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Gene expression profile and presentation of novel gene variants of *COX7B* and *COX7C* in a cohort of patients with chronic myeloid leukemia

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ABSTRACT

Chronic myeloid leukemia (CML) is a common blood malignancy awaiting decisive cure. Understanding the genomic nature of the disease could help to improve treatment strategies and introduce novel therapeutic targets. The purpose of this study was to sequence the entire transcriptome of a cohort of CML patients to understand differences in gene expression profiles and gene variants compared to healthy controls. RNA was extracted from 10 CML and four healthy control subjects and sequenced by the DNBSEQ platform. Differential gene expression was evaluated and confirmed by the q-RT-PCR technique. Gene variants were also analyzed. The results showed that *COX7B* and *COX7C* were upregulated in CML patients than in controls; this was confirmed by quantitative PCR. In addition, novel single nucleotide and insertion/deletion variants were also found in both genes. In conclusion, the results of this study recommend further exploration of *COX7B* and *COX7C* and their novel variants in myeloid leukemogenesis.

Keywords: CML, *COX7B*, *COX7C*, RNA sequencing

INTRODUCTION

Chronic myelogenous leukemia (CML) is a multiphasic hematologic malignancy that begins with a chronic phase. This phase is followed by two phases: an accelerated phase and a progression phase. The accelerated phase is characterized by enhanced leukocytosis, while blasts count increases in the progression phase. The molecular pathophysiology of the disease involves B-cell receptor-ABL tyrosine kinase fusion (BCR-ABL). This kinase forms following chromosomal translocation t(9;22) (q34; q11.2) (Philadelphia chromosome) and is a hallmark of disease progression [1]. It was reported that autophosphorylation potential of the resulting BCR-ABL complex initiates phosphorylation of ty177 of BCR, which is known to mediate leukemogenesis [2]. The oncogenic potential of BCR-ABL occurs mainly through formation of adaptor molecules complexes, for example GRB2-BCR-ABL complex. This complex is known to recruits other downstream effectors; such as son of sevenless (SOS), and then activates cell survival pathways; such as ras-mitogen activated protein (MAP) extracellular signal-regulated kinase (ERK1/2) (MEK) and PI3K/Akt pathways that promote cell proliferation and sustains the oncogenic potential [3]. Despite that, the driving force of the BCR-ABL abnormality in CML as a single genetic factor suggests that there are molecular downstream effectors that advocate this fusion oncogenic potential. In this context, although introduction of tyrosine kinase inhibitors (TKI) showed promising results in treating CML and stops the disease progression, resistance of TKI remains a clinical concern in ~13% of all treated patients [1]. Hence, we should keep in mind that during the phases of CML progression from chronic phase till blast phase, accumulation of secondary cytogenetic abnormalities known as additional chromosomal abnormalities (ACAs) contribute to uncontrolled proliferation of an BCR-ABL-initiated myeloid progenitor [4]. Therefore, monitoring these additional cytogenetic signatures in CML patients may provide an advantage to better understanding of the disease genetic framework and open doors for successful therapeutic interventions. In this respect, response to treatment in CML patients falls into three categories: optimal, warning and failure. Otimal response means that the quality of health of patients when continue the same treatment regimen will be as good as the general population. Warning response means that patients get benefits of the treatment, but the overall outcome is not favorable, and disease might progress. The failure response means that the treatment regimen is not beneficial for patients, and they should shift to another treatment [5]. Following up response to treatment is done either by cytogenetics or quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), or both can be employed to confirm complete cytogenetic remission or major molecular remission (MMR) after treatment. Moreover, cytogenetics is crucial in cases of disease progression and/or failure or warning response to treatment [5]. Likewise, mutation analysis is recommended upon imatinib failure [6]. In this context, it was recently reported that employing single-cell sequencing to conclude expression profiles in CML patients

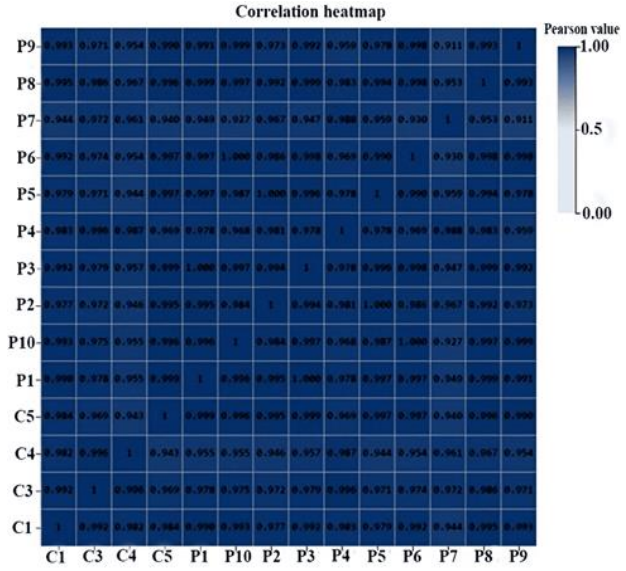

Figure 1. Correlation heatmap showing the degree of similarity of gene expression between samples (Source: RNA sequencing report)

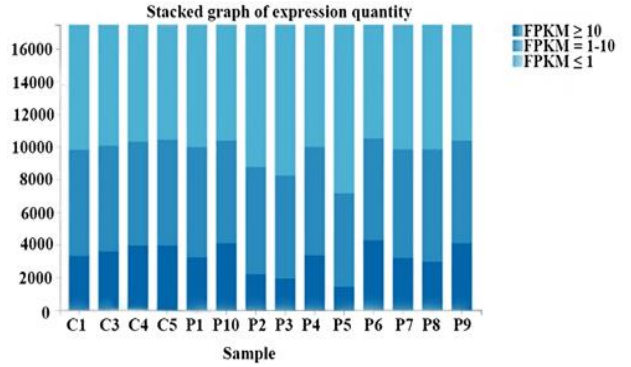

Figure 2. Gene expression quantification in the study sample (More than 16,000 genes have been detected in each sample and gene expression was expressed as fragment per kilo base million (FPKM); the darker the color, the higher the quantity of these genes) (Source: RNA sequencing report)

Table 1. Primers used in q-RT-PCR experiments

Gene	Forward primer	Reverse primer
COX7B	5'CACCAGAAACGTACACCTGA3'	5'TCCGACTTGTGTTGCTACAT3'
COX7C	5'GTAGGAGCCACTATGAGGAG3'	5'TGTTTTAAGCAGTTGGTGTC3'
GAPDH	5'AGCCACATCGCTCAGACAC3'	5'GCCCAATACGACCAAATCC3'

could be used as a diagnostic approach to predict potential responsiveness to TKIs [7]. In addition, the use of single-cell sequencing identified novel markers for TKI resistance as reported by another study [8]. In this study, we employed RNA sequencing to conclude differential gene expression profile in a small cohort of "treated" CML patients in comparison to corresponding healthy controls. In addition, we sought to analyze gene variants derived from single nucleotide polymorphism and insertion/deletion (INDEL) analysis performed on the RNA sequencing data. This could help to identify novel gene markers and unexplored gene variants in CML patients.

METHODS

Patients and Controls

Patients were recruited from hemto-oncology follow-up clinic at King Fahd University Hospital between late 2017 and 2021. 10 patients with confirmed diagnosis of CML were enrolled in the study along with four healthy controls including one female. All patients with other hematological or solid cancers and/or chronic conditions were excluded from participation. All participants signed an informed consent before enrollment.

Blood Collection and RNA Extraction

Blood samples were collected at the phlebotomy unit of the hospital into PAXgene blood RNA tubes (Preanalytix, Switzerland). Samples were mixed and equilibrated at room temperature, and then total RNA was extracted as described in the instruction manual of PAXgene Blood RNA Kit (#762164, Preanalytix, Switzerland). RNA concentration and quality were then assessed by Agilent 2100 bioanalyzer (Agilent RNA 6000 Nano kit #5067-1511, Agilent Technologies, Germany).

RNA Sequencing and Bioinformatics

Following passing quality control checks, qualified samples were depleted from ribosomal RNA to enrich mRNA, and then cDNA library was created and amplified and got prepared for DNBSEQ platform. Pearson's correlation coefficient was calculated to assess the similarity of gene expression levels between samples (**Figure 1**).

Gene expression was quantified after mapping the clean reads to the reference transcriptome sequence using Bowtie2 [9]. Gene expression level was quantified in each sample using RNA sequencing by expectation-maximization (RSEM) [10]. Differential gene expression analysis was then performed using DESeq2 method [11] to recognize the differentially expressed genes (DEGs) between the patients' group and the control group. DEGs were then clustered hierarchically using R package pheatmap. The number of genes that are expressed in each sample were classified by their expression level, which was estimated as fragment per kilo base million (FPKM), where the higher the FPKM number, the higher the expression level of genes (**Figure 2**).

Quantitative Polymerase Chain Reaction

Gene expression of the selected genes was quantified by q-RT-PCR to confirm the results of RNA sequencing. Starting with RNA, a single-step master mix (MQ one step probe qPCR mix; Cat. No. OCQ-M-001-100) was used to synthesize the cDNA in the same PCR reaction tube starting by 100 ng of RNA. Primer concentration was adjusted to 200 nM/reaction in a final reaction volume of 20 µl. The cycling program was set to reverse transcription at 45 °C for 10 minutes, initial denaturation at 95 °C for two minutes, then 45 cycles of denaturation at 95 °C for 10 seconds, annealing and elongation at 65 °C for 30 seconds, and then melting analysis. Amplification and melting curves were created.

GAPDH was used as an internal control and the gene expression level was calculated using the $2^{-\Delta\Delta CT}$ method; where $2^{-\Delta\Delta CT}$ represents the fold change level as calculated in controls and patients relative to the expression level in control subject #1 (C1). The primers used in the study were designed by SnapGene software (Dotmatics, Boston, MA, the USA) and are presented in **Table 1**.

Table 2. Fold change values of *COX7B* & *COX7C* in CML patients

Gene symbol	log_2fold change	Fold change	Q-value
COX7B	1.547469977	2.92304082	0.014882277
COX7C	1.141885959	2.20669304	0.011545679

Single Nucleotide Variant and Insertion/Deletion Analysis

Gene alignment to the reference genome (GCF_000001405.38_GRCh38.p12, homosapiens, NCBI) was done using Hierarchical Indexing for spliced alignment of transcripts (HISAT2) [12]. Duplicate reads and low quality locus were filtered and the single nucleotide variant (SNV) and INDEL information were then retrieved using GATK package, which is an efficient processing and computing data engine developed by the Broad Institute Software package for next generation sequencing data analysis. SNV and mutations were detected using haplotype caller according to [13]. Gene variants were verified at https://www.ncbi.nlm.nih.gov/dbvar/.

Statistical Analysis

Comparison between the relative gene expression levels concluded from q-RT-PCR was done using Mann-Whitney u test. Also, Spearman rank correlation was done between *COX7B* and *COX7C* gene expression levels and BCR-Abl (%) in patients using graphpad prism (version 7). Statistical analysis of RNA sequencing was done as a part of the Bioinformatics package and Q value (p-value adjusted for false discovery rate) was calculated to indicate statistically significant findings during analysis; for example as done for differential gene expression analysis.

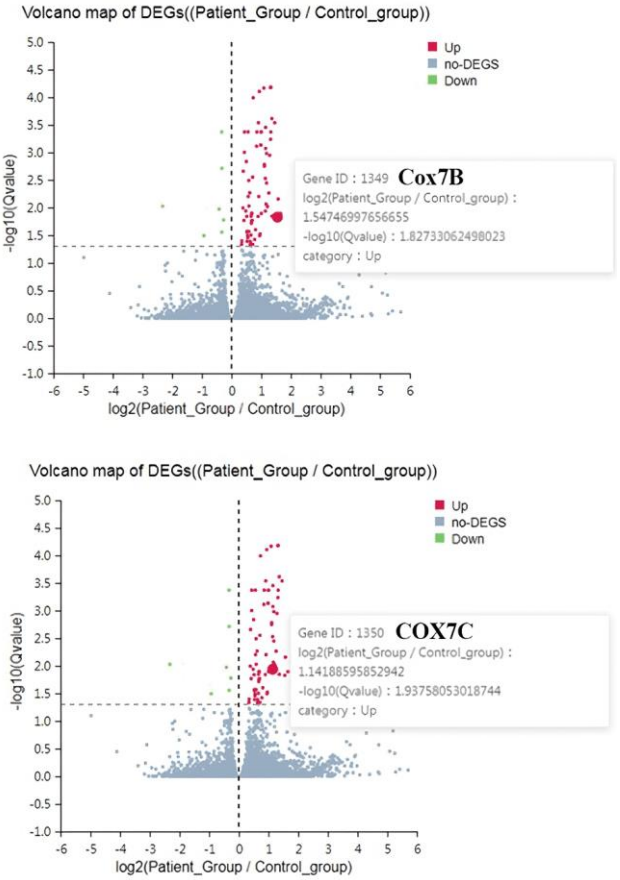

Figure 3. Differential gene expression plots showing position of *COX7B* & *COX7C* amongst the upregulated genes (Source: RNA sequencing report)

RESULTS

Patients' Demography and Characteristics

The study cohort included 10 CML patients (three females and seven males) with mean age of 55 years at the time of enrollment. Four healthy participants (one female and three males) with mean age of 39 years were recruited as the corresponding controls. The patients and the control group were Arabs and nationals of Saudi Arabia. The average duration of diagnosis for patients is 12 years. All patients were presented to the hospital with common similar symptoms including general weakness, fatigue and abdominal pain. Ultrasonography of abdomen revealed splenomegaly, which ranged from mild to severe splenomegaly. Two patients were diagnosed incidentally from complete blood count (CBC) results that were performed during general check-ups. The first white blood cell count (WBC) of each patient revealed remarkable high numbers in some patients with an average WBC of "183.477 (k/ul)" that suggested the initial diagnosis, which was then confirmed by the molecular analysis through detection of BCR-Abl (p210). All patients showed the CML characteristic translocation between chromosome 9 and 22 (Philadelphia chromosome), and some patients had low scores for leukocytes alkaline phosphatase (LAP score) when requested for them. All patients were treated with *imatinib* (200 mg once a day (QD)) as a standard treatment until major molecular remission (MMR; BCR-Abl <0.1% by international standard) is achieved. Only one patient had received *imatinib* for 12 years, where BCR-Abl was at >10% after treatment, before shifting to *nilotinib* for two years, and then prescribed *imatinib* before achieving the MMR after doubling the medicine dose to 400 mg BID. Another patient who still in an active disease stage was prescribed *nilotinib* once before changing to *imatinib*. The BCR-Abl level was reduced in this patient from 11% to 1.83% in 9/2021, but she did not achieve MMR. Notably, the mean BCR-Abl values in patients during the follow-up period ranged from 0.0005% to 5.5%.

Whole Transcriptome Sequencing

Differential gene expression analysis

The results showed that 6.9 gigabytes of bases were generated from sequencing of each sample. The average mapping to the reference genome was 89.5%. The gene mapping ratio was 80.13%, and 17,469 genes were identified. The bioinformatics revealed a differential number of 78 indicating that the number of genes that were differentially expressed, with statistically significant Q value, between controls and the patient group is 78. Amongst these 78 genes, 71 genes were upregulated, and seven genes were downregulated. In this article, we will present two upregulated genes whose fold change values were >2 (**Table 2** and **Figure 3**) and are closely related; these genes are Cytochrome C Oxidase Subunit 7B (*COX7B; gene NCBI ID: 1349)* and Cytochrome C Oxidase Subunit 7C (*COX7C; gene NCBI ID: 1350)*.

These results of RNA sequencing was further confirmed by q-RT-PCR results presented in **Table 3** and **Figure 4**. The data of q-RT-PCR of the two genes in each subject of the control and patients and the average gene expression level (fold change) in patients vs. controls. The results indicated that both genes were remarkably higher in CML patients, though the difference

Table 3. q-RT-PCR fold change values of *COX7B* & *COX7C* in CML patients vs. controls

Sample	C1	C2	C3	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
							COX7B						
Fold change relative to C1	1	2.938	9.882	8.631	6.223	2.568	3.421	32.392	14.821	17.439	32.986	36.449	44.422
							COX7C						
	1	4.391	7.784	7.069	4.999	2.075	2.767	17.299	6.706	12.046	19.344	19.564	30.821

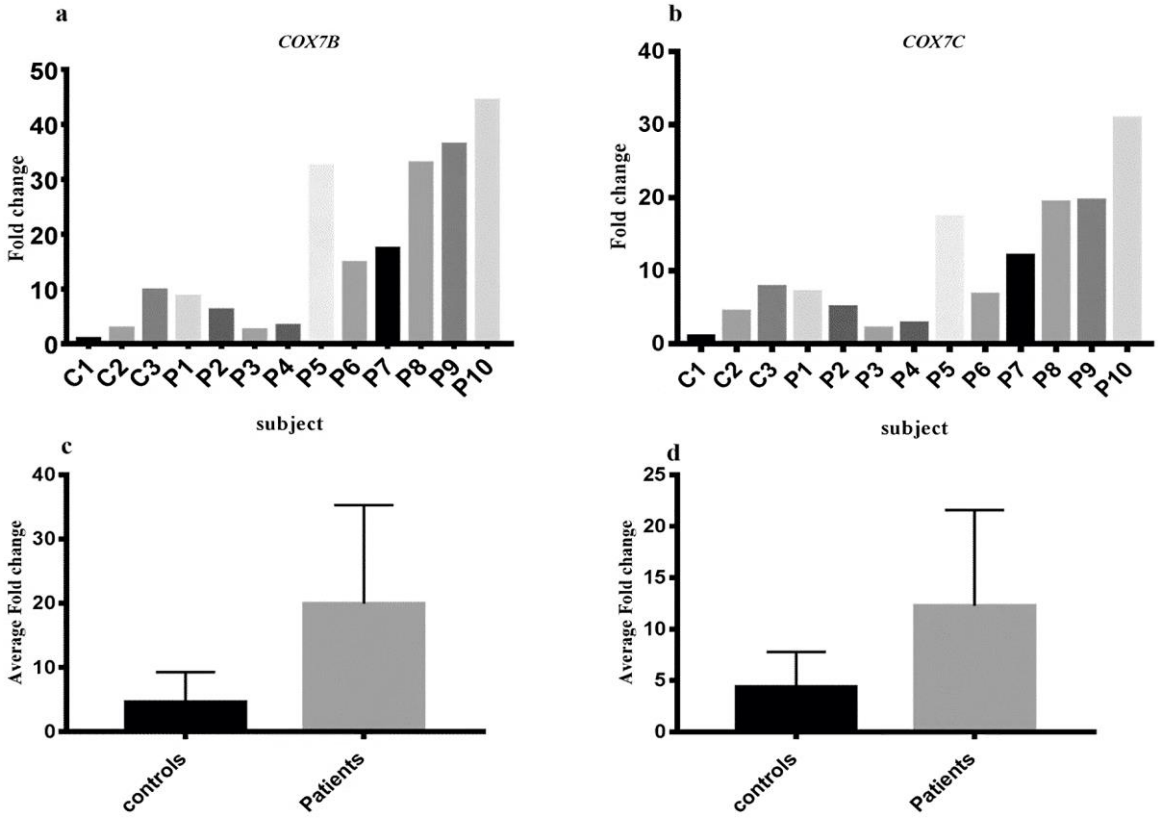

Figure 4. Quantitative PCR gene expression levels in CML patients & controls (Results of q-RT-PCR revealed that gene expression level of *COX7B* & *COX7C* in CML patients was higher than that in control subjects [a & b]. Results were presented for each participant [a & b] & as an average of expression values in patients & control groups [c & d]) (Source: Author's own elaboration using graphpad prism, version 7)

was not statistically different, with a mean fold change value (patients vs. controls) of 19.93 vs. 4.6 and 12.3 vs. 4.4 for *COX7B* and *COX7C*, respectively. Additionally, correlation analysis showed a weak, non-significant inverse correlation between patients' gene expression levels and the mean value of BCR-Abl (%) (p-values=0.31 and 0.33 for *COX7B* and *COX7C*, respectively [correlation graph is not shown]).

Single Nucleotide Variant and Insertion/Deletion Analysis

The results of SNV analysis showed the variants reported in *COX7B* and *COX7C* in CML patients. The data summarized in **Table 4** shows that there are many variants that were previously reported in literature in *COX7B* and *COX7C* genes; however, clinical significance of these variants have not been previously explored and were not reported in "ClinVar" database (https://www.ncbi.nlm.nih.gov/clinvar/docs/clinsig/). On the other hand, the results of this study revealed novel variants in both genes. These include 11 single nucleotide variants in *COX7B* and two variants in *COX7C*. Indel analysis (**Table 5**) revealed six novel variants in *COX7B* and one novel variant in *COX7C*. Also, the clinical significance of these variants was not confirmed. The variants presented herein are known to have a modifying effect on protein.

DISCUSSION

Cancer is a disease known with its complex genomic architecture and poorly understood mechanisms of pathogenesis, perhaps due to involvement of a large number of genes. Blood cancers including CML are similar to other malignant diseases with regard to complexity of genomic landscape and possible contribution of multiple driving factors that might influence disease origin, progression and response to treatment. Therefore, continuous search for candidate proteins in disease could be useful to identify novel disease markers and therapeutic targets; and this was the purpose of this study. The results of whole transcriptome sequencing showed a differential gene expression pattern in CML patients when compared to the healthy controls. Amongst DEGs, *COX7B* and *COX7C* expression was confirmed by quantitative PCR, and gene variants analysis revealed new variants in both genes. This might introduce these two genes for further exploration in CML. *COX7B* encodes a structural subunit of the metabolic enzyme cytochrome c oxidase (COX), also known as mitochondrial respiratory chain complex IV, which may be involved in drug metabolism [14].

Table 4. Novel & reported single nucleotide variants in *COX7B* & *COX7C* in the study sample

Gene	GENE NCBI ID	Location	Variant position	SNP reference	RN	NC	CS
COX7B	1349	77899498	5_prime_UTR_variant	**Novel**	G	T	Not reported
		77899611	Intron	**Novel**	C	T	Not reported
		77900648	Intron	rs111761876	G	T	Not reported
		77901310	Intron	rs1235437578	A	G	Not reported
		77901763	Intron	**Novel**	T	C	Not reported
		77901971	Intron	**Novel**	A	G	Not reported
		77903456	Intron	**Novel**	A	G	Not reported
		77903462	Intron	rs1603367193	A	G	Not reported
		77903545	Intron	**Novel**	T	C	Not reported
		77903546	Intron	rs1194334312	A	T	Not reported
		77903689	Intron	**Novel**	C	T	Not reported
		77904179	Intron	**Novel**	A	G	Not reported
		77904193	Intron	**Novel**	A	G	Not reported
		77904194	Intron	**Novel**	A	G	Not reported
		77904725	Intron	**Novel**	G	A	Not reported
		77905280	3 prime UTR variant	rs2077131363	T	C	Not reported
		77905343	3 prime UTR variant	rs41304464	G	T	Not reported
		77905778	Downstream gene variant	rs5912528	A	G	Not reported
		77906460	Downstream gene variant	**Novel**	A	G	Not reported
COX7C	1350	86618745	Intron	rs6452719	A	G	Not reported
		86618966	Intron	rs150799572	G	T	Not reported
		86618994	Intron	rs200439068	G	A	Not reported
		86619385	2KB_upstream_synonymous_ variant	rs147693864	G	A	Not reported
		86619533	Intron	rs77975079	T	C	Not reported
		86620005	intron	rs16902283	T	C	Not reported
		86620740	3_prime_UTR_variant	rs1750103958	T	A	Not reported
		86620760	3_prime_UTR_variant	**Novel**	A	G	Not reported
		86621776	Downstream _ gene	**Novel**	A	G	Not reported
		86622655	Downstream _ gene	rs2410718	G	A	Not reported

Note. RN: Reference nucleotide; NC: Nucleotide change; &CS: Clinical significance

Table 5. Novel & reported INDEL variants in *COX7B* & *COX7C* in the study sample

Gene	GENE NCBI ID	Location	Variant position	SNP reference	RN	NC	CS
COX7B	1349	77901706	Intron variant	**Novel**	C	CT	Not reported
		77903028	Intron variant	**Novel**	GT	G, GTT, & GTTT	Not reported
		77903911	Intron variant	**Novel**	G	GT	Not reported
		77904641	Intron variant	**Novel**	C	CA	Not reported
		77905657	Downstream gene variant	**Novel**	A	AT	Not reported
		77906991	Downstream gene variant	**Novel**	GT	G, GTT, & GTTT	Not reported
COX7C	1350	86618978	Intron variant	rs368187717	C	CA	Not reported
		86619650	Intron variant	**Novel**	CTA	C	Not reported

Note. RN: Reference nucleotide; NC: Nucleotide change; &CS: Clinical significance

COX7B is required for the assembly and activity of COX enzyme. Hence, *COX7B* is indispensible for electron transport chain reactions and energy production [15, 16]. A recent study reported *COX7B* as a sensitizing agent for bladder cancer cells to platinum-based therapy indicating its possible role in good prognosis [17].

As platinum-based therapy is not the treatment of choice of CML, the role of *COX7B* in CML needs further clarification. It was also reported that *COX7B* potentiates brain tropism of metastasized breast cancer cells, which enhances migration of breast cancer cells towards brain tissue as extensively investigated in [18]. In line with this, *COX7B* was also reported amongst other genes as a possible marker for nasopharyngeal cancer confirming its probable pro-carcinogenic effect [19]. In a similar context, a rare variant in *COX7B2* gene, an isoform of *COX7B* was found to be conserved in a family with nasopharyngeal cancer [20]. Moreover, different single nucleotide variants in *COX7B2* was found to be associated with increased risk of nasopharyngeal cancer (reviewed in [21]). This suggests the importance of examining the clinical significance of the genetic variants identified in this study. In addition, *COX7B* was recently reported as an epithelial-mesenchymal-transition gene amongst others and was found to be associated with chemoresistance in esophageal squamous cell carcinoma [22]. Hence, *COX7B* could be a candidate whose role should be further investigated in CML to understand the reason beyond its upregulation in the current study cohort.

COX7C, the second candidate of this study, is another structural subunit of COX enzyme whose regulatory and catalytic function is yet to be described (reviewed in [21]). *COX7C* upregulation was reported in skin squamous cell carcinoma in comparison to normal skin tissue [23]. Likewise, the gene was upregulated in colon cancer patients after receiving five-fluorouracil treatment suggesting a possible role of this gene in patients response to chemotherapy [24]. It was also recently reported that *COX7C* is implicated in development of venous thromboembolism in patients with colon cancer [25]. This is in concordance with another study, which reported that *COX7C* is associated with venous thromboembolism via its ability to inhibit miR-200a-5p [26].

Moreover, a recent study presented *COX7C* as a biomarker for sepsis-related diabetes mellitus [27]. There is not much more reported on the relationship between *COX7C* and cancer pathogenesis suggesting importance of future studies to explore the implications of *COX7C* upregulation in CML. However, another study indicated the relationship between one *COX7C* gene variant and poor outcome of intracerebral hemorrhage in men [28]. In addition, single nucleotide variants in *COX7C* and other COX-related subunits were found to be associated with increased risk of Alzheimer's disease [29]. This also suggests the importance of studying genetic variations in the COX gene family and understanding their role in the pathophysiology of various human diseases, including CML.

CONCLUSION

Based on the association between *COX7B* and *COX7C* genes and cancer, this study sheds light on the possible involvement of both genes in the development, progression and prognosis of CML. This is because their expression is elevated in patients with CML compared to healthy controls. Therefore, it is recommended that the role of both genes be further studied in larger cohorts at diagnosis and during follow-up of the disease. This may help to understand the interaction between both genes and current therapies. Also, the impact of genetic variants reported in this, and previous studies should also be investigated to assess their influence on disease susceptibility or preventive effects, if any.

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Ethical statement: The author stated that the study was reviewed and approved by the Institutional Review Board at Imam Abdulrahman Bin Faisal University (IRB-2017-03-147).

Declaration of interest: No conflict of interest is declared by the author.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the author.

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