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Original Research / Özgün Araştırma

Diagnostic efficiency of miR-21 and miR-34a serum levels in malign and benign prostate diseases

Malign ve benign prostat hastalıklarında miR-21 ve miR-34a serum düzeylerinin tanısal etkinliği

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Özet

Amaç: Bu çalışmada, benign prostat hiperplazisi, kronik prostatit ve prostat kanseri ayrımında miR-21 ve miR-34a serum seviyelerinin tanısal etkinliğinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntemler: Prostat iğne biyopsisi yapılan toplamda 70 hastadan (25 benign prostat hiperplazisi, 10 kronik prostatit ve 35 prostat kanseri) kan örnekleri alındı. Uygun koşullarda serum eldesinden sonra RNA izolasyonu, cDNA sentezi ve qRT-PCR analizi Rotor-Gene® Q (Qiagen, Germany) cihazında Qiagen marka kitler kullanılarak gerçekleştirildi. Normalizasyon için referans gen olarak RNU6 kullanılarak -ΔCt değerleri hesaplandı. Tüm istatistiksel hesaplamalarda -ΔCt değerleri kullanıldı.

Bulgular: Benign prostat hiperplazisine kıyasla kronik prostatit ve kanser gruplarında miR-21 serum seviyelerinin upregüle olduğu ve gruplar arasındaki farklılığın istatistiksel olarak anlamlı olduğu görülmüştür (sırasıyla p=0.021 ve p=0.001). miR-21'in tek başına spesifisite ve sensitivite değerleri benign prostat hiperplazisi ile prostat kanseri ayrımında %56 ve %86 olarak tespit edilmiştir. miR-21'in miR-34a ile kombinasyonunun spesifisite ve sensitivite değerleri benign prostat hiperplazisi ile prostat kanseri ayrımında %84 ve %71 olarak hesaplanmıştır.

Sonuç: Bu çalışma ile miR-21 ve miR-21/miR-34a kombinasyonunun prostat kanseri tanı-

Abstract

Objective: In this study aimed to determine the diagnostic efficiency of miR-21 and miR-34a serum levels in the discrimination of benign prostatic hyperplasia, chronic prostatitis, and prostate cancer.

Materials and Methods: Blood samples were taken from 70 patients (25 benign prostatic hyperplasias, 10 chronic prostatitides, and 35 prostate cancer) who underwent prostate needle biopsy. After obtaining serum under suitable conditions, RNA isolation, cDNA synthesis, and qRT-PCR analysis were performed using Qiagen brand kits on Rotor-Gene® Q (Qiagen, Germany) device. -ΔCt values were calculated using RNU6 as a reference gene for normalization. -ΔCt values were used in all statistical calculations.

Results: It was observed that miR-21 serum levels were upregulated in chronic prostatitis and cancer groups compared to benign prostatic hyperplasia and the difference between the groups was statistically significant (p = 0.021 and p = 0.001, respectively). The specificity and sensitivity of miR-21 and miR-21/miR-34a combination was calculated as 56% and 86%; 84% and 71% in discriminating benign prostatic hyperplasia and prostate cancer groups, respectively.

Conclusion: In this study, it has been shown that miR-21 and miR-21/miR-34a combination has diagnostic performance that can be a biomarker candidate in diagnosing prostate cancer.

The study was approved by the Dışkapı Yıldırım Beyazıt Research and Training Hospital Ethics Committee (Approval number: 06/34 . Date: 17 Dec 2012).
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All research was performed in accordance with relevant guidelines/regulations, and informed consent was obtained from all participants.

sında biyobelirteç adayı olabilecek tanısal perfomansa sahip olduğu gösterilmiştir. Ayrıca, miR-21 seviyelerinde benign prostat hiperplazisine kıyasla kronik prostatit ve prostat kanserinde kademeli bir yüksekliğin olması, moleküler düzeyde gerçekleşen inflamasyon ve kanser dönüşümü süreçlerinin dolaşımdaki mikroRNA profiline de yansıdığını düşündürmektedir.

Anahtar Kelimeler: Prostat kanseri, prostatit, benign prostat hiperplazisi, mikroRNA, tanısal etkinlik.

In addition, the presence of a gradual increase in chronic prostatitis and prostate cancer at miR-21 levels compared to benign prostatic hyperplasia suggests that inflammation and cancer transformation processes taking place at the molecular level are also reflected in the circulating microRNA profile.

Keywords: Prostate cancer, prostatitis, benign prostatic hyperplasia, microRNA, diagnostic efficiency.

INTRODUCTION

Prostate cancer is one of the most common types of cancer, especially in older men (1). Prostate cancer mortality is closely related to the stage of disease at diagnosis. The 5-year survival rate in localized prostate cancer is approximately 100%, while it is less than 40% in metastatic prostate cancer (2, 3).

Measurement of serum prostate-specific antigen (PSA) levels in the diagnosis of prostate cancer has increased the chance of diagnosing prostate cancers at an early stage. However, the PSA test is insufficient in determining the prostate cancer type and prognosis. Especially, the diagnosis of indolent prostate cancers and the application of treatment processes, which have a very slow growth rate and will not pose a life-long fatal risk, have revealed the idea that PSA test causes overdiagnosis and overtreatment in prostate cancer (4). Therefore, investigations for new biomarkers other than PSA have begun in prostate cancer, and micro RNAs are among the molecules on which studies continue for this purpose.

MicroRNAs (miRNA) are 20-22 nucleotide long RNA molecules. They bind to target mRNA, controlling gene expression by degradation of the mRNA or suppressing translation. miRNAs have positive or negative effects on cancer development by regulating tumor suppressor and oncogene genes. Studies on the diagnostic and prognostic use of tumor suppressor or oncogenic miRNAs detected in materials such as tissue, serum, plasma, urine, or saliva in cancer are ongoing (5).

miR-21 is an oncogenic miRNA that has been shown to act by targeting PTEN in prostate cancer. miR-34a is a miRNA reported to have tumor suppressor effects in prostate cancer via CD44 (6-8). This study aimed to determine the diagnostic efficiency of miR-21 and miR-34a serum levels to diagnose prostate cancer.

MATERIAL AND METHODS

This study is derived from a medical specialty thesis conducted in Ankara Diskapi Yildirim Beyazit Training and Research Hospital, Department of Medical Biochemistry. Ethical approval for this study was obtained from Diskapi Yıldırım Beyazit Research and Training Hospital (2012-06/34). Written informed consent was obtained from all volunteers before the study.

Sample Collection

The study samples consist of blood samples collected from patients who applied to the Department of Urology between 2012 and 2013 due to lower urinary tract symptoms or PSA elevation. These patient were performed 8-12 quadrant transrectal ultrasound (TRUS) guided prostate biopsy. The patients signed informed consent forms. Before the biopsy, blood samples were drawn from the patients. Anticoagulant-free gel tubes were used for blood collection. After the coagulation was completed, the samples were centrifuged in the NF800 centrifuge device (nüve®) at 2100xg for 10 minutes. PSA levels were measured in the Advia Centaur XP (Siemens) device. Serum was then aliquoted and stored at -80 °C until the miRNA analysis.

Based on the biopsy results, three study groups were formed: benign prostatic hyperplasia (BPH, n= 25), chronic prostatitis (CP, n= 10), and prostate cancer (PCa, n= 35). Volunteers with a previous diagnosis of neoplastic disease were not included in the study.

RNA isolation

MiRNeasy Mini Kit (Qiagen, Germany) was used for RNA isolation. The samples were dissolved and mixed at room temperature. First, 700 µL of Qiazol Lysis Reagent and 200 µL of serum were added to the tubes prepared for each sample. The tubes were mixed with a vortex device and left to stand at room tempera-

ture for 5 minutes. Then, 140 µL of chloroform was added to each tube, mixed well, and left to stand at room temperature for 2-3 minutes. Samples were centrifuged at 21.000xg at 4 ° C for 15 minutes. After centrifugation, the colorless upper part was pipetted with a 350 µL pipette and transferred to a new tube. The RNA isolation was completed by placing the tubes in the QIAcube (Qiagen, Germany) device following the procedure. RNA amounts were measured spectrophotometrically at 260 nm wavelength in a Nanodrop 2000 device (Thermo Scientific, USA), and it was observed that there was sufficient RNA content for the study. The RNAs obtained were either taken immediately for cDNA synthesis or stored at -20 ° C until cDNA synthesis was performed.

cDNA Synthesis

The miScript II RT Kit (Qiagen, Germany) was used for cDNA synthesis. First, 0.2 ml 8-strip tubes were numbered. In the tube were added miScript HiSpec Buffer, miScript Nucleics Mix, and miScript Reverse Transcriptase Mix at 4 µL, 2 µL, and 2 µL per sample to prepare the reaction mix, respectively. 8 µL of the reaction mixture and 12 µL of the patient's RNA sample were added to 0.2 mL tubes previously enumerated for each patient. After gentle mixing (without vortexing), the tubes were spin centrifuged. Later, the tubes were placed in a thermal cycler device at 37 °C for 60 minutes and at 95 °C for 5 minutes, and cDNA synthesis was performed. The synthesized cDNAs were stored at + 4 °C for a short time until the PCR step.

qRT-PCR Phase

Qiagen (Germany) brand kit based on the SYBR Green method was used to measure miRNA levels. miScript Primer Assays and cDNAs were reconstituted using 550 µL and 80 µL of RNase free water, respectively. For the PCR reaction, the reaction mix consisting of 12.5 µL SYBR Green PCR Master Mix, 2.5 µL RNase free water, 2.5 µL Universal Primer and 2.5 µL Primer Assay per sample number was prepared. Then, 20 µL of the prepared mixture for each sample and 5 µL of the cDNAs were added to the 0.2 ml PCR tubes. These procedures were repeated in the same way for miR-21, miR-34a, and RNU6 measurements. Tubes prepared with a total volume of 25 µL for each patient sample were placed in the PCR device (Rotor-Gene Q). Initial activation was applied at 95 ° C for 15 minutes. Then a reaction program was set up for 40 cycles at 94 ° C for 15 seconds, at 55 ° C for 30 seconds, and at 70 ° C for 30 seconds. Ct (threshold cycle) values greater than 40 were not included in the calculation.

Statistical Analysis

The SPSS program was used for statistical calculations. Whether the parameters were compatible with the normal distribution was evaluated using the Shapiro-Wilk test. While PSA values were not normally distributed. miR-21 and miR-34a values were normally distributed. The PSA values did not conform to the normal distribution; therefore, Kruskal-Wallis Variance Analysis specified the differences between groups. In order to determine the source of difference, the Mann-Whitney U test was used, in which the p value obtained by Bonferroni correction was used for significance.

RNU6 was used as a reference gene for normalization. Ct values of miR-21 and miR-34a were normalized with the formula -ΔCt = - (Ct Target miRNA - Ct Reference gene). -ΔCt values were used in all statistical analyzes.

ANOVA analysis was applied to examine the differences in miR-21 and miR-34a values between the groups, LSD test was used in post-hoc analysis. Box plot graphics were used to see the differences visually. The correlation between normally distributed parameters was analyzed by Pearson correlation analysis and did not show normally distributed parameters were analyzed by Spearman correlation analysis. ROC analysis was used to determine the diagnostic efficacy of miR-21 and miR-34a. Binary logistic regression analysis was performed to determine the diagnostic capability of a combination of miR-21 and miR-34a. The determination of the most appropriate cut-off value was performed using the Youden index. In statistical analysis, $p \leq 0.05$ was considered to be significant.

RESULT

Age and PSA values

The mean age of the study groups was calculated as 64 (50-79) for the BPH group, 66 (57-75) for the CP group, and 69 (51-82) for the PCa group. The differ-

ence between BPH and PCa groups' mean ages was significant (p = 0.020). The difference between the other groups in terms of age was not statistically significant (p> 0.05). The PSA values do not distribute normally; therefore, median values have been used for statistical analysis. PSA levels were determined as 5.3 μg/L (2.2-13.2) for the BPH group, 8.9 μg/L (4.7-22.6) for the CP group, and 23.1 μg/L (2.8-1654.0) for the PCa group. PCa median PSA value was different from BPH and CP groups (p <0.001 and p =0.017, respectively).

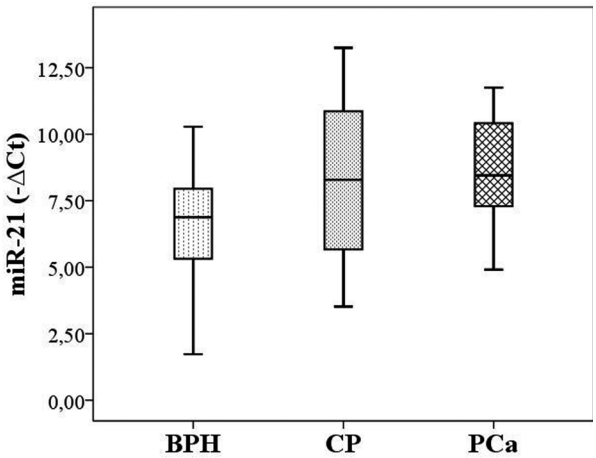
miR-21 and miR-34a levels between the groups

Serum levels of miR-21 were significantly upregulated in CP and PCa groups compared to BPH (p = 0.021 and p = 0.001, respectively). The difference between the groups in serum levels of miR-34a was not statistically significant (p> 0.05). Serum levels of miR-21 in the groups are presented in Figure 1.

In our study, there were 39 patients (22 BPH, 7 CP, 10 PCa) with PSA values between 2.5-10 μg/L (grey zone). When statistical analysis was performed on this subgroup, it was observed that miR-21 levels were significantly upregulated in the CP and PCa groups compared to BPH (p<0.001 and p=0.005, respectively).

In the correlation analysis, no statistically significant correlation was found between serum miR-21 levels and PSA, age, Gleason score (p> 0.05).

Figure 1. The box-plot of miR-21 serum levels between the groups



Diagnostic efficiency of serum miR-21 and miR-34a levels

Numerical data on the efficiency of serum miR-21 and miR-34a levels in diagnosing PCa and CP are presented in Table 1. Accordingly, in the discrimination between the benign group consisting of BPH and CP patients and the malignant group consisting of PCa patients, the AUC value of miR-21 alone was 0.682, while that of its combination with miR-34a was 0.765. Conversely, the AUC value of miR-21 in the discrimination of PCa from BPH was 0.746, while the AUC value of its combination with miR-34a increased to 0.840. In the discrimination of CP from BPH, the AUC value of the

Table 1. ROC analysis results for miR-21 and miR-34a in discrimination of prostate cancer

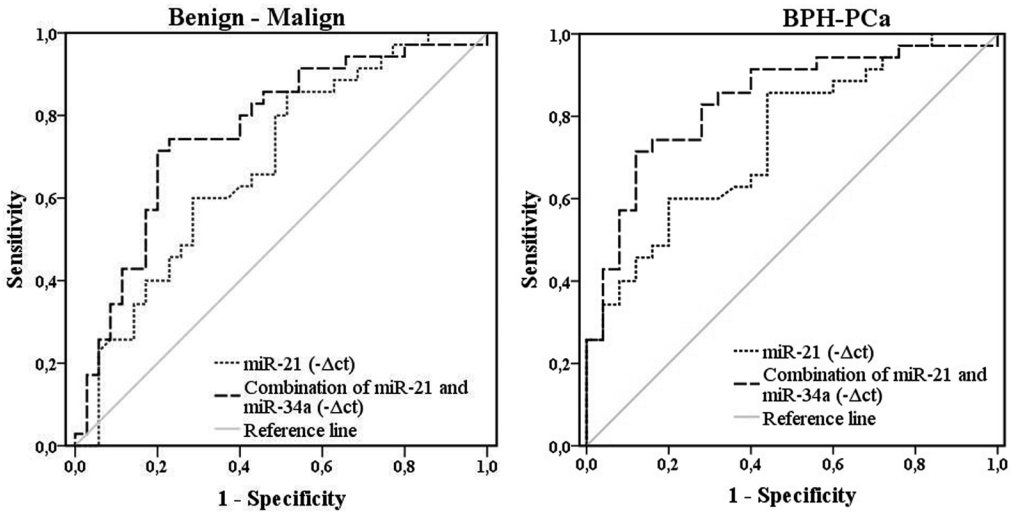
Groups	Variables	AUC	SE	P value	%95 CI
Benign vs Malign	miR-21	0.682	0.064	0.009	0.557 - 0.808
	miR-34a	0.433	0.069	0.335	0.298 - 0.568
	miR-21ve miR-34a	0,765	0.058	<0.001	0.651 - 0.879
BPH vs PCa	miR-21	0.746	0.063	0.001	0.622 - 0.869
	miR-34a	0.427	0.075	0.341	0.281 - 0.574
	miR-21 ve miR-34a	0.840	0.051	<0.001	0.739 - 0.941
BPH vs CP	miR-21	0.672	0.116	0.116	0.446 - 0.898
	miR-34a	0.452	0.110	0.661	0.237 - 0.667
	miR-21ve miR-34a	0.756	0.094	0.019	0.572 - 0.940

***AUC:** area under the curve; SE: standard error*

miR-21 and miR-34a combination was 0.756, which was considered statistically significant. There was no diagnostic efficiency of miR-34a alone for discrimination between the groups (p> 0.05). The sensitivity and specificity values were calculated for the parameters with significant diagnostic value in the ROC analysis. The specificity and sensitivity values of miR-21 alone were 49% and 86% for the discrimination between benign and malignant groups, respectively, while they were 56% and 86% for the discrimination between BPH and PCa groups, respectively. The specificity and sensitivity values of the combination of miR-21 with miR-34a were 80% and 71% in the discrimination between benign and malignant groups, 84% and 71% in the discrimination between BPH and PCa groups, and 72% and 80% in the discrimination between BPH and CP, respectively. Figure 2 shows the ROC curves.

According to the results of the ROC analysis performed on the subgroup of 39 patients (22 BPH, 7 CP, 10 PCa) with PSA values between 2.5-10 μg/L (grey zone) in our study, to discriminate CP and PCa from BPH the AUC values of miR-21 were calculated as 0.818 and 0.825, respectively. In this subgroup, the sensitivity and specificity of miR-21 for the discrimination between BPH and CP were 71% and 91%, respectively, while the sensitivity and specificity of miR-21 for the discrimination between BPH and PCa were 100% and 59%, respectively.

Figure 2. ROC curves in discrimination of prostate cancer



DISCUSSION

The survival rates in PCa are closely related to early diagnosis. There is clearly a need for a new diagnostic biomarker for PCa, as the PSA test has low sensitivity, and biopsy, an invasive procedure, is still required for definitive diagnosis. Therefore, since the 2000s, there has been a continuous rise in the number of studies investigating miRNAs as highly stable biomarker candidates. It has been previously reported that miR-21 serum levels are upregulated in PCa compared to healthy controls or BPH. In a study by Ağaoğlu et al., miR-21 serum levels were measured to be upregulated in localized/localized advanced PCa compared to the healthy control group, and miR-21 levels in the metastatic patient group were upregulated compared to those in patients with localized PCa (9). Watahiki et al. showed that plasma levels of miR-21 are upregulated in castration-resistant PCa compared to localized PCa, and miR-21, along with some other miRNAs, may also be a potential biomarker for discriminating between these two groups (10). The most important problem encountered in PCa screening in clinical practice is the insufficient sensitivity of the PSA test. In a study with large participation, when the cut-off for PSA measurements was defined as 4.1 ng/mL, the specificity and sensitivity of PSA were calculated as 93.8% and 20.5%, respectively (11). Therefore, it is necessary to develop new biomarkers, especially to discriminate between BPH

and PCa correctly. In our study, serum miR-21 levels increased in PCa compared to BPH, and the AUC value of miR-21 in the discrimination between BPH and PCa was 0.746 (Table 1). In our study, the specificity and sensitivity of miR-21 alone in the discrimination between BPH and PCa were determined to be 56% and 86%, respectively. This result is consistent with other studies that measured the serum levels of miR-21 in BPH and PCa groups. Kotb et al. determined that serum miR-21 levels were upregulated in PCa compared to BPH, and the specificity and sensitivity of miR-21 were both 90% to discriminate between PCa and BPH (12). A study by Endzelins et al., investigating the diagnostic efficacy of miR-21 measured in extracellular vesicles (EVs) and plasma samples, reported that miR-21 levels measured in EVs could be used to discriminate between BPH and PCa, with an AUC value of 0.670. However, the plasma levels of miR-21 were reported to be not statistically significant in discriminating PCa (13). In our study, miR-34a alone did not have statistically significant efficacy in the diagnosis of PCa. However, the diagnostic efficacy of miR-21 combined with miR-34a (AUC = 0.838) was determined to be higher than the miR-21 alone (AUC = 0.746). Accordingly, evaluating multiple miRNAs rather than single molecules could provide better diagnostic efficiency. An important finding in our study is that while there was not found a statistically significant difference in serum miR-21 levels between CP and PCa, there was a significant increase in CP compared to BPH. In a study by Chen et al. in accordance with our findings, miR-21 levels in the prostate secretions of patients with chronic prostatitis/chronic pelvic pain syndrome were upregulated more than two-fold (14). In addition, miR-21 was shown to take place effectively in PCa formation and growth.

Once activated, the androgen receptor directly interacts with the regulatory regions of miR-21. It indicates that miR-21 is an androgen-dependent molecule. miR-21 alone has also been shown to be sufficient for androgen-independent PCa formation. Thus, miR-21 plays a role in both androgen-dependent and androgen-independent PCa development (15). A study by Fabbri et al. revealed the relationship between miRNAs and the "toll-like receptor" (TLR) family. They reported that the binding of miR-21 and miR-29a as ligands to murine TLR-7 and human TLR-8 receptors might mediate prometastatic inflammatory responses, leading to tumor growth and metastasis (16). In summary, these studies revealed a possible role of miR-21 in cancer formation based on chronic inflammation. Our study is also consistent with these results.

CONCLUSION

In conclusion, with this study, we showed that miR-21 and the combination of miR-21 / miR-34a have diagnostic value as biomarker candidates for the diagnosis of PCa. In addition, the gradual elevation of miR-21 levels in CP and PCa compared to BPH gives rise to the thought that chronic inflammation and cancer transformation processes taking place at the molecular level is also reflected in the circulating miRNA profile. However, comprehensive investigations are needed to demonstrate the act of miRNAs in cancer development from CP and to determine the combined diagnostic efficiency of circulating miRNAs in the diagnosis of PCa.

Acknowledgment

The first results of this study are presented as an oral presentation. Oral presentation information is as follows. Dülgeroğlu Y, Erden G, Ekici M, Yeşilyurt A, Odabaş Ö, Uçar F, Öztürk G. Investigating the Circulating Mir-21 and Mir-34A Expression in Bph, Prostatitis and Prostate Cancer. 25th National Biochemistry Congress. 3-6 September 2013. İzmir, Turkey.

Conflict of Interest

The authors declare to have no conflicts of interest.

Financial Disclosure

Dışkapı Yıldırım Beyazıt Research and Training Hospital, Scientific Studies Support Board, Decision Date: 05 Dec 2012, Decision No: 36.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Ethical Approval

The study was approved by the Dışkapı Yıldırım Beyazıt Research and Training Hospital Ethics Com-

mittee (Approval number: 06/34) (Date: 17 Dec 2012). The study protocol conformed to the ethical guidelines of the Helsinki Declaration.

Author Contributions

Conception and design; YD, GE, Data acquisition; YD, ME, Data analysis and interpretation; YD, ME, AY, ÖO, Drafting the manuscript; YD, Critical revision of the manuscript for scientific and factual content; GE, FU, GÖ, Statistical analysis; YD, Supervision; GE.

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