ROLE OF CYSTATIN C GENE POLYMORPHISM AS A RISK FACTOR OF PROSTATE CANCER A CASE-CONTROL STUDY

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AUTHORS’ CONTRIBUTIONS

All authors have made substantial contributions to conception and design, and/or acquisition of data, and/or analysis and interpretation of data. All authors participated in drafting the article or revising it critically for important intellectual content and all authors gave final approval of the version to be submitted, and any revised version. Each author participated sufficiently in the work to take public responsibility for appropriate portions of the content. All authors read and approved the final manuscript.

ARTICLE INFORMATION

Reviewers:
(1) Innocent Damudu Peter, University of Maiduguri, Nigeria.
(2) P. Fernandez, Stellenbosch University, South Africa.
(3) Ugwu Melvin Nnaemeka, Cross River University of Technology, Nigeria.
(4) Alok Nahata, Ying Zhi Agricultural and Industries, Malaysia.

Received: 12 September 2018
Accepted: 02 November 2018
Published: 07 December 2018

ABSTRACT

Background: Cystatin C is found to be down regulated in prostate cancer and modifies invasion of prostate cancer cells via MAPK/Erk and androgen receptor pathways. In this work, we detected the frequency of cystatin C gene single nucleotide polymorphism different variants and its relation to the serum levels of both total testosterone and PSA.

Methods: The study was conducted on (50) cases of newly diagnosed Prostatic cancer patients and another age and sex-comparable (50) healthy individuals as a control group. All were investigated for the frequency of Cystatin C gene polymorphism, serum total testosterone and serum PSA.

Results: Cystatin C gene (BB) variant was detected in 16.0% of cases and 4.0% in controls which showed significant difference with p value=0.046 as compared with (AA) and (AB) variants where (AA) variant was detected in 56.0% of cases and 64.0% in controls which showed no significant difference with p value =0.414, and (AB) variant was detected in 28.0% of cases and 32.0% in controls which also showed no significant difference with p value = 0.663. The serum PSA levels in cases were from 30 to 150 (ng/ml) and in controls were from 0.0 to 3.4 (ng/ml) which showed high significant difference between both groups with p value = 0.000. Serum PSA Levels was higher in the mutant type (BB) than the wild type (AA). As in wild type (AA) the serum PSA levels ranged from 31.5 to 150 (ng/ml) and mutant type (BB) the serum PSA levels ranged from 65 to 150 (ng/ml).

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1. INTRODUCTION

Prostate cancer is the second commonest cancer in men, found in 30% of men at age 50, 60% of men at age 80 – 90 and up to 100% in those over age 90. Cancer prostate is often adenocarcinoma with varying grades. Symptoms and signs of prostatism, occasionally haematuria may present [1].

There are three non-modifiable risk factors for prostate cancer age, race and a positive family history of prostate cancer, a number of modifiable or behavioural factors have been found to be associated with prostate cancer risk. Several factors including smoking and obesity are only weakly related to prostate cancer onset but are positively associated with prostate cancer mortality [2]. Several studies reported that gene polymorphisms are associated with hereditary, sporadic and familial-based prostate cancer. Several genome-wide linkage studies reported that some pathways might be involved in tumour development or progression [3].

Cystatin C is a basic protein (isoelectric point at pH 9.3) encoded by CST3 gene. It is a non-glycosylated and low molecular mass (13.3 kDa) gamma traces, post gamma-globulin or neuro-endocrine polypeptide. It is a member of cystatin super-family of cysteine protease inhibitors. It is produced by all nucleated cells and even in inflammatory conditions [4].

The gene has two intron sequences of 2252 and 1254 bp, resulting in an overall gene size of approx. 4.3 kb. The introns are positioned between the nucleotide triplets encoding amino acid residues 55-56 and 93-94 of the mature protein respectively [5]. The locus of cystatin is in the short arm of chromosome 20, which contains the majority of type II cystatin genes and pseudogenes [6].

Patients with low expression of Cystatin C and high expression of androgen receptor (AR) tend to have worse overall survival than patients with high expression of Cystatin C and high AR expression, induced over-expression of AR in PC3 cells (Human prostate cancer cell line, cell culture) greatly enhanced the invasiveness of PC3 cells, so there may be a crosstalk between Cystatin C and AR-mediated pathways [7].

Some prospective studies have reported that higher testosterone levels are associated with increased risk for prostate cancer, although the majority has not found a statistically significant association. However, many of the latter studies have failed to consider the problem of competing risks [8].

Moslemi et al. [9], has found that PSA alone can be used efficiently as a test for PCa screening of Iranian males. In addition, they found that the incidence of PCa increases with increasing age, lesser weight of prostate and increased tPSA. There was no difference in the Gleason score in their two studied groups of different PSA levels (4–10 and 10–20 ng/mL).

High serum PSA levels and high tissue testosterone (T) levels in men with prostate cancer are significantly associated with poor prognosis, including advanced clinical stage and high Gleason score. Tissues testosterone levels determined from biopsy specimens, in combination with pathological features, may be one of several useful diagnostic tools for predicting the prognosis and determining a suitable therapeutic program for patients with prostate cancer [10].

This work aimed to detect the frequency of single nucleotide polymorphism in Cystatin C gene in prostatic cancer patients in comparison to normal control group and to find out if there is correlation between the presence of single nucleotide polymorphism in Cystatin C gene and the serum level of both testosterone and PSA in both prostatic cancer patients and normal control group.

2. SUBJECTS AND METHODS

2.1 Study Design

In our Case Control Study, we examined samples of (50) cases of newly diagnosed Prostatic cancer patients who presented to the Assiut University Hospital, as well as (50) age and sex comparable apparently healthy individuals (outpatient clinic) as control group. They were investigated for the frequency of Cystatin C gene polymorphism, serum total testosterone and serum PSA in each of the two studied groups. Diagnosis of prostate cancer was done depending on the routine work up which includes clinical examination, laboratory, radiological and...
histopathological investigations. Formal consent was obtained from patients and controls.

The study was approved by Ethical Committee no. 258 of Azhar-Assiut School of Medicine.

2.1.1 Inclusion criteria

(1) Egyptians origin residing in Egypt area as judged by their names, languages and places of birth.
(2) Availability of biological material.
(3) Availability of clinical and follow up data.

2.1.2 Exclusion criteria

1- Male patients with any other type of malignant or benign tumors have been excluded from our study.
2- Past history of Chemotherapy or Surgical treatment of cancer.

All cases were subjected to the following:

1. Medical history, full clinical examination and general laboratory investigations were done:

Including Random blood sugar, Kidney functions test, Liver functions test, Prothrombin time and concentration, complete blood picture. Clinical examination, particularly for prostate enlargement, symptoms, signs and its clinical manifestations were performed. Radiological investigations including Transrectal Ultrasound and prostate MRI (magnetic resonance imaging) were done. As well as Histopathological investigations for Prostate Biopsy by Transrectal Ultrasound-Guided Prostate Biopsy Techniques for confirmation of cancer prostate.

Approximately 6 ml venous blood was collected from each patient and control and divided into two tubes, one containing EDTA for the molecular testing, and the other plane tube used to separate serum by centrifugation at 3000 r.p.m. to be used for analysis and stored at -4 °C .

Detection of Cystatin C gene polymorphism by PCR restriction fragment length polymorphism:

2. Genotyping:

DNA isolation: DNA isolation from peripheral blood at diagnosis using salt precipitation, as described by Determination of DNA concentration by Spectrophotometry determine the nucleic acid concentration by measuring the optical density at 260 nm of the prepared diluted DNA (1 O.D unit = 50 µg/ml). The purity of the nucleic acid (the absence of proteins contamination) is determined by the ratio of absorbance at 260 nm to the absorbance at 280 nm. The ratio of 260/280 should be between 1.7 – 2.0.

DNA concentration: Absorbance at 260 nm X 50 (nucleic acid factor) X dilution = concentration of DNA in µg/ml [11].

Cystatin C gene SNP genotype analysis: We used PCR-RFLP technique to discriminate between wild type, heterozygous and homozygous allele.

(PCR-RFLP) Assay: The Cystatin C gene polymorphism was determined by using the method described by Zurdel et al. [12]; Polymerase chain reaction (PCR) products (318 bp) from genomic DNA were generated by using primer 024, TGGGAGGGACGAGGCGTTCC/and1206R, TCCATGGGGCCTCCCACCAG. The thermo profile was 95°C 45 seconds, 13 ´ (95°C 15 seconds, 68°C 30 seconds -1°C per cycle, 72°C 30 seconds), 23 ´ (95°C 15 seconds, 55°C 30 seconds, 72°C 30 seconds), and 72°C 5 minutes. Three polymorphic KspI restriction sites in the 5’ region of CST3 were covered by the 318 bp PCR fragment. Through strong linkage disequilibrium between the three polymorphisms only two haplotypes were observed. The haplotypes are defined by either concomitant KspI restriction endonuclease cleavage both 80 bp upstream of the mRNA transcription start site and in the penultimate codon of the signal peptide (haplotype A) or by an exclusive cleavage downstream of the transcription start site (haplotype B). Haplotypes were confirmed by direct sequencing of PCR products from individuals with the genotypes A/A, A/B and B/B. Restriction digestion of the PCR product with KspI (MBI Fermentas, Vilnius, Lithuania) at 37°C overnight revealed fragment sizes of 41/226/51 bp (homozygote haplotype A), or 127/191 bp (homozygote haplotype B), or all five fragments in A/B heterozygotes, the digestion products were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide and visualised under ultraviolet light.

3. Hormonal analysis detection of Testosterone and Prostatic specific antigen (PSA) serum levels; Enzyme-linked immune-sorbet assay (ELISA) technique was used to determine the serum level of both testosterone and PSA to determine their role in the susceptibility to prostate cancer. Both were analysed using DRG ELISA kit by Mindray (Germany).
2.2 Statistical Analysis

A data entry file using EXCEL 2007 program was prepared. Data were analysed using SPSS (version 19). The frequencies, percentages, mean and standard deviation were computed. Chi-square test was used to compare qualitative variables between groups. Mann-Whitney test was used as the test of significance to compare quantitative data between groups. Spearman correlation was done to measure correlation between quantitative variables. P-value considered statistically significant when P < 0.05.

3. RESULTS

Patients and control groups were investigated for the frequency of Cystatin C gene A>B polymorphism regarding the wild (AA), heterozygous (AB) and homozygous (BB) types, in addition to the hormonal analysis of the routine markers (serum total testosterone & serum PSA).

According to the age distribution in the two studied groups, the age of cases in our study was ranged from 60 to 70 years with mean of $64.58 \pm 3.10$ years while the age of controls was ranged from 60 to 70 years with mean of $65.14 \pm 3.39$ years which showed no significant difference with p value = 0.421. The sex distribution in the two studied groups was the same as the disease specific for the prostate in adult males.

3.1 Cystatin C Gene Polymorphism Results

Cystatin C gene wild type (AA) was detected in 56.0% of cases and 64.0% in controls which showed no significant difference with p value = 0.414. Heterozygous type (AB) was detected in 28.0% of cases and 32.0% in controls which showed no significant difference with p value = 0.663 and homozygous type (BB) was detected in 16.0% of cases and 4.0% in controls which showed significant difference with p value=0.046 as compared with the wild type (AA). The frequency of both polymorphisms (AB+BB) versus the wild type (AA) was found to be 44.0% versus 56.0% in the Patient group and 36.0% versus 64.0% in the control group with p value = 0.415 which showed no significant difference as compared with the wild type (AA). The B allele was detected in 30.0% of cases and in 20.0% of controls which also showed no significant difference with p value =0.104 as compared with the wild type (AA). Regarding Cystatin C polymorphism genotypes in relation to age distribution, there was no significant difference in age distribution among various groups of cases genotypes compared to wild type. As in wild type (AA) the age was ranged from 60 to 70 years with mean of $(65.14 \pm 3.41)$ years with p value =0.217, in hetero type (AB) the age was ranged from 60 to 69 years with mean of $63.79 \pm 2.83$ years with p value =0.421, and in mutant type (BB) the age was ranged from 61 to 67 years with mean of $(64.00 \pm 2.14)$ years with p value =0.809 (Table 1, Figs. 1 & 2).

3.2 Hormone Results (Total Testosterone & PSA)

Total testosterone: Table 2 illustrated the hormonal level of total testosterone in the two studied groups. The serum total testosterone level of patient group was ranged from 2 to 7 (ng/ml) with mean of $(4.21 \pm 1.43)$ ng/ml while the serum total testosterone level of control group was ranged from 2 to 7 (ng/ml) with mean of $(4.11 \pm 1.49)$ ng/ml which showed no significant difference with p value = 0.574.

Cystatin C gene polymorphism in relation to testosterone hormone: There was no significant difference in total testosterone hormone levels among various groups of cases genotypes compared to wild type. As in wild type (AA) the serum total testosterone hormone was ranged from 2 to 7(ng/ml) with mean of $(4.47 \pm 1.49)$ ng/ml with p value =0.169, hetero type (AB) the serum total testosterone was ranged from 2 to 6.2 (ng/ml) with mean of $(3.78 \pm 1.40)$ ng/ml with p value =0.543 and mutant type (BB) the serum total testosterone was ranged from 2.2 to 5.8 (ng/ml) with mean of $(4.05 \pm 1.21)$ ng/ml with p value =0.633 as shown in Fig. 3.

PSA: Table 3 illustrated the hormonal levels of PSA in the two studied groups. The serum PSA levels in cases of our study was ranged from 30 to 150 (ng/ml) with mean of $(90.02 \pm 36.54)$ ng/ml while the serum PSA levels of controls was ranged from 0.0 to 3.4 (ng/ml) with mean of $(0.84 \pm 0.95)$ ng/ml which showed high significant difference with p value < 0.0001.

Cystatin C polymorphism in relation to PSA: There was no significant difference in PSA levels among various groups of cases genotypes compared to wild type. As in wild type (AA) the serum PSA levels was ranged from 31.5 to 150 (ng/ml) with mean of $(90.90 \pm 39.92)$ ng/ml with p value =0.594 , hetero type (AB) the serum PSA levels was ranged from 30 to 137.5 (ng/ml) with mean of $(85.19 \pm 36.15)$ ng/ml with p value =0.704 and mutant type (BB) the serum PSA levels was ranged from 65 to 149.3 (ng/ml) with mean of $(95.38 \pm 26.22)$ ng/ml with p value =0.759 (Fig. 4).
Table 1. Cystatin C gene polymorphism in the two studied groups

<table>
<thead>
<tr>
<th>Cystatin C gene Polymorphism</th>
<th>Cases group (No.=50)</th>
<th>control group (No.=50)</th>
<th>P-value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>AA (wild)</td>
<td>28</td>
<td>56.0%</td>
<td>32</td>
<td>64.0%</td>
<td>0.414</td>
</tr>
<tr>
<td>AB (hetero)</td>
<td>14</td>
<td>28.0%</td>
<td>16</td>
<td>32.0%</td>
<td>0.663</td>
</tr>
<tr>
<td>BB (mutant)</td>
<td>8</td>
<td>16.0%</td>
<td>2</td>
<td>4.0%</td>
<td>0.046</td>
</tr>
<tr>
<td>AB+BB</td>
<td>22</td>
<td>44.0%</td>
<td>18</td>
<td>36.0%</td>
<td>0.415</td>
</tr>
<tr>
<td>BB allele</td>
<td>30</td>
<td>30.0%</td>
<td>20</td>
<td>20.0%</td>
<td>0.104</td>
</tr>
</tbody>
</table>

Fig. 1. PCR product of Cystatin C gene polymorphism pre-digestion by KspI shows bands at 318 bp, at lane 2,3,4,5,6,7
1st column in left side shows: 50 bp ladder, white lines on left side of ladder vertically from below to above refer to bands at 50, 100, 150, 200, 250, 300, 400, 500, 600 bp and so on, at lane 1
Fig. 2. Cystatin C gene polymorphism after KsPl digestion

Lane 1: 50 bp ladder, white lines on left side of ladder vertically from below to above refer to bands at 50,100,150,200,250,300,400,500,600 bp and so on.
Lane 2,3,6,9: Wild type (AA) showing three bands at 41, 51, 226 bp
Lane 4,8: Mutant type (BB) showing two bands at 127, 191 bp
Lane 5,6,7: Heterozygous variant (AB) showing five bands at 41, 51, 127, 191, 226 bp.

PCR product of Cystatin C gene polymorphism pre-digestion by KsPl (restriction enzyme) showed bands at 318 bp (base pair). While PCR product of Cystatin C gene polymorphism after digestion by KsPl showed three bands at 41, 51, 226 bp in wild type (AA), two bands at 127, 191 bp in mutant type (BB) and five bands at 41, 51, 127, 191, 226 bp in heterozygous type (AB).

Table 2. Serum total testosterone hormone levels in the two studied groups

<table>
<thead>
<tr>
<th>Testosterone hormone (ng/ml)</th>
<th>Cases (No.= 50)</th>
<th>Control (No.= 50)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>4.21 ± 1.43</td>
<td>4.11 ± 1.49</td>
<td>0.574</td>
</tr>
<tr>
<td>Range</td>
<td>2.0 - 7.0</td>
<td>2.0 - 7.0</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Cystatin C polymorphism in relation to serum PSA levels

Table 3. Serum PSA levels in the two studied groups

<table>
<thead>
<tr>
<th>PSA (ng/ml)</th>
<th>Cases (No.= 50)</th>
<th>Control (No.= 50)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>90.02 ± 36.54</td>
<td>0.84 ± 0.95</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Range</td>
<td>30.0 - 150.0</td>
<td>0.0 - 3.4</td>
<td></td>
</tr>
</tbody>
</table>
4. DISCUSSION

Cystatin C expressed widely in the male reproductive system, so its presence might be significantly important in the regulation of normal physiological proteolysis, inflammatory diseases and malignancies in the male genital tract [13].

Allelic variation of a gene may be related to the biological function of its encoded protein; therefore our study hypothesized a potential association of allelic variants of CST3 gene in patients having prostate cancer and determine if any of the CST3 genotypes are associated with prostate cancer. In this hospital-based case-control study, a functional Cystatin C gene polymorphism was genotyped and the association between this genetic variant, the risk of prostate cancer and the serum levels of both total testosterone and PSA among the normal Egyptian population was investigated.

In our study, Cystatin C gene wild type (AA) was detected in 56.0% of cases and 64.0% in controls which showed no significant difference with p value =0.414 , heterozygous type (AB) was detected in 28.0% of cases and 32.0% in controls which showed no significant difference with p value = 0.663 and homozygous type (BB) was detected in 16.0% of cases and 4.0% in controls which showed significant difference with p value =0.046 as compared with the wild type (AA). The frequency of both polymorphisms (AB+BB) versus the wild type (AA) was found to be 44.0% versus 56.0% in the Patient group and 36.0% versus 64.0% in the control group with p value = 0.415 which showed no significant difference as compared with the wild type (AA). The B allele was detected in 30.0% of cases and in 20.0% of controls which also showed no significant difference with p value =0.104 as compared with the wild type (AA).

Our study showed that: The distributions of CST3 gene genotypes in the Prostate cancer cases were AA= 56.0%, AB= 28.0% and BB=16.0%. With B allele freq.=30.0 % where AB (hetero) genotype showed no significant difference as compared with the control group with p value=0.663 and BB(mutant) genotype showed the significant difference as compared with the control group with p value = 0.046 . Also there was no significant difference in age distribution among various groups of cases genotypes compared to wild type.

The results suggested that: Homozygosity for haplotype B (BB mutant) of Cystatin C genotype was significantly associated with Prostate cancer, therefore Cystatin C gene polymorphism in our studied group was associated with the risk of Prostate cancer particularly allelic variant B (CST3 BB ) and so the Cystatin C gene (CST3) is genetically associated with increased risk of prostatic cancer.

Significant association was found between one CST3 gene haplotype and deep white matter hyper intensity (DWMH) (p=0.002), this haplotype was also associated with lower plasma CST3 levels (p = 0.01) that SNPs in the CST3 gene confer a risk for developing ischemic white matter lesions in Japanese population possibly through altered secretion of CST3 [14,15].
In contrary to our study: Cystatin C gene polymorphism was not associated with increased risk of different diseases in different populations in a hospital-based case-control studies; Lack of an association between Cystatin C gene polymorphisms and Alzheimer's disease, associations between polymorphisms of the Cystatin C gene (CST3) at 5’ flanking region and exon 1 in Caucasian patients with late onset AD and exon 1 in a US study of late onset AD have been reported. In their study, the occurrence of CST3 -157 G/C and +73 G/A polymorphism distributions did not differ between AD patients and controls [16].

In our study we found that: The serum total testosterone levels of cases showed no significant difference as compared to controls. Where the serum total testosterone levels of cases was ranged from 2 to 7 (ng/ml) with mean of (4.21 ± 1.43) ng/ml while the serum total testosterone levels of controls was ranged from 2 to 7 (ng/ml) with mean of (4.11 ± 1.49) ng/ml with p value = 0.574. Also, there was no significant difference in serum total testosterone levels among various groups of cases genotypes compared to wild type in relation to Cystatin C gene polymorphism.

The results suggested that: There was no association between the risk of prostate cancer and the serum levels of total testosterone among our study of Egyptian population.

The serum levels of testosterone was not associated with increased risk of prostate cancer in a hospital-based case-control studies, a direct relationship between circulating testosterone levels and PCa aggressiveness and prognosis is not evident in all circumstances and patient settings [17].

In a collaborative analysis of the worldwide data of 18 prospective studies on endogenous hormones and prostate cancer risk, serum concentrations of sex hormones including (total testosterone, calculated free testosterone, dihydrotestosterone, estradiol, calculated free estradiol, etc.) were not associated with risk of prostate cancer [18].

In contrary to our study: The serum levels of testosterone was associated with increased risk of prostate cancer in a hospital-based case-control studies; High serum PSA levels and high tissue testosterone levels in men with prostate cancer are significantly associated with indicators of poor prognosis, including advanced clinical stage and high Gleason score [10].

Their study shows that low free and bioavailable testosterone levels are associated with an increased risk of PCa in a rebiopsy after HGPIN diagnosis. Men with low testosterone levels and High grade prostatic intraepithelial neoplasia (HGPIN) could, therefore, be considered a high-risk cohort for developing PCa [19,20].

Higher testosterone levels represent a risk factor for PCa appears to have little evidentiary support. By contrast, some studies have described relationships between lower testosterone levels and more advanced disease [21]. The majority of the studies did not adhere to the guidelines published by professional societies concerning TT measurements; many of the studies were limited by the small size of the cohorts and duration of follow-up [22].

In our study we found that: The PSA of cases showed high significant difference as compared to controls, where the serum PSA levels of cases ranged from 30 to 150 (ng/ml) with mean of (90.02 ± 36.54) ng/ml while the serum PSA levels of controls ranged from 0.0 to 3.4 (ng/ml) with mean of (0.84 ± 0.95) ng/ml with p value = 0.000. Also there was no significant difference in PSA serum levels among various groups of cases genotypes compared to wild type in relation to Cystatin C gene polymorphism.

The results of PSA in our study suggested that: There was high association between the risk of prostate cancer and PSA serum levels among our study of Egyptian population, which has become worldwide diagnostic tool for PCa and has been documented by a lot of different studies and articles [23].

5. CONCLUSION

There was an association between SNP in Cystatin C gene with genetic susceptibility to prostate cancer. Also, the results showed that Cystatin C gene polymorphism particularly allelic variant B (CST3 BB) mutant type was significantly associated with the susceptibility to prostate cancer.

Cystatin C gene polymorphism particularly allelic variant B (CST3 BB) mutant type and PSA were associated with the susceptibility to prostate cancer, but testosterone was not associated.

ETHICS APPROVAL AND CONSENT

A) The study has been carried out on samples and not on patients. However, informed consents have been obtained from all subjects included in the study before sampling.
B) No risks to the subjects included in the study.
C) No harmful investigations.
D) The study was approved by Ethical Committee no. 258 of Azhar- Assiut School of Medicine.
FUNDING
This research is partially funded by Azhar University Hospital- Assiut, Faculty of Medicine, Azhar University- Assiut.

ACKNOWLEDGEMENTS
This research was partially supported by [Assiut University Hospital, Faculty of Medicine, Assiut University]. We thank our colleagues from [Assiut University Hospital, Faculty of Medicine, Assiut University] who provided us insight and expertise.

COMPETING INTERESTS
Authors have declared that no competing interests exist.

REFERENCES


