained as same procedure as patients involved.

**Genomic DNA extraction**

The genomic DNA (gDNA) from CML patients and healthy individuals were extracted using a commercial QIAamp DNA Blood kit (QIAGEN, Germany). Purity and yields of the gDNA were determined spectrophotometrically at 260 nm to 280 nm using a NanoDrop® ND-100 spectrophotometer (Thermo Scientific, USA). The gDNAs were ensured to have an absorbance 260 nm/280 nm ratio greater than 1.7. One μl of each gDNAs were then analyzed on 1% of agarose gel to ensure its integrity. All gDNAs concentration were equalized to 5 ng/μl diluted in nuclease-free-water (Ambion, Life Technologies, Canada) and stored at -20°C until used.

**TERC copy number analysis using taqman® qPCR assay**

TERC gene in CML patients and healthy individuals were analysed using custom Taqman® copy number quantitative real-time polymerase reaction chain (qPCR) assay. The ID assay of the target gene was selected from GeneAssist™ Copy Number Assay Workflow Builder (Hs03458156_cn, a use of FAM reporter and non-fluorescence quencher probe; Applied Biosystems, Foster City, CA, USA), together with the TaqMan® Copy Number Reference Assay RNase P, a use of VIC reporter and TAMRA quencher probe (Applied Biosystems, Foster City, CA, USA) as reference gene. Each single reaction with a total volume of 20 μl consisted of a 20 ng gDNA of CML patients or healthy individuals, and no-template control (NTC). A duplex reaction was utilized in this assay between the target gene and the reference gene. For each well contained the gDNAs or the NTC, a Taqman® Copy Number Assay comprised of the forward and reverse primer specifically for the target gene and also the minor groove binder (MGB) probe labelled with the FAM dye carry out synchronously with the Taqman® Copy Number Reference Assay in which it also consisted of specific forward and reverse primer for RNase P and a VIC probe labelled with the TAMRA dye. The assays were run accordingly to the manufacturer’s protocol. The
qPCR was performed in 96 well plates using the ABI PRISM 7500 Real Time PCR Sequence Detection System (SDS) v1.3.1 (AppliedBiosystems, UK) with utilization of absolute quantitation settings. The thermal cycling of the qPCR conditions were 2 minutes at 50°C and 10 minutes at 95°C, 15 seconds at 95°C for 40 cycle followed by 1 minute at 60°C. All of the CML patient samples, healthy individual samples and NTC were done in four replicates.

The raw quantification cycle (Cq) values collected by SDS were analysed for the copy number calculation with the assistance of the Applied Biosystems CopyCaller™ Software v2.0. The analysis settings used the copy number experiment data with the selected calibrator sample to ensure the consistency of the calculated copy number in each samples. The results were exported into the software and the copy numbers were calculated by using a comparative Cq relative quantification method. The results of the samples were only acceptable if the value of confidence interval were >80%, the standard deviation of ΔCq were <0.20, the RNase P Cq was <32 (Graf et al., 2012) and the z-score calculation were <2.65. Results for the samples with gene amplification were considered acceptable even though the confidence interval were <80% with the value of its standard deviation of ΔCq were <0.20 and the z-score value were <2.65. Meanwhile, the qPCR amplification curves were standardized based on two different criteria: 1) autobaseline and 2) 0.2 Cq threshold as recommended by manufacturer.

The results of this assay was determined by examining both copy number predicted (CNP) and also copy number calculated (CNC) reviewed by the CopyCaller™ Software. TERC gene was considered to have amplification if the CNP were more than 2 copies (>2) and CNC were above the normal threshold level.

**Fluorescence in situ hybridization (FISH)**

One of the healthy individual was selected to be the calibrator in Taqman® Copy Number Assay. Verification of the calibrator whether it had normal quantity of TERC gene copy number was performed by using FISH technique to confirm the normal copy number of TERC gene.

**Isolation of mononuclear cells:** The mononuclear cells from the selected calibrator were prepared by using cytospin method for later used in FISH. Freshly collected peripheral blood was mixed and diluted with the phosphate buffer saline (PBS, Sigma Aldrich, UK) in 1:1 ratio. The cells were then isolated by density-gradient centrifugation at 1400 g for 15 minutes with the use of Lymphoprep solution (AXIS-SHIELD PoC AS, Oslo, Norway). The separated layers of the cells were harvested by Pasteur pipette and the collected cells were directly used for cytospin slide preparation.

**FISH Analysis:** FISH was performed on the slide prepared by cytospin technique. The sample was span at 450 r.p.m for 5 minutes using Shandon cytospin® 4 cytocentrifuge (Thermo Scientific, US). The slide was denatured at 75°C for 10 minutes in humidified HYBrite machine (Vysis, USA) after POSEIDON™ hTERC (3q26)/3q11 probe FISH kit (Kreatech Diagnostics, The Netherlands) was applied onto the area containing cell and subsequently hybridized for 20 hours at 37°C on the same hot plate chamber. The hybridized slide was then washed using washing buffer (2X SSC/0.3% Tween 20, Sigma Aldrich, UK) at 73°C +/- 1°C for 2 minutes and re-washed in 0.4X SSC/0.1% Tween 20 (Sigma Aldrich, UK) at room temperature for 1 minute. Slide was allowed to air dry in darkness for 15 minutes just after the slide was soaked into gradually increased concentration of ethanol, each for 1 minute (75%, 85%, 100%). 5 μl of 4,6-diamidino-2-phenylindole (DAPI) Counterstain (Kreatech Diagnostics, The Netherlands) was dropped onto the slides, a glass coverslip was applied and it was sealed with fixogum. Slide was viewed using either single or multiple bandpass filter under Olympus BX51 fluorescent microscope with cytovision software version 4.5.1 Build 5 (Olympus, USA). The FISH signal for TERC gene scored for the number of red (3q26) TERC locus and green (3q11) control signals. The TERC (3q26) probe was designed as a dual- colour assay to detect amplification at 3q. Two single colour red (R) and green (G) signals (2R2G) were identified as the normal copy number amplification of TERC gene.

**Statistical analysis**

GraphPad Software Prism® version 6 was used to compare the difference or the similarity in the distribution pattern of the CNC value TERC gene copy number status between groups. Mann-Whitney test was used to determine the differences in CNC of TERC gene between CML-IM Resistant patients and CML-IM Respond patients. The test was conducted by Statistical Package for Social Sciences (SPSS) version 18.0 software with P values as two-tailed.

**Results**

**FISH Results for Calibrator**

FISH analysis for the C4 indicated that this selected healthy individual was suitable to employ C4 as calibrator for Taqman® copy number qPCR assay since it showed 2 signals of red and 2 signals of green in more than 200 interphase nuclei (1:1 ratio). It was considered as trustable as it did not show any TERC gene amplification (Figure 1).

**TERC gene amplification status**

A total of 126 samples from CML patients and 30 samples from healthy individuals were evaluated. The copy number predicted (CNP) and copy number calculated (CNC) were determined. Out of the CML-IM Resistant (n=63), 5 patients showed TERC gene amplification (CNP=3) and the remaining 58 patients had CNC value of 2. Whereas, 4 CML-IM Respond patient (n=63) showed TERC gene amplification (CNP=3) and the remaining 59 patients exhibited CNC value of 2. Meanwhile, the CNC of TERC gene in all healthy individuals was not exceeded than 2. Based on the CNC of healthy individuals, value of 2.31 was considered as a normal threshold level of the normal range TERC gene without having gene amplification (Figure 2). According to this figure, 52 CML-IM Resistant patients and 57 CML-IM Respond...
patients were demonstrated had normal level of TERC gene which their CNC values were below than 2.31. A total of 11 CML-IM Resistant patients and 6 CML-IM Respond patients had more than 2.31 TERC CNC value suggested TERC gene amplification. All CNC values of healthy individuals DNA samples were fell below 2.31.

**Statistical evaluation of TERC gene amplification**

Comparing of the mean CNC TERC gene between CML-IM Resistant patients (mean=2.07) and CML-IM Respond patients (mean=1.99) to the healthy individuals (mean=1.94), there was a slightly increased of CNC value in CML-IM Resistant patients compared to CML-IM Respond patients and healthy individuals (Table 1 and Figure 3). The median CNC of TERC gene between CML-IM Resistant patients and CML-IM Respond patients were evaluated and compared to each other. There was no significant difference of the median CNC value between CML groups (p=0.148). The median CNC value of TERC gene among CML groups was closely similar, with CML-IM Resistant (2.06, interquartile range 0.34) while CML-IM Respond (1.98, interquartile range 0.36) (Table 2).

### Table 1. Mean of CNC TERC Gene copy Number, Range of CNC TERC Gene and Standard Deviation in the Healthy Individuals, CML-IM Respond Patients and CML-IM Resistant Patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Mean CNC TERC Gene</th>
<th>CNC Range</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Individuals</td>
<td>1.94</td>
<td>1.59-2.31</td>
<td>0.17</td>
</tr>
<tr>
<td>CML-IM Respond</td>
<td>1.99</td>
<td>1.57-2.69</td>
<td>0.29</td>
</tr>
<tr>
<td>CML-IM Resistant</td>
<td>2.07</td>
<td>1.68-2.75</td>
<td>0.29</td>
</tr>
</tbody>
</table>

### Table 2. Comparison of Median CNC TERC Gene between CML-IM Resistant Patients and CML-IM Respond Patients

<table>
<thead>
<tr>
<th>TERC Gene Copy No.</th>
<th>CML-IM Resistant</th>
<th>CML-IM Respond</th>
<th>Z stat*</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.06</td>
<td>1.98</td>
<td>-1.45</td>
<td>0.148</td>
<td></td>
</tr>
</tbody>
</table>

*Mann-Whitney test was applied, it is significant when p value < 0.05.
Discussion

Imatinib mesylate (IM), a tyrosine kinase inhibitor belongs to the 2-phenylaminopyrimidine group is a well-known standard drug in treatment of CML. IM is the recommended initial therapy for majority of CML patients diagnosed with chronic phase and used in minority diagnosed in advanced phase (accelerated and blast phase) (Brecia et al., 2010). However, resistance to IM treatment observed in some patients in chronic phase point that all patients do not respond to the same degree of the treatment. Bakalova et al., (2003) suggested the development of resistant towards IM treatment among CML patients potentially due to the implications of telomerase activation.

The increment of telomerase activity nearly 90% in variety types of human cancers (Kim et al., 1994) propose that normal somatic cells have regulator(s) to suppress the telomerase regulation activity, yet the regulator(s) is dysfunctional during cell immortalization (Kyo et al., 2008). Since telomerase activity is being regulated by expression of its two main core components, TERC and TERT genes, clarification of control mechanisms for both genes is crucial in understanding telomerase activation in human cancers. Investigation of telomerase component’s amplification might be useful in understanding the molecular mechanisms involved especially in resistant to the IM treatment. Cao et al. (2008) reviewed some evidences that increased copy number of gene or gene amplification of telomerase core components may give a possible answer to the telomerase up-regulation.

Here, in our study, we examined amplification of TERC gene. Previous study done by Nowak et al., (2006) showed that the different groups of leukaemic cells possess TERC gene amplification ranging from 2 to 12 copies and proposed that this amplification was related to the high expression of TERC gene and high telomerase activity in leukaemic cells. However, in this study we only found 5 CML-IM Resistant and 4 CML-IM Respond patients (based on their copy number predicted) and 11 CML-IM Resistant and 6 CML-IM Respond patients (based on their copy number calculated) exhibited TERC gene amplification with low level of TERC gained. We found out that the mean copy number calculated of TERC gene between CML-IM Resistant patients and CML-IM Respond patients and from those from healthy individuals were apparently not so much difference although the distribution of the TERC gene copy number calculated showed that CML-IM Resistant patients a bit higher compared to other groups. When comparing the CNC TERC gene among both groups of CML patients, there is no significant difference observed and this might speculate that TERC gene amplification was not the potential cause of resistant to the IM therapy.

A report from Özer et al., (2011), they examined TERC gene amplification in the bone marrow samples from acute myeloid leukaemia patients and discovered none of the patients had TERC gene amplification. Other study done by Serakinci and Koch, (2002) also found there was no evidence of TERC gene amplification in human leukaemic cells. Our study showed almost all of study participants did not have TERC gene amplification and support the fact that haematologic disorders dissimilar from other cancers as they might originate from a cell with low-level of telomerase activity.

Several studies evaluated the value of TERC gene amplification as a prognostic marker during the development of human cancers and it was needed to discriminate the transition of the cancer phases. Heselmeyer-Haddad et al., (2005) found TERC gene was amplified from those who had cervical intraepithelial neoplasia (CIN) 3 or the invasive phase of carcinoma and that gained was required to distinguish from CIN 1 and CIN 2 phases. In other recent finding in different grouping of laryngeal lesions produced by Liu et al., (2012) they stated a significant elevated percentage of amplified TERC gene in moderate dysplasia, severe dysplasia, carcinoma in situ and invasive carcinoma compared to normal epithelium and mild dysplasia (p<0.0001). Moreover, Andersson et al., (2006) showed TERC gene amplification in cervical adenocarcinomas with absolute mean copy number ranged between 2.3 and 5.2 comparable to the mean of cervical adenocarcinomas without infection of human papillomavirus (HPV was 3.3). It has to be taken into account that all these mentioned TERC gene amplification studies were done in the solid tumours. Recurrent balanced of gene aberrations and gene rearrangements have been identified in haematological disorders, while on the contrary various genomic imbalances have been found in solid tumours (Albertson et al., 2003; Mitelman et al., 2004) hence create a distinct difference between amplification in solid tumours are due to aneuploidy and haematological disorders generally related to the structural abnormalities in which its karyotype closer to diploid. Soder et al., (1997) reported 97% increased of TERC gene copy number in cervical carcinomas and squamous cell carcinoma of the head and neck were caused by polysomy of chromosome 3 and formation of isochromosome 3q. Other than that, Liu et al., (2012) stated that chromosome aneuploidy was a common event in laryngeal lesions as they found most of the increased TERC gene copies attributed with 2:3 ratio (control signal versus TERC signal) of amplification pattern. Bignold (2006) also stated that chromosome number abnormalities are commonly found in solid tumours especially with presence of extra gene copy or known as aneuploidy. From that facts, the possible mechanism of increase TERC genes in solid tumours may cause by extra chromosomal gain due to chromosome number abnormality such as aneuploidy, polysomy or formation of isochromosome which is differ from haematological malignancies.

Thus, the present results are in concordance with the statement that increased of telomerase activity via TERC gene amplification may not be a property of haematological disorders and not practically influence the treatment in chronic myeloid leukaemia compared to the solid tumours. It is possible to conclude that TERC gene amplification may not be one of the characteristic of development of resistant towards IM treatment as the molecular mechanism of resistant may exist in different pathway.

There is possibility that when FISH technique was...
applied to detect gene amplification, there have been difficulties to detect the signal from the probes due to the overlapping of the nuclei (Gozzetti and Le Beau, 2000; Reichard et al., 2006). Application of commercialized Taqman® Copy Number qPCR Assay in this study perhaps may overcome the sensitivity limitation since qPCR ability is to detect as low as 10⁻⁴ copy number (Gabert et al., 2003). TERC gene Taqman® Copy Number Assay run as a duplex quantitative real-time polymerase chain reaction (qPCR) since it works together with the Taqman® Copy Number Reference Assay (RNase P). Copy number calculated (CNC) values were calculated using the formula: CNC of sample=relative quantification of sample X copy number of calibrator whereas copy number predicted (CNP) values were determined based on the closest integer value given by the CNC. Previous studies calculated the copy number values based on the maximum likelihood algorithm without the use of calibrator and only CNP values were published (Graf et al., 2012; Liu et al., 2012). In our study, to estimate the copy number of TERC gene amplification, we utilized a calibrator and used both results (CNC and CNP) produced from the CopyCaller™ Software. CNC gave the actual TERC gene amplification values in all samples while CNP gave the nearest integer values produced by the CNC (Figure 4). Thus, by using both results, we could compare the similarities and the differences of TERC gene values between CML groups and healthy individuals as well as differentiate the samples contained high and low copy number values. With the utilization of selected healthy individuals (C4) as a calibrator in all experiments, the CopyCaller™ software analysed and calculated copy number values based on the comparative Cq (ΔΔCq) method of relative quantification. In order to avoid false-positive results, we chose, evaluated and confirmed the TERC gene copy number from the selected calibrator by validated its TERC gene copy number using fluorescence in situ hybridization (FISH) analysis.

In this study, we used samples from peripheral blood that might be contained a mixture of normal and malignant cells, and the results also might be interfered and influenced by that possible reason. Even though we used different origin of samples which are from peripheral blood rather than bone marrow, former study revealed that there was no significant variance of telomerase activity either in bone marrow or peripheral blood samples in both leukemic control and normal cells (Gurkan et al., 2005). No proven study stated the rate of TERC gene amplification whether in peripheral blood or bone marrow samples are significantly different, thus it is assume that the level of TERC gene in both samples are closely similar.

In conclusion, to the best of our knowledge, this present study was the first written report of copy number status particularly amplification of TERC genes in CML patients who are being treated with IM. We utilized Taqman® copy number quantitative real-time polymerase reaction chain (qPCR) assay approaches to analyze TERC gene amplification status in CML which had never been reported previously. The value of TERC in CML patients with amplification of TERC gene were all showed in low level of gained and this might speculated that amplification of TERC were not a common phenomenon in CML. This present work also did not show any significant changes in TERC gene copy number in CML resistant to the IM compared to the CML respond to the IM treatment. This could consider that any TERC gene amplification in haematological disorders might have different contributions in disease development and therapy management compared to the solid tumours. Still, much intention need to understand including the regulator that control TERC gene to be amplified, mechanisms involve in increment of TERC component and its beneficial input towards cancer diagnostics and prognostics. Further investigations of TERC gene amplification study in various types of human tumours are warranted to highlight the significant of this oncogenic event in such cancers.

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