

New indicator of cellular ischemia in coronary slow-flow phenomenon: Cell-free DNA

Koroner yavaş akım fenomeninde hücresel iskemi için yeni gösterge: Serbest DNA

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ABSTRACT

Objective: Coronary slow-flow phenomenon (CSFP) is defined as the delayed arrival of coronary blood flow to the distal vascular bed in at least 1 major epicardial coronary artery. Cell-free DNA (cfDNA) is a type of DNA that circulates freely in the blood once released from nucleated cells. The aim of this study was to determine if the level of cfDNA, which is an indicator of ischemia at the cellular level, was increased in CSFP.

Methods: The study included 46 patients in total: 23 patients with CSFP and 23 with a normal coronary angiogram (NCA). The level of cfDNA, and clinical, biochemical, and angiographic features of the groups were compared.

Results: The mean age was 53.8±10.3 years for the CSFP patient group and 56.6±9.4 years for the NCA patient group. There was no statistically significant difference between the groups in terms of basal clinical characteristics or laboratory data. The plasma cfDNA level was 5.04±2.37 ng/μL in the CSFP patients and 2.28±1.09 ng/μL in the NCA group (p<0.001).

Conclusion: Several invasive and noninvasive studies conducted on patients with CSFP have revealed myocardial ischemia. The results of this study demonstrated that the level of cfDNA was significantly increased in patients with CSFP as a result of ischemia at the cellular level caused by microvascular disruption.

ÖZET

Amaç: Koroner yavaş akım fenomeni (KYAF) en az bir majör epikardiyal koroner arterde kan akımının distal damar yatağına geç ulaşması olarak tanımlanır. Serbest hücresel DNA (shDNA), hücre nükleuslarından serbestleşen ve dolaşımda serbest olarak tespit edilebilen DNA tipidir. Bu çalışmada, KYAF'de hücresel düzeyde iskeminin göstergesi olan shDNA düzeylerinin artıp artmadığını göstermeyi amaçladık.

Yöntemler: Bu çalışmaya, 23 KYAF'lı ve 23 anjiyografik olarak normal koroner arterlere (NKA) sahip toplam 46 hasta alındı. Grupların shDNA düzeyleri, klinik, biyokimyasal ve anjiyografik özellikleri karşılaştırıldı.

Bulgular: Ortalama yaş KYAF grubunda 53.8±10.3 ve NKA grubunda 56.6±9.4 idi. Laboratuvar bulguları ve bazal klinik karakteristikleri yönünden gruplar arasında istatistiksel anlamlı fark yoktu. Plazma shDNA düzeyleri KYAF'da 5.04±2.37 ng/μL, NKA'da 2.28±1.09 ng/μL olarak tespit edildi (p<0.001).

Sonuç: Farklı invaziv ve noninvaziv çalışmalar KYAF'da miyokardiyal iskeminin varlığını göstermiştir. Çalışmamızda KYAF'lı hastalarda mikrovasküler bozukluğa bağlı olarak, hücresel düzeyde iskemi ve hasarın göstergesi olan shDNA düzeyinin anlamlı şekilde arttığını gösterdik.

Coronary slow-flow phenomenon (CSFP) is defined as the delayed arrival of coronary blood flow to the distal vascular bed in at least 1 major epicardial

artery without any stenosis in the epicardial coronary arteries.^[1] Routine coronary angiographies have revealed a CSFP incidence of 1% to 7%.^[2-4] Tambe et

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Abbreviations:

CAD	Coronary artery disease
CSFP	Coronary slow-flow phenomenon
cfDNA	Cell-free DNA
DM	Diabetes mellitus
FFS	Fractional flow reserve
HT	Hypertension
LAD	Left anterior descending artery
LCX	Left circumflex artery
MI	Myocardial infarction
MPS	Myocardial perfusion scintigraphy
NCA	Normal coronary angiogram
RCA	Right coronary artery
ROC	Receiver operating characteristic
TFC	TIMI frame count
TIMI	Thrombolysis in Myocardial Infarction

cardiac death, life-threatening arrhythmias, myocardial ischemia, recurrent acute coronary syndromes, and congestive heart failure.^[6–8] Though a number of studies performed in biopsies of the left and right ventricles of the patients with CSFP have shown that CSFP can cause luminal narrowing, changes in normal morphology of the nucleus, thickening of the capillary endothelium, and small vessel disease, such as pyknosis, the possible role of dynamic components affecting the microvascular structure remains to be demonstrated in order to support these findings.^[9,10]

Cell-free DNA (cfDNA) is a type of DNA that circulates freely in the blood after being released from nucleated cells.^[11,12] The origin of cfDNA is not clear; however, it has been proposed that cfDNA is released into circulation as a consequence of cell lysis, necrotic death, apoptosis, and active release.^[13] In 1948, Mandel et al.^[14] reported that some nucleic acids circulated in the blood. In several studies conducted shortly afterwards, it was suggested that cfDNA may occur as a result of metastasis. The quantitative measurement of cfDNA isolated from serum and plasma samples of patients with diseases such as rheumatoid arthritis, systemic lupus erythematosus, leukemia, chronic glomerulonephritis, and cancer has been observed to be generally higher than in normal individuals.^[15–19] A review of the literature demonstrates that, with the development of technical measurements, the quantitative value of cfDNA has been investigated in many diseases, as well as whether genetic analyses such as next-generation sequencing can be performed using cfDNA.^[20,21]

CSFP is an angiographic observation defined by the delayed passage of contrast media in the epicar-

al.^[5] described CSFP in 1972 in 6 patients who presented with chest pain and they suggested that this phenomenon could develop due to abnormalities in coronary microcirculation.

Several studies have reported an association between CSFP and sudden

dial vessels. Various studies have shown an increased risk of sudden cardiac death, life-threatening arrhythmias, and congestive heart failure in CSFP. These risks are increased even without the presence of coronary ischemia-induced stenosis, suggesting different etiopathogenetic conditions in these patients. The level of cfDNA is an important parameter that indicates cellular ischemia and may be a valuable sign demonstrating silent myocardial ischemia in these patients. The aim of the present study was to demonstrate whether the level of cfDNA was increased in CSFP.

METHODS**Study population**

This prospective study included 8350 patients who underwent coronary angiography between August 2017 and May 2019. CSFP was detected in 23 consecutive patients. An additional 23 subjects with a normal coronary angiogram (NCA) were randomly selected for comparison. In total, the study population consisted of 46 patients. The research was designed as cross-sectional, observational study. Patients with CSFP had slow flow in all 3 of their coronary arteries; patients who had slow flow in 1 or 2 coronary arteries were not included in the study. The indication for coronary angiography was typical angina and a positive exercise test or a perfusion defect observed with myocardial perfusion scintigraphy (MPS). The patients' clinical characteristics, including age, sex, smoking status, and dyslipidemia, hypertension (HT), and diabetes mellitus (DM) findings were recorded. Hemogram findings; kidney and liver function tests; troponin, C-reactive protein, lipid parameters; and thyroid hormones were evaluated in all of the patients. Potential study participants in both groups were excluded for a previous history of percutaneous coronary intervention, moderate-severe valvular disease, left ventricular dysfunction (ejection fraction <50%), left ventricular hypertrophy, myocardial infarction (MI), rhythm other than sinus, chronic systemic illness, chronic obstructive lung disease and/or cor pulmonale, renal failure, congenital heart disease, active infection, or neoplastic disease, with the concomitant presence of slow flow and stenotic lesions. Patients using nitrate medication before or during the coronary angiography and those with unstable angina pectoris or acute coronary syndrome were also excluded from the study if the coronary angiography detected

CSFP. HT was defined as a blood pressure of $\geq 140/90$ mmHg or the use of antihypertensive medication. DM was defined as a fasting blood glucose value of ≥ 126 mg/dL or the use of hypoglycemic agents. Hypercholesterolemia was defined as a baseline cholesterol level of >200 mg/dL and/or a low-density lipoprotein cholesterol level of >130 mg/dL or the use of statin medications. Those who had regularly smoked in the previous year were considered current smokers.

This study was approved by the institutional review committee and medical ethics committee (date: 30/04/2019, no: 04/11). Written informed consent was obtained from all of the patients.

Coronary angiography

A femoral arterial approach was used for selective left and right coronary angiography and an independent expert interventionalist analyzed the angiographic data. Iohexol was used as the opaque substance in all patients. Left heart catheterization was performed using the Judkins technique. Coronary arteries were displayed at the cranial and caudal angles in the right and left oblique view at 15 frames per second. The patient angiograms were re-evaluated by 2 interventional cardiologists to determine the Thrombolysis in Myocardial Infarction (TIMI) frame count (TFC) for each coronary artery. The first frame used was the frame in which the opaque material was delivered to the coronary artery ostium with visualization of the coronary artery, and the last frame was the frame necessary to image the distal landmark of the opaque material. The first posterolateral branch of the right coronary artery (RCA), left circumflex artery (LCX), and distal bifurcation of the left anterior descending artery (LAD) were defined as the distal section. The difference between the first and last frames was evaluated as the TFC. The frame counts of the LAD were divided by 1.7 to correct for the longer length of this vessel. After the correction, a value of 25 or more for all vessels was accepted as CSFP.^[22]

An NCA was defined as the lack of angiographic atherosclerosis during routine coronary angiography. These patients had angiographically normal coronary arteries and no provocative vasoreactivity test was performed.

Cell-free circulating DNA measurement

Angiography was performed after 12 hours of fasting.

Blood samples were taken during angiography immediately after diagnosis and centrifugation was initiated. Samples of approximately 10 mL of peripheral blood were centrifuged at 2500xg for 10 minutes. The supernatant was transferred to a new sterile tube which was again centrifuged at 16,000xg for 10 minutes, and this new supernatant was put into a new sterile tube and kept at -80°C . The Plasma/Serum Cell-Free Circulating DNA Purification Micro Kit (Cat. 55500; Norgen Biotek Corp., Thorold, Ontario, Canada) was used for the DNA isolation. The quantity of DNA was measured using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Statistical analysis

Statistical analysis was performed using SPSS for Windows, Version 16.0 (SPSS Inc., Chicago, IL, USA). Percentages, means, and SD were calculated. The Kolmogorov-Smirnov test was used to determine the normality of distribution. A t-test was used to compare parametric variables between groups. A chi-square test was used to compare non-parametric values and percentages. Statistical significance was established as $p \leq 0.05$. Receiver operating characteristic (ROC) curves were generated and used to determine the sensitivity and specificity of cfDNA and the cut-off value for predicting coronary slow flow.

Power analysis was conducted for cfDNA for both the CSFP and NCA groups. To obtain a precision of 10% at a type I error level of 5% with 80% power, the necessary sample size was determined to be 24. The study plan was to include at least 46 patients.

RESULTS

Results were obtained from 46 patients who underwent coronary angiography: 23 patients with isolated CSFP and 23 with an NCA. The mean age was 53.8 ± 10.3 years in the CSFP patients and 56.6 ± 9.4 years in the NCA patients. There was no statistically significant difference between the groups in terms of HT, hyperlipidemia, DM, family history of CAD, smoking status, age, or gender. The serum glucose, urea, creatinine, triglycerides, low-density lipoprotein, high-density lipoprotein, total cholesterol, thyroid stimulating hormone, and hematocrit levels were not significantly different between groups (Table 1).

The indication for coronary angiography was a positive treadmill exercise test (ST depression) or

the presence of ischemia observed with MPS. In the CSFP group, 13 patients had a positive treadmill exercise test and 10 patients had ischemia findings on MPS. In the NCA group, 11 patients had a positive treadmill exercise test and 12 patients had ischemia findings on MPS. There was no statistically significant difference between the groups with regard to the treadmill exercise test or the MPS ischemia. All of the patients had slow flow in all 3 coronary arteries: the mean corrected TFC for the LAD was 36.2 ± 8.2 , the TFC for the CX was 33.2 ± 6.9 and 32.9 ± 7.5 for the

RCA in patients with CSFP. The mean corrected TFC for the LAD was 18.1 ± 2.7 , the TFC for the CX was 17.9 ± 2.9 and the TFC for the RCA was 18.0 ± 2.7 in the NCA group (Table 2).

The mean plasma cfDNA level was 5.04 ± 2.37 ng/ μ L in the CSFP patients and 2.28 ± 1.09 ng/ μ L in the NCA group ($p<0.001$) (Table 2, Fig. 1). According to ROC analysis, the area under the curve for cfDNA was 0.887. The cut-off value of cfDNA to detect coronary slow flow was 3.21, with a sensitivity of 82.6 and

Table 1. Clinical and biochemical features of the patients

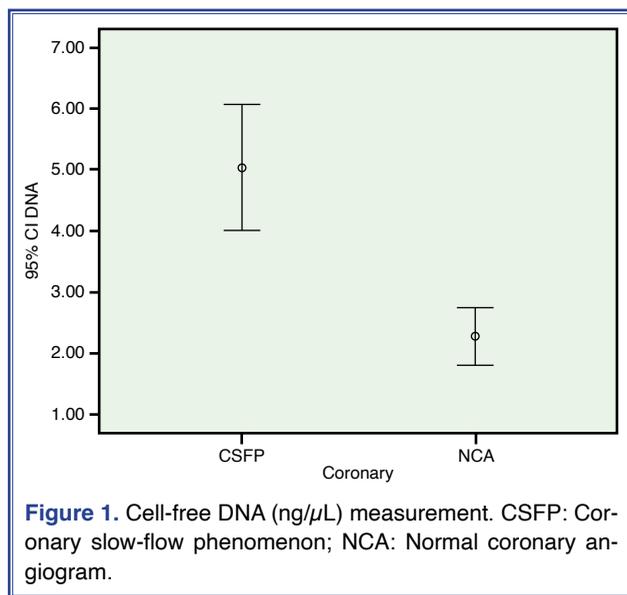
	CSFP (n=23)	NCA (n=23)	p-value
Age (years)*	53.8 \pm 10.3	56.6 \pm 9.4	0.337
Gender (male/female)*	13/10	11/12	0.559
Hypertension, n (%)*	8 (35)	9 (39)	0.763
Diabetes mellitus, n (%)*	7 (30)	6 (26)	0.746
Hyperlipidemia, n (%)*	8 (35)	7 (30)	0.756
Family history of CAD, n (%)*	6 (26)	5 (22)	0.732
Smoking, n (%)*	10 (43)	9 (39)	0.767
Fasting blood glucose (mg/dL)**	109.6 \pm 11.7	106.1 \pm 10.8	0.528
Urea (mg/dL)**	34.3 \pm 10.5	34.1 \pm 8.6	0.952
Serum creatinine (mg/dL)**	0.86 \pm 0.19	0.86 \pm 0.18	0.994
Total cholesterol (mg/dL)**	198 \pm 39	190 \pm 38	0.506
Triglycerides (mg/dL)**	165 \pm 56	148 \pm 55	0.304
LDL cholesterol (mg/dL)**	125 \pm 33	120 \pm 23	0.526
HDL cholesterol (mg/dL)**	40 \pm 9	41 \pm 8	0.667
TSH (mIU/L)**	1.57 \pm 0.96	1.46 \pm 0.82	0.671
Hematocrit**	42.6 \pm 2.8	42.9 \pm 2.8	0.714

*Chi-square test; **t-test. CAD: Coronary artery disease; CSFP: Coronary slow-flow phenomenon; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; NCA: Normal coronary angiogram; TSH: Thyroid-stimulating hormone.

Table 2. Treadmill exercise test, myocardial perfusion scintigraphy, TFC, and cfDNA measurement

	CSFP (n=23)	NCA (n=23)	p-value
Positive treadmill exercise test, n (%)	13 (57)	11 (48)	0.382
Ischemia on MPS, n (%)	10 (43)	12 (52)	
LAD-corrected TFC*	36.2 \pm 8.2	18.1 \pm 2.7	<0.001
Circumflex artery*	33.2 \pm 6.96	17.9 \pm 2.9	<0.001
Right coronary artery*	32.9 \pm 7.5	18.0 \pm 2.7	<0.001
Hs-troponin T*	7.83 \pm 3.83	7.96 \pm 3.23	0.902
cfDNA (ng/ μ L)*	5.04 \pm 2.37	2.28 \pm 1.09	<0.001

*T-test. cfDNA: Cell-free DNA; CSFP: Coronary slow-flow phenomenon; Hs-troponin: High-sensitivity troponin; LAD: Left anterior descending artery; NCA: Normal coronary angiogram; TFC: Thrombolysis in Myocardial Infarction frame count.



a specificity of 91.3% (Fig. 2).

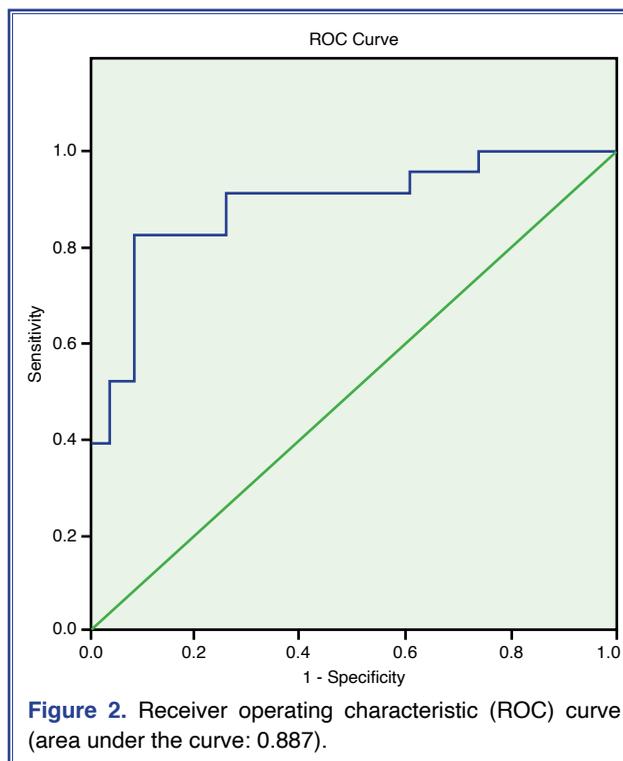
DISCUSSION

cfDNA is DNA found in plasma that is released from cells, particularly as a result of ischemic and apoptotic events. In the present study, it was found that the level of cfDNA was significantly increased in cases of CSFP.

CSFP can be detected through delayed dye opacification in the epicardial coronary arteries during an angiography, in the absence of obstructive CAD.^[23] The exact mechanism of action in CSFP is not clear. Various disorders, such as diffuse atherosclerosis, increased platelet aggregation, increased microvascular inflammation, and endothelial and microvascular dysfunctions have been proposed as responsible for the development of CSFP.^[24,25]

Mosseri et al.^[10] reported that the small coronary arteries of patients with CSFP can reveal proliferation of the myointimal layer, hypertrophy of the media layer, hyperplasia of the fibromuscular layer, swelling of the capillary endothelial cells, and degeneration of endothelial cells. Furthermore, there are lipofuscin deposits and the myocardium is hypertrophic.

Yilmaz et al.^[26] analyzed the high incidence of metabolic syndrome in patients with CSFP and the proposition that pathogenesis may be an early phase of atherosclerosis. They suggested that metabolic disorders disrupt the vascular structure. Sezgin et al.^[27]



reported that coronary resistance may be increased due to endothelial dysfunction in the small coronary arteries, which results in myocardial ischemia in patients with normal coronary arteries presenting with angina. Cin et al.^[28] found that patients with CSFP had diffuse intimal thickening and widespread calcification along the vessel wall. With respect to these findings, we suggest that CSFP can be considered diffuse atherosclerosis involving the epicardial coronary arteries and the microvascular system.

Pekdemir et al.^[29] examined epicardial coronary arteries that looked normal in cases of CSFP using the fractional flow reserve (FFR) and intravascular ultrasound, and it was suggested that a decreased FFR may be related to increased resistance in the epicardial coronary arteries due to diffuse atherosclerotic disease. Camsari et al.^[30] and Pekdemir et al.^[31,32] assessed high levels of endothelin-1 and low nitric oxide concentration in the peripheral blood and the coronary sinus both at rest and after atrial pacing with exercise-induced stress and demonstrated that endothelial functions were disrupted. Mangieri et al.^[33] detected glycogen content, mitochondrial abnormalities, and thickening of vessel walls with luminal size reduction in the endomyocardial biopsies of 20 patients with CSFP. Narimani et al.^[23] observed that the

lateral *s'* and *e'* waves were lower in patients with CSFP in a tissue Doppler study.

Studies of myocardial perfusion scan and cardiac stress tests have already shown ischemia in 30% to 80% of these patients.^[34] Goel et al.^[35] observed a positive stress test more commonly in patients with CSFP than NCA patients (71% vs 42%; $p < 0.01$). Cesar et al.^[35] have found perfusion abnormalities in 13 of 17 CSFP patients using MPS. Sadr-Ameli et al.^[36] noted ischemia in 109 of 217 patients in a long-term follow-up study using noninvasive techniques such as myocardial perfusion imaging, stress echocardiography, and exercise stress testing, and reported that CSFP cannot be considered a totally benign condition.

Acute cellular injury or programmed cell death are the main reasons for circulating cfDNA and it can be used as a marker of cellular damage. After apoptosis, it can be detected in the plasma or serum of healthy individuals.^[37,38] Recent studies have reported a high level of cfDNA in cases of autoimmune diseases, sepsis, trauma, pregnancy-associated disorders, infection, and a variety of malignancies.^[37,38]

Chang et al.^[39] found that the average concentration of cfDNA in 55 patients with MI was >10-fold higher than that of 274 normal controls. Jing et al.^[40] also found that the cfDNA level was significantly higher in patients with MI compared with controls. Furthermore, Destouni et al.^[41] reported that the concentration of cfDNA was significantly higher in patients with MI than in a healthy control group using real-time polymerase chain reaction testing of the β -globin gene during hospitalization. In a study performed using a DNA-based Alu assay, it was demonstrated that the level of cfDNA was significantly higher in patients with acute coronary syndrome when compared with the controls.^[42]

CSFP is characterized by delayed passage of contrast substance to the epicardial vessels and the etiopathogenesis has not yet been fully determined. Studies of MI have revealed that the level of cfDNA released from the myocardium after ischemia was statistically significantly high. Previous studies have also shown that CSFP is associated with different diseases, such as subclinical atherosclerosis, endothelial dysfunction, inflammation, coronary microvascular dysfunction, and small vessel disease. Invasive and noninvasive studies conducted on patients with

CSFP have demonstrated the presence of myocardial ischemia. In our study, we showed that the level of cfDNA, a marker of cellular ischemia and damage, was significantly increased as a result of ischemia at the cellular level caused by microvascular disruption in patients with CSFP.

Conclusion

Although it was thought to be a benign phenomenon by many clinicians, our study has shown that CSFP actually reveals subclinical findings. A high level of cfDNA in patients with CSFP suggests that impaired myocardial perfusion and the resulting ischemia cause myocardial cell lysis or rapid apoptosis, which leads to earlier programmed cell death. The high level of cfDNA in these patients might be a sign of increased cardiovascular risk. As reported in recent studies, evidence of ischemia at the cellular level indicates that this phenomenon cannot be considered a totally benign condition.

Limitations

Although the present study provided significant results, it has also some limitations. A larger number of patients could better represent the population. The lack of follow-up is a second limitation. In addition, exclusion of isolated coronary artery slow flow is another limitation of the study. Investigation of the relationship between isolated CSFP and cfDNA is a matter for future study.

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