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ORIGINAL RESEARCH

The Effect of Hormone Therapy on the Expression of Prostate Cancer and Multi-Epigenetic Marker Genes in a Population of Iranian Patients

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Background and Aim: Many recent studies have shown a direct relationship between the decrease in the expression of *GSTP1* and *RASSF1* with the incidence and progression of prostate cancer. Moreover, the expression level of these genes is greatly affected by epigenetic factors and their methylation pattern. Given the prevalence of prostate cancer and the importance of choosing the best method to inhibit the progression of the disease and provide specific treatment, it is important to evaluate the effect of hormone therapy on the expression of effective prostate cancer genes and epigenetic markers.

Patients and Methods: In this case–control study, 35 prostate cancer samples were examined before and after hormone therapy. Following the blood sampling, RNA extraction, and cDNA synthesis, the expression of *GSTP1, RASSF1, HDAC, DNMT3A*, and *DNMT3B* was assessed by real-time PCR.

Results: The results analysis showed that the expression of *GSTP1, RASSF1*, and *DNMT3B* was significantly increased, *DNMT3A* was significantly decreased (P value<0.05) and *HDAC* expression did not change significantly (P value=0.19) after hormone therapy.

Discussion: Significant changes in the expression of *GSTP1, RASSF1, DNMT3B* and *DNMT3A* in the studied samples indicate that these genes are susceptible targets for cancer hormone therapy in Iranian men like in the other populations. Evaluation of gene activity in a larger population of patients may support these findings.

Keywords: *GSTP1*, *RASSF1*, *HDAC*, *DNMT3A*, *DNMT3B*, prostate cancer, hormone therapy, real-time PCR

Introduction

Prostate cancer (PCa) is one of the most mortal malignancies in American men with an approximate rate of one in nine with a higher incidence in African origin people.[1] Approximately 192,000 individuals were diagnosed with prostate cancer in the United States in 2009, while 27,000 were expected to die of the disease.[2] The disease is rarely diagnosed in men under the age of 50 (less than 0.1% of all patients). The maximum incidence of the cancer is between 70 and 74 years of age, and 85% of cases are diagnosed after the age of 65 years.[3]

GSTs are a group of isozymes that perform detoxification by binding glutathione to electrophilic compounds. Of this family, the role of *GSTP1* has been identified more prominently in cancers, especially in prostate cancer.[4] The Ras-association domain family 1 (*RASSF1*) gene is an important RAS signaling pathway effector which is located in the 3p21.3 chromosomal region and plays an important role in

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the tumorigenesis of cancers.[5] Previous studies have demonstrated *GSTP1* and *RASSF1* important role in prostate malignancies and introduced them as candidate biomarkers for diagnosis of the disease through their expression changes.[6,7] Moreover, epigenetic markers such as *HDAC* and DNMTs are among the factors that influence the methylation and consequent expression of these genes.[8]

Researchers have shown that in the prostate cancer tissue, testosterone levels are higher than in normal non-cancerous tissue and sex hormones are of high importance in the pathogenesis of prostate cancer, which is less prevalent in infertile men.[9] It has long been recognized that prostate cancer is dependent on androgens for growth and proliferation. Therefore, androgen deprivation has been suggested as an effective treatment and is commonly used in clinical practice for androgen-dependent patients.[10,11]

Numerous studies have confirmed the accumulation of testosterone and dihydrotestosterone in the enlarged prostate stroma and its relation to the prostate mass. Reducing male sex hormones by hormone therapy can decrease the prostate mass,[12] and affect the expression of many genes and microRNAs associated with the cancer development.[13,14]

Given the inhibitory effects of hormone therapy and the multifactorial nature of prostate cancer and its significant prevalence in Iran, this study could provide an effective guide to the treatment of the cancer in Iranian men.

In this cross-sectional study, we aimed to investigate the effect of hormone therapy on the expression of several candidate biomarker genes such as *GSTP1* and *RASSF1* and some epigenetic markers that have been shown to be effective on the incidence and progression of prostate cancer in a group of patients nominated for advanced treatment. Therefore, we evaluated the expression of GSTP1, RASSF1, HDAC, DNMT3A and DNMT3B in understudy patients at diagnosis time and after Bicalutamide therapy for a period of 3 months.[11]

Patients and Methods

Patients

Seventy blood samples from prostate cancer patients were collected before and after treatment in EDTA-containing tubes and stored at 20°C. The diagnosis has been reported by a specialist in the urology department following biopsy and pathology confirmation for prostate cancer (Grades 1–5). Appropriate blood samples were obtained from Shohadaye Tajrish and Modares hospitals (Tehran, Iran) for gene expression analysis. Clinical and pathological data were collected from patients after PCa diagnosis due to high prostate-specific antigen (PSA) levels and the presence of cancer cells in histological analysis following ultrasound rectal biopsy. Sampling was performed following a three-month hormone therapy with the Bicalutamide family drugs. By default, all stages of this research were covered by the Hospital Ethics Committees and SBMU. Written informed consent was obtained from the patients.

RNA Extraction

Five milliliters of each blood sample was lysed by RiboEx TMLS, centrifuged at 13,000 × g for 12 min at 4°C. RNA was extracted according to the manufacturer's instructions (Cat.No.315–150). A spectrophotometer (NanoDropTM 2000/2000c, USA) was used to quantify RNA concentration. Agarose gel electrophoresis (1%) was used to evaluate the quality of the extracted RNA.

cDNA Synthesis and Quantitative RT-PCR

HyperScript First-Strand Synthesis Kit GeneAll (Cat No: 605-005) was applied to synthesize cDNA from the extracted total RNA following the manufacturer's guidelines. Primer3 and BLAST web sites were used to design gene-specific primers. Qiagen Rotor-Gene Q was used for the cDNA denaturation at 95°C for 15 mins, then amplification at 95°C for 10 s and annealing for 35 s at an appropriate amplicon temperature for 45 cycles (Table 1). Negative controls were used to confirm that no genomic DNA contamination existed. Beta-2- macroglobulin (*B2M)* was selected as a housekeeping gene for normalization and 2% agarose gel was used for verification of amplification products accuracy.

Statistical Analysis

In this study, $2^{-\Delta\Delta CT}$ was used to analyze the relative gene expression changes. REST 2009 Software was used for gene expression analysis and normalization using Real-Time PCR data. Significant differences between samples' gene expression before and after therapy were determined by two-tailed Student's *t*-test using GraphPad Prism 8.0.2 (GraphPad prism Software, Inc. San Diego CA, USA). To determine the efficiency of the examined genes as diagnostic PCA biomarkers, receiver operating characteristic (ROC) curve analyses was performed by using MedCalc-version19.1.2. In statistical analysis, a P value of <0.05 was considered significant.

Table 1 Details on Primers Used for qRT-PCR Analysis.

Amplicon Tm(°C)	Amplicon Length(bp)	Primer Sequence	Gene Name
58	174	F-5′CCAGTCCAATACCATCCT3′ R-5′GCCTTCACATAGTCATCC 3′	*GSTP1*
60	70	F-5′GACCTCTGTGGCGACTTCAT3′ R-5′CTCCACAGGCTCGTCGC 3′	*RASSF1*
58	173	F-5′ACATCTCCATCCTACAAGT3′ R- 5′ GTGACAACATTTCCATCCT 3′	*HDAC*
59	100	F-5′CTACTACATCAGCAAGCG 3′ R-5′TTCCACAGCATTCATTCC 3′	*DNMT3A*
60	162	F-5′ GCTCTTACCTTACCATCG 3′ R-5′ACTCTGAACTGTCTCCAT 3′	*DNMT3B*
58	117	F-5′ AGATGAGTATGCCTGCCGTG 3′ R-5′ GCGGCATCTTCAAACCTCCA 3′	*B2M*

Results

Patient Dataset

Blood samples were obtained from 35 PCa patients, 52 to 85 years old with a mean of 71 years, before and after hormone therapy. A summarized clinicopathological feature of understudy patients is shown in (Table 2).

Expression Variation of the Examined Genes

The expression of prostate cancer genes *GSTP1, RASSF1, HDAC*; and *DNMT3A*, and *DNMT3B* epigenetic markers were evaluated by qRT-PCR at the time of diagnosis and after 3 months of hormone therapy by Bicalutamide. The mRNA expression level of *GSTP1, RASSF1*, and *DNMT3B* in PCa patients who responded to hormone therapy was significantly upregulated ($p<0.05$), *HDAC* gene expression did not show any significant changes (P=0.19) and *DNMT3A* gene expression was significantly decreased after the treatment (P=0.02) (Figure 1). The qRT-PCR results were used for assessment of the relative expression of the examined genes in two evaluated groups.

Table 2 Clinicopathological Characteristics of Patients with Prostate Cancer

Parameter	Group		Value
N	PCA (Past treatment) PCA (Post hormone therapy)		35 35
Age (year)	PCA	<60 >60	12.5% 87.5%
PSA	PCA	<4 >4	7.5% 92.5%
Exercise	PCA	Positive Negative	47.5% 52.5%
Smoke	PCA	Positive Negative	77.5% 22.5%
Grade	1 2 3 4 5		27.5% 42.5% 12.5% 7.5% 10%

ROC Curve Analysis of Examined Genes

The receiver operating characteristic curve (ROC) was plotted to find the value of evaluating the expression of the under study genes with the drug response of patients after hormone therapy. ROC analysis showed area under the ROC curve of A) 0.78 for *GSTP1* (Sensitivity 80%, Specificity75% and *P* value =0.001), B) 0.73 for *RASSF1* (Sensitivity 77.5%, Specificity 70% and *P* value =0.0004), C) 0.77 for *DNMT3A* (Sensitivity 72.5%, Specificity 82.5% and *P* value<0.0001), D) 0.75 for *DNMT3B* (Sensitivity 85.37%, Specificity 62% and *P* value<0.0001) and E) 0.89 for *HDAC* (Sensitivity 81%, Specificity 76% and *P* value <0.0001) (Figure 2).

Discussion

Since the clinical significance of *GSTP1, RASSF1, HDAC, DNMT3A* and *DNMT3B* genes expression in PCa patients has not been thoroughly studied in Iran, we aimed to investigate the effect of hormone therapy on their expression to introduce a possible combination of genetic and

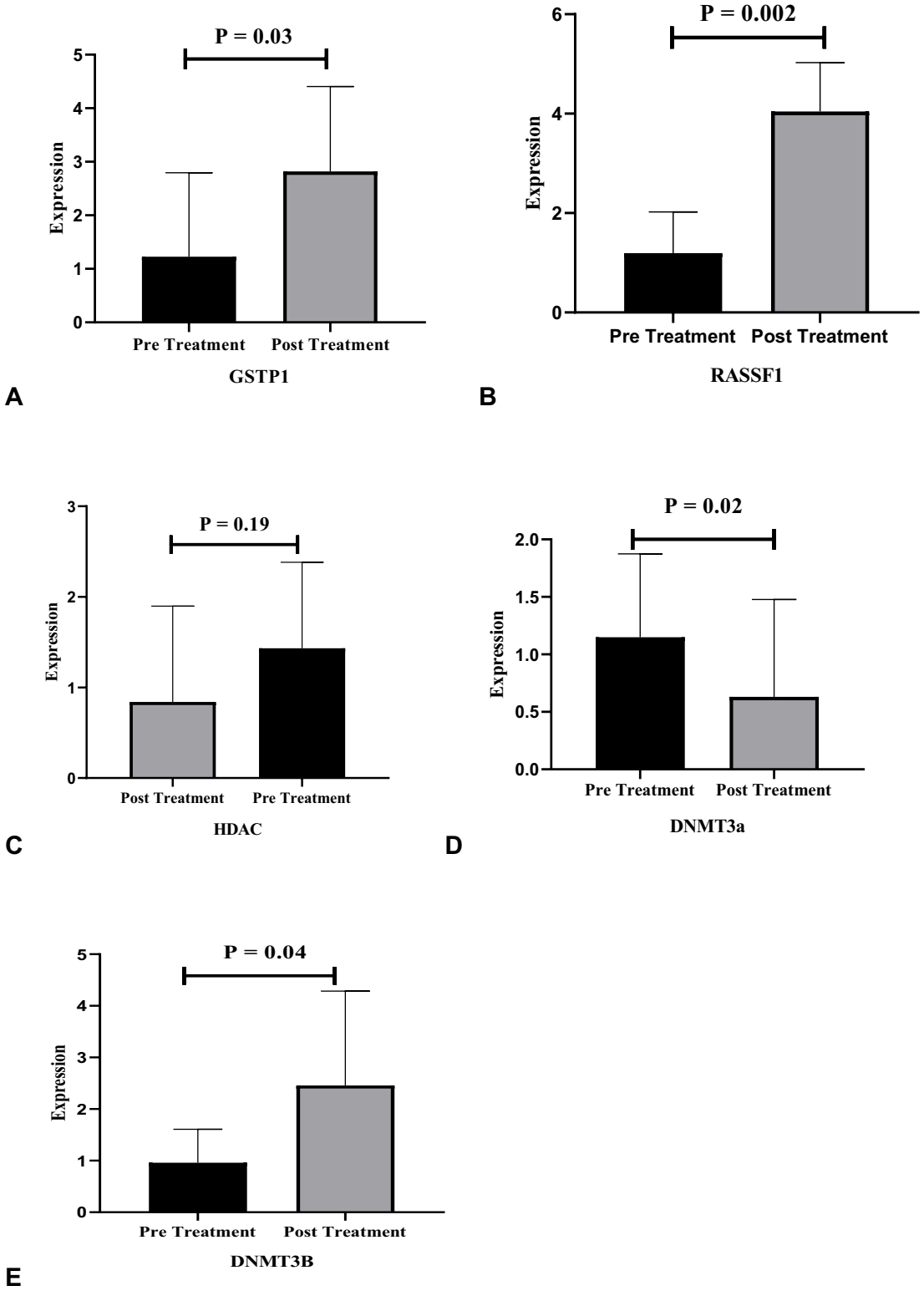

Figure 1 The relative mRNA expression level of *GSTP1*, *RASSF1*, *HDAC*, *DNMT3A*, and *DNMT3B* in two different groups of PCA patients (n: 70). Results were normalized with *B2M* gene. (**A**) Upregulation in the expression level of GSTP1 after treatment (P=0.03), (**B**) Upregulation in the expression level of GSTP1 after treatment (P=0.002), (**C**) No significant change in HDAC expression before and after treatment (P=0.19), (**D**) Downregulation of DNMT3A gene expression (P=0.02), and (**E**) upregulation of DNMT3B gene expression after hormone therapy (P=0.04).

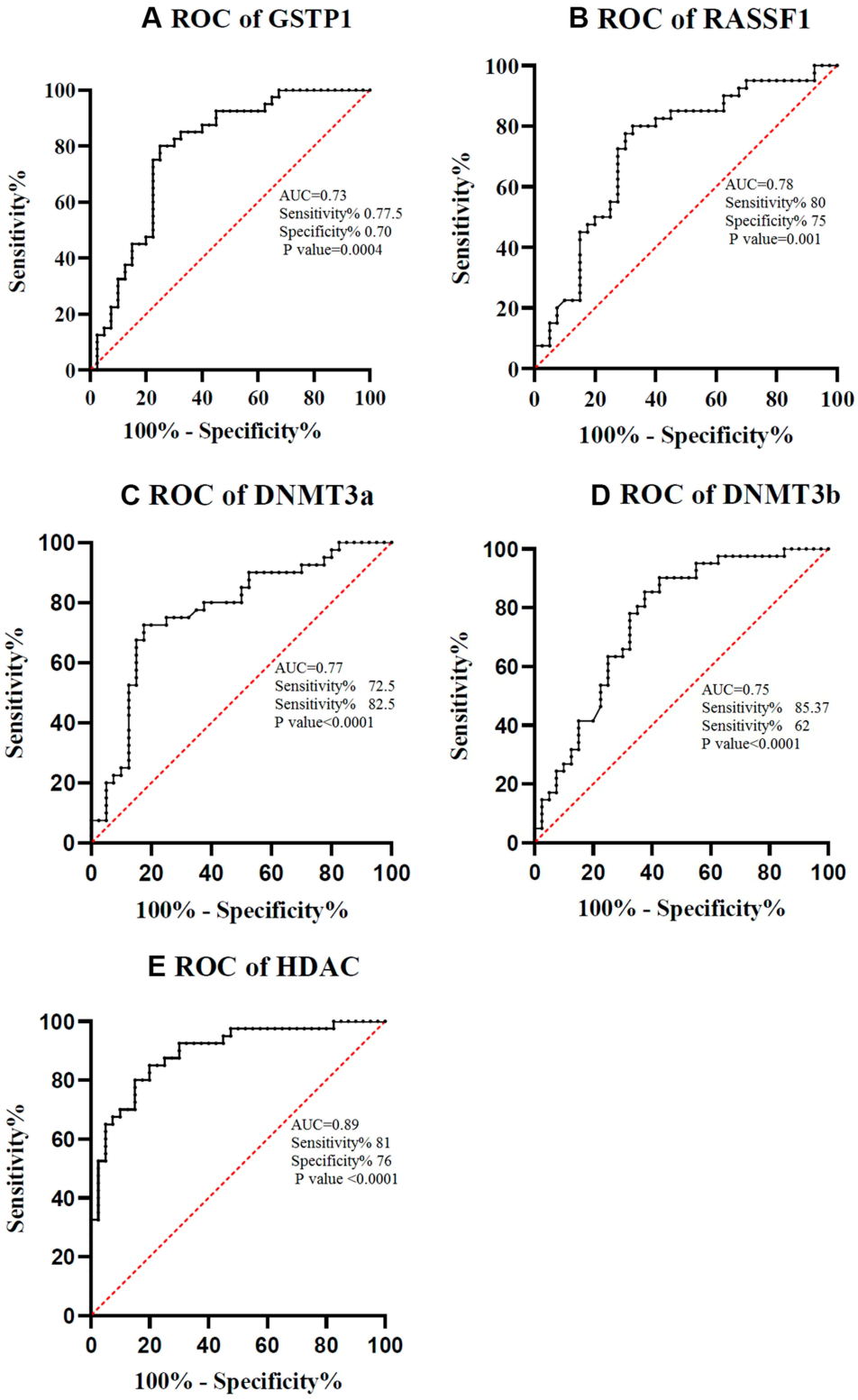

Figure 2 ROC curve analysis showed that GSTP1 (**A**), RASSF1 (**B**), DNMT3A (**C**), DNMT3b (**D**) and HDAC (**E**) expression assays have high specificity and sensitivity to detect patients' response to the treatment.

epigenetic biomarkers that can be effective in the disease treatment and to evaluate the drug efficacy and follow-up the patients during hormone therapy.

Over the past decades, researchers have focused on identifying the genetic background of prostate cancer. A number of genetic alterations in association with

epigenetics and gene expression have made a better prediction of the disease.[15] *GSTP1 and RASSF1* are two genes whose expression in healthy and cancerous groups has been discussed in various articles on the incidence and progression of prostate cancer.[16] It is shown that the expression of these genes is affected by changes in the methylation pattern through which their expression is reduced by hypermethylation.[17] Therefore, they are among the candidate genes as biomarkers for early detection of prostate cancer in Kashmir, Vietnam, and a large number of other populations.[16,18,19] Inês Graça (2016) stated that potential therapeutic agents for managing prostate cancer are epigenetic changes of the disease which highlights the important role of epigenetic modulators in pre-trial and clinical trials.[20] In addition, it is well understood that *HDAC, DNMT3A*, and *DNMT3B* are major epigenetic factors regulating methylation patterns of *GSTP1* and *RASSF1* genes which alter the expression of the genes in prostate cancer.[7,21,22]

Christopher J. Luskeb in 2002 discussed the association of *GSTP1* glutathione S-transferase genotypes with the response to androgen deprivation therapy in patients with advanced prostate cancer.[23] Our results showed a significant increase in *GSTP1* and *RASSF1* expression in post-treatment specimens ($P < 0.05$). These results confirm the specificity of these two genes for an earlier and faster detection of cancer and their use as possible biomarkers tools.

Giovanni Luca Gravina in 2011 showed that a significant increase in the expression of *DNMT* including *DNMT3A* and *DNMT3B* in the advanced stages of the disease was a great achievement to apply hormone therapy to the development of the hormone resistance phenotype in cancer patients.[24] Tarek K. Motavi in 2018 highlighted the important role of HDAC in the progression of prostate cancer and suggested that a combination of HDAC inhibitors and hormone therapy could be ideal for the treatment of advanced prostate cancer.[25] Due to the effect of methyltransferases on the methylation of *GSTP1* and *RASSF1* genes, and their divergent expression in PCa patients in comparison to BPH group, an elevation in *DNMT3A* and *DNMT3B* expression level could be expected. Our study had some interesting results, when *DNMT3A* expression decreased significantly ($P = 0.02$) and *DNMT3B* expression was enhanced after treatment ($P = 0.04$). HDAC also did not show significant expression changes ($P = 0.19$). This contradiction in the predicted results could be attributed to drug resistance in patients or sample size.

Our results indicated a decrease in the expression of *GSTP1* and *RASSF1* as candidate markers for the prognostic profile of PCa patients in Iran. *GSTP1* and *RASSF1* may act as risk factors in the diagnosis of the patients with poor prognosis and can also be known as therapeutic targets for killing cells that are specifically running and causing the recurrence. Increased expression of both *GSTP1* and *RASSF1* genes after treatment may indicate a favorable patient response to hormone therapy and remission.

Conclusion

In conclusion, the analysis of our data suggests that reduced levels of *GSTP1* and *RASSF1* expression in patients with prostate cancer before hormonal treatment in comparison to the post-treatment stage indicate that these genes are potential cancer markers in Iranian population as was reported in the previously approved data. Therefore, evaluating the patient at different stages of the disease can determine the severity and/or progression as well as the response to appropriate treatment.

Ethics and Consent Statement

This work was approved by the Ethics and Clinical Studies Committee of Shahid Beheshti University of Medical Sciences and the ethics committee of Men's Health and Reproductive Health Research Center (Tehran, Iran) and Islamic Azad University, Science and Research Branch, Tehran, Iran, Review board. Each patient signed a written consent according to the Declaration of Helsinki.

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Disclosure

The authors declare that they have no conflicts of interest.

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