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Makale / Article

The Influence of Radiotherapy on Circulating Mirna Expression Levels and Hemorheological Properties in Prostate Cancer

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Abstract

Background: In prostate cancer, radiotherapy has therapeutic effect and increase treatment efficacy and thus the survival rate. However radio-resistant tumors may relapse and metastases. In cancer cells, some genes effected by radiation has direct effects on results of radiotherapy. MikroRNAs (miRNAs) are the molecules which regulate some processes related with internal and external stresses. It has been showed that radiation resulted in some changes on synthesis of miRNAs and so, cellular responses in tumor cells. The aim of our study was to investigate expression levels of miR-20a and miR-106b and some biochemical parameters, and plasma viscosity in patients with prostate cancer, before and after radiotherapy.

Methods: 35 patients who admitted to Radiation Oncology Department for prostat cancer radiotherapy were included in this study. Blood samples were obtained before and after radiotherapy. miR-20a and miR-106b expressions were analyzed by using quantitative reverse-transcription polymerase chain reaction (qRT-PZR). Plasma viscosity values were measured by using Harkness capillary viscometer. Blood albumin and total protein levels were analyzed by autoanalyser system. Leukocyte, lymphocyte, erythrocyte, platelet and neutrophil counts were measured by automatic cell counter.

Results: We found that miR-20a and miR-106b expression levels, and plasma viscosity values increased in the patients after radiotherapy. We also found that erythrocyte and platelet counts, albumin and total protein levels did not significantly change while PSA, fPSA, leukocyte, lymphocyte and neutrophil counts significantly decreased in patients after radiation treatment. From Pearson's rank correlation analysis, we found that miR-20a expression levels positively correlated with miR-106b expression levels after radiotherapy group (r=0.722, p=0.01). The levels of fPSA correlated significantly with miR-106b expression levels before radiotherapy group (r=-0.598, p=0.014). Additionally, the leukocyte count correlated significantly with miR-20a expression levels before radiotherapy group (r=–0.474, p=0.035).

Conclusion: Our findings showed that expression levels of some miRNAs such as miR-20a and miR-106b and hemorheological parameters were changed in prostate cancer patient after radiation treatment. These changes might be an important factor for cancer treatment and metastasis. Effects of these changes on prostate cancer patients must be clarified with further studies.

Keywords: Radiotherapy, prostate cancer, miR-20a, miR-106b, plasma viscosity, blood cells

Özet

Amaç: Prostat kanserinde, radyoterapi hayatta kalma oranını ve tedavi etkinliğini arttıran terapötik yaklaşımlatdan birisidir. Ancak radyo-dirençli tümörler nüks edebilir ve metastaz yapabilir. Kanser hücrelerinde, radyasyondan etkilenen bazı genler, radyoterapi sonuçları üzerinde doğrudan etkiye sahiptir. MikroRNA'lar (miRNA'lar), iç ve dış streslerle ilgili bazı süreçleri düzenleyen moleküllerdir. Tümor hücrelerinin radyasyona hücresel yanıtında miRNA'ların sentezinde bazı değişikliklere neden olduğu gösterilmiştir. Çalışmamızın amacı radyoterapi öncesi ve sonrası prostat kanseri olan hastalarda miR-20a ve miR-106b ekspresyon düzeyleri, bazı biyokimyasal parametreler ve plazma viskozitesindeki değişiklikleri araştırmaktır.

Materyal ve Metod: Çalışmaya radyoterapisi için Radyasyon Onkolojisi Bilim Dalı'na başvuran 35 prostat kanserli hasta dahil edildi. Radyoterapiden önce ve sonra kan örnekleri alındı. miR-20a ve miR-106b ekspresyonları kantitatif ters transkripsiyon polimeraz zincir reaksiyonu (qRT-PZR) kullanılarak analiz edildi. Plazma viskozite değerleri Harkness kapiler viskometre kullanılarak ölçüldü. Kan albumin ve toplam protein seviyeleri otoanalizör sistemi ile analiz edildi. Lökosit, lenfosit, eritrosit, trombosit ve nötrofil sayımı, otomatik hücre sayımı cihazında ölçüldü.

Bulgular: Radyoterapi sonrası hastalarda miR-20a ve miR-106b ekspresyon seviyelerinin ve plazma viskozite değerlerinin arttığı bulundu. Radyoterapi sonrası hastalarda PSA, fPSA, lökosit, lenfosit ve nötrofil sayıları önemli derecede azalırken, eritrosit ve trombosit sayıları, albumin ve total protein düzeyleri arasında anlamlı bir değişiklik olmadığı bulundu. Pearson korelasyon analizi ile, radyoterapi sonrasında miR-20a ile miR-106b ekspresyon düzeylerinin pozitif korelasyon gösterdiği bulundu (r = 0.722, p = 0.01). Radyoterapi öncesinde fPSA ile miR-106b ekspresyon düzeyleri arasında anlamlı negatif bir korelasyonun olduğu görüldü (r = -0.598, p = 0.014). Ayrıca, radyoterapi öncesinde lökosit sayısı ile miR-20a ekspresyon düzeyleri arasında anlamlı negatif bir korelasyonun varlığı gösterilmiştir (r = -0.474, p = 0.035).

Sonuç: Bulgularımız, prostat kanserli hastalarda radyoterapi sonrasında miR-20a ve miR-106b gibi bazı miRNA'ların ekspresyon düzeylerinin ve hemorheolojik parametrelerin değiştiğini göstermektedir. Bu değişiklikler, kanser metastazı için önemli bir faktör olabilir. Bu değişikliklerin prostat kanserli hastaların üzerindeki etkileri daha ileri çalışmalarla açıklanmalıdır.

Anahtar Kelimeler: Radyoterapi, prostat kanseri, miR-20a, miR-106b, plazma viskozitesi, kan hücreleri

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Introduction

Prostate cancer is the most common cancer among American men, with 220.800 estimated new cases in 2015. Typically, patients with localized prostate cancer are treated with radical prostatectomy or primary radiation therapy and around 70% of those patients are cured.[1]

In prostate cancer, radiotherapy has therapeutic effect and increase treatment efficacy and thus the survival rate. However radio-resistant tumors may relapse and metastases. In cancer cells, some genes effected by radiation has direct effects on results of radiotherapy. Ionizing radiation (IR) influence human health as they break chemical bonds of the molecules and damage DNA by the production of free radicals and hence proliferative cells can undergo apoptosis.[2] Failure to repair this type of damage leads directly or indirectly to cell death. While some cells undergo apoptosis immediately after irradiation, it is generally thought that tumor eradication occurs by mitotic (or "reproductive" or "delayed") cell death resulting from IR-induced injury that causes cell inactivation and becomes lethal after a few cell divisions. In this case, the most relevant processes to cellular radiosensitivity are the double-strand break repair pathways: homologous recombination (HR) and nonhomologous end-joining (NHEJ).[3-6]

MikroRNAs (miRNAs), molecules composed approximately 19-23 nucleotides, regulate gene expression at the post-transcriptional levels related with internal and external stresses. It has been showed that radiation resulted in some changes on synthesis of miRNAs and so, cellular responses in tumor cells. In the literature, although there are some information about changes of miRNA profiles in prostate cancer cell lines, there are very limited number of human studies. It has been reported miRNAs levels have established functions, especially in cell cycle and apoptosis regulation as response to radiotherapy, in vivo and in vitro. Numerous studies have identified many dysfunctional miRNAs by using a high-throughput approach, which contributed to prostate cancer progression, including the let-7 family, -20a, -21,-34a, -106b, -125b, -205 and -521.[7]

Li et al. found that miR-106b was dysregulated after radiation treatment and radiation-induced p21 activation was suppressed, suggesting it may override radiation-induced cell cycle arrest and cell growth inhibition.[8] miR-20a is one of the members of the mir-17- 92 cluster. Sylvestre et al. described an overexpression of miR-20a in the human prostate cancer cell line PC3 using PCR.[9] Volinia et al. recorded an up-regulation of miR-20a in PCa tissue using a microarray assay.[10] The identified function of miR-20a is the modulation of the E2F2 and E2F3 mRNAs translation via binding sites in their 3'-untranslated region, this supports the oncogenic behavior of miR-20a. Pesta et al. showed that the more dedifferentiated cancer cells (Gleason score 7-10) have a higher expression of oncogenic miR-20a.[11]

Stem cells are mostly radiosensitive and their damage by radiations can cause transformation of the functional cells into non-functioning cells. Long term exposure to even low doses of radiation can affect proliferating cells.[12] IR exposure is sensitive for those tissues (i.e., bone marrow, the gastrointestinal tract and skin) which turnover rapidly as well as to those tissues (i.e., central nervous system, lung, heart, liver, kidney and gonads) which turnover slowly.[13] Hematopoietic system is one of the most radiosensitive systems because its functional cells transport oxygen, which is the source of free radicals after IR.[14] Blood forming cells are located in bone-marrow and such cells are highly susceptible to radiation damage.[15] Changes of plasma viscosity values, miRNAs levels and blood cells counts affect the blood hemodynamic properties after radiotherapy.

The aim of the study was to examine the effects of IR on plasma viscosity, the blood cells count parameters and miR-20a and miR-106b expression levels of prostate cancer patients who admitted to Radiation Oncology Department for radiotherapy.

Materials and Methods

Our study group consisted of 35 patients with prostate cancer (PCa) who underwent external beam radiotherapy for prostate cancer was included in this study between March 2014 and December 2016 in Clinic of Radiation Oncology (Ministry of Health, Istanbul Research and Training Hospital, Istanbul, Turkey). Intensity-modulated radiotherapy (IMRT) used linear accelerators to safely and painlessly deliver precise radiation doses to a tumor while minimizing the dose to surrounding normal tissue in the patient with prostate cancer. According to the histopathological evaluation, all of patients were adenocarcinoma patients with PCa. The protocol was approved by the Ethics Committee of Sakarya University, Medical Faculty, and was conducted in accordance with

the Declaration of Helsinki. Informed consent was obtained from each study subject. Blood samples were taken on admission before initiation of radiation therapy and after completion of radiation therapy.

Whole blood samples were collected into EDTA containing and without antikoagulant vacutainers in the morning after a 12 h fasting. Then, the samples were immediately centrifuged for plasma and serum (without anticoagulant) separation at 3000×g for 10 min. Whole blood samples were collected and divided into two aliquots, and stored at –80 °C until RNA isolation. Total protein and albumin levels in serum were measured by colorimetric methods with commercially available kits (Cobas 8000, Roche Diagnostics GmbH, Mannheim, Germany). Blood cells count were measured by automated hematology analyzer (Beckman Coulter, CA, USA). Blood RNA was isolated using total RNA Purification Kit (Jena Bioscience Building Blocks Of Life, Thüringen, Germany, Cat. no: PP-210S) according to manufacturer's instructions. Thermo Scientific Nanodrop 2000c spectrophotometer was used to determine the purity and concentration of RNA samples. 1 µg of isolated RNA was used for cDNA synthesis with Cohesion Biosciences kits (Cat No: CPK1027 for miRNA-20a; CPK1076 for miRNA-106b; London, UK) according to manufacturer's instructions. Real-time PCR assays of the transcribed cDNA were performed using the Cohesion Biosciences microRNA assays. Circulating levels of miRNAs expression were analyzed by quantitative reverse transcription-PCR. The copies number of each samples are determined according to the standard curve after the RT-PCR. The copy number of 0.1 µM synthetic RNA, our working standard, is 6×107. Thus, the copy numbers of samples are given by the device according to the standard curve.

Plasma viscosity were made according to the recommendations of the International Committee for Standardization in Haematology (ICSH) using a Harkness capillary viscometer (Coulter Electronics, Ser. No. 6083, Luton, England) and evaluated in relation to distilled water (relative viscosity), the water bath of which was maintained at 37°C.[16,17]

Statistical Analysis

Results are shown as mean±standart deviation and mean±standart error. Statistical analysis was performed using SPSS 17.0 statistical software for Windows (SPSS, Chicago, IL, USA). The distribution of variables was assessed by the Kolmogorov-Smirnov test. Non-parametric (Mann-Whitney U test) and parametric (t-test) tests were used for quantitative analyses of the biochemical parameters, plasma viscosity and miRNAs expression levels. Pearson correlation test was used for the detection of the relationship between variables. The results were evaluated in confidence interval of 95 % and statistical significance of $p<0.05$.

Results

In total, 35 patients were included in the analysis. miR-20a, miR-106b expression levels, total proteins levels, blood count and plasma viscosity were available for these patients.

Table 1. Some biochemical parameters in patients with prostate cancer before and after radiotherapy groups (mean±SD) (Student t- test or Mann-Whitney U test)

Parameters	Before Radiotherapy	After Radiotherapy	p
Total Protein (g/dl)	7.17±0.27	7.04±0.38	0.217
Albumin (g/dl)	4.25±0.31	4.13±0.30	0.082
Leukocyte (103/mm3)	7.02±2.02	5.51±1.11	0.012
Erythrocyte (106/mm3)	4.84±0.36	4.62±0.59	0.065
Platelet (103/mm3)	256.353±61.977	231.706±70.826	0.069
Neutrophil (%)	60.93±8.09	50.58±5.89	0.006
Lymphocyte (%)	23.19±7.84	17.08±3.84	0.007
Plasma Viscosity (mPas)	1.22±0.11	1.42±0.05	0.018

The mean (±standard deviation) age of the patients was 71.76±7.02 years, ranging from 60 to 87 years. The mean BMI was 26.96 (range: 20–35). The majority of patients (60 %) presented with stage T2c (high risk), other patients presented with stage T2b (20%) (intermediate risk) and T2a (20%) (low risk) disease (risk assessment D'Amico classification).

There was no significant difference in total protein (7.17±0.27 vs. 7.04±0.38, p=0.217), albumin (4.25±0.31 vs. 4.13±0.30, p=0.082), erythrocyte (4.84±0.36 vs. 4.62±0.59, p=0.065) and platelet (256.353±61.977 vs. 231.706±70.826, p=0.069) counts among the patients before and after radiotherapy (Table 1). However, there were significant differences between PSA, fPSA, plasma viscosity, leukocyte, lymphocyte and neutrophil counts among the patients before and after radiotherapy (p=0.035, p=0.020, p=0.018, p=0.012, p=0.007 and p=0.006, respectively) (Figs. 1 and 2) (Table 1). miR-20a and miR-106b expression levels were

increased in patients after radiotherapy (p<0.001 and p<0.001, respectively) (Figs. 3 and 4).

From Pearson's rank correlation analysis, we found that miR-106b expression levels positively correlated with miR-20a expression levels (r=0.954, p<0.01) and negatively correlated with fPSA levels (r=-0.598, p=0.014) before radiation therapy. Additionally, the leukocyte count was negatively correlated with miR-20a expression levels before radiotherapy group (r=–0.474, p=0.035). miR-20a expression levels was also positively correlated with miR-106b expression levels after radiotherapy (r=0.722, p=0.01)

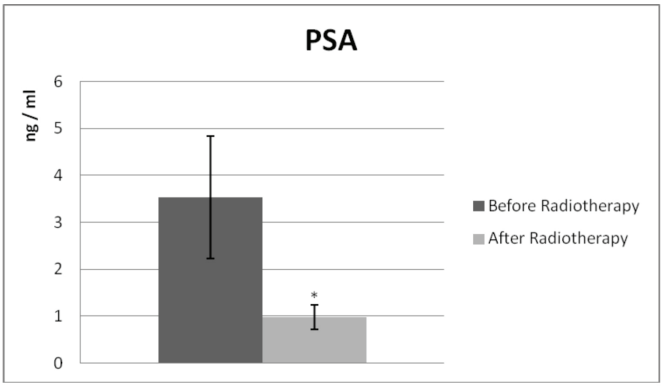

Figure 1. The mean levels of PSA in patients with prostate cancer before and after radiotherapy groups (mean±SE).*p<0.05, significantly different from the before radiotherapy group (Mann-Whitney U test)

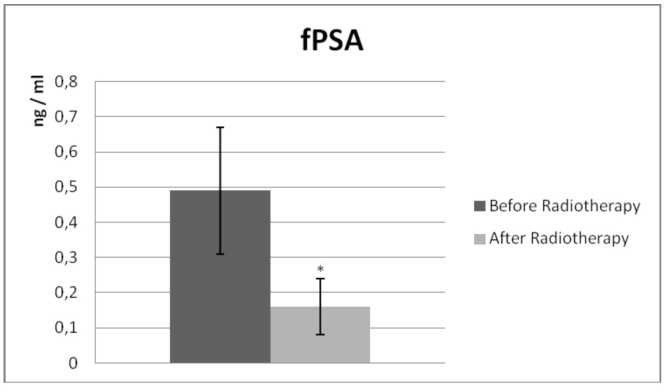

Figure 2. The mean levels of fPSA in patients with prostate cancer before and after radiotherapy groups (mean±SE).).*p<0.05, significantly different from the before radiotherapy group (Mann-Whitney U test)

Discussion

In the present study, we investigated ionizing radiation-response of miRNAs such as miR-20a and miR-106b. Previously, these miRNAs have been studied in prostate cancer cell lines. We investigated that expression levels of miR-20a and miR-106b before and after radiotherapy in patients with prostate cancer for the first time. We also searched relationship between miRNAs expression levels and some biochemical parameters, plasma viscosity in the same patients group. In the literature, no studies have examined the effects of radiation on miRNA expression levels and hemorheological parameters in prostate cancer. Although there are some information about changes of miRNA profiles in prostate cancer cell lines, there are very limited number of human studies. miRNAs expression levels have contradictory results in these studies. A wide variety of factors, advanced as a source of these contradictory findings.

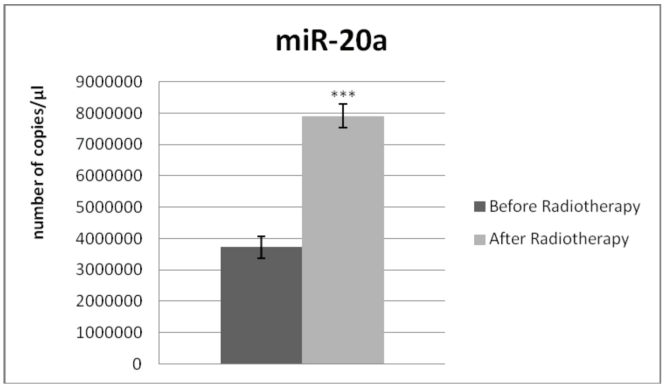

Figure 3. The mean expression levels of miR-20a in patients with prostate cancer before and after radiotherapy groups (mean±SE).).***p<0.001, significantly different from the before radiotherapy group (Mann-Whitney U test).

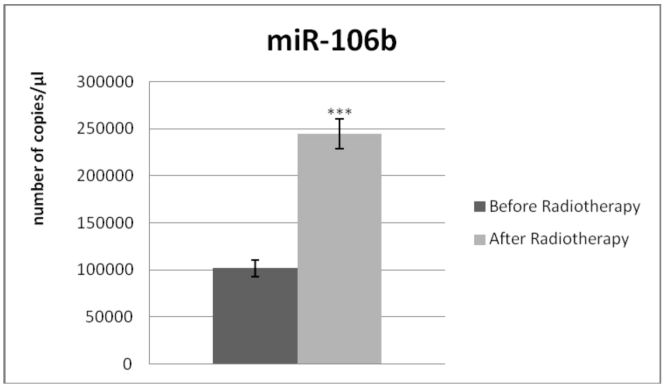

Figure 4. The mean expression levels of miR-106b in patients with prostate cancer before and after radiotherapy groups (mean±SE).).***p<0.001, significantly different from the before radiotherapy group (Mann-Whitney U test).

IR is one of the primary modalities used in cancer therapy. Radiation induces considerable DNA damages, which, if not repaired, cause cancer cells to prog¬ress to apoptosis and cell cycle ar-

rest. Some cancer cells are resistant to radiation treatment due to activation of complex signaling pathways that counteract these damages, including ErbB, nuclear factor B (NF B), MAPK, PI3K/AKT and trans¬forming growth factor-(TGF-) signaling pathways. Several radiation-related miRNAs have been identified that contribute to the radiosensitivity of cancer cells by modu¬lating the radiation-response signaling pathway. miRNAs are known to function as gene silencers and are involved in modulating biological functions, including apoptosis, the cell cycle and the metastasis of multiple types of cancers. Since miRNAs are generally slightly repressed by their target genes, the alteration of an individual miRNA is insufficient for accomplishing a biological function. Several studies have investigated the role of miRNAs to develop prostate cancer therapy; few studies have determined the roles of miRNAs in radiation response in prostate cancer.[18-21]

Comprehensive miRNA profiling of prostate cancer has indicated that several miRNAs are differentially expressed between prostate cancer and the adjacent normal, which contributes to prostate cancer progression. The expression levels of ionizing radiation-induced miRNAs are frequently dysregulated in prostate cancer. Some of miRNAs expressions are increased or decreased due to radioresistence or radiosensitivity. In this case, microRNAs have played an important role in determining the efficacy of radiotherapy in prostate cancer. Thus, understanding the function of miRNAs may provide practical benefits for clinical treatments of multiple types of cancers. Predicting the outcome of cancer treatment may be the most promising application of miRNAs.

Sylvestre et al. reported that increased expression levels of miR-20a in the human prostate cancer cell line.[9] Additionally, Volinia et al. recorded an up-regulation of miR-20a in PCa tissues.[10] The identified function of miR-20a is the modulation of the translation of the E2F2 and E2F3 mRNAs via binding sites in their 3'-untranslated region, this supports the oncogenic behavior of miR-20a. The same authors also observed that miR-20a overexpression reduced apoptosis in the PC3 cell line.[9] Pesta M et al. showed that the more dedifferentiated cancer cells (Gleason score 7-10) have a higher expression of oncogenic miR-20a. They found no statistical differences in the miRNA expressions (mir-20a,let-7a, miR-15a and miR-16) in the PCa tissue samples in comparison with the BPH tissue samples.[11]

We found that the levels of fPSA were negatively correlated with miR-106b expression levels, the leukocyte counts were negatively correlated with miR-20a expression levels before radiotherapy group. Additionally, we found that miR-20a expression levels positively correlated with miR-106b expression levels after radiotherapy group. These correlations between miRNAs and fPSA and leukocyte counts in this patient groups may reflect responsiveness of cancer cells to radiotherapy. In the present study, we found that miR-20a and miR-106b expression levels were increased in patient with PCa after radiotherapy. In terms of microRNA expression levels, there was a significant difference between the two groups before and after radiotherapy, suggesting roles of these miRNAs in radiotherapy of human prostate cancer. Underlying mechanisms of this role is not clear; it might be a reason or a result. It should be kept in mind that individual differences in changes of these miRNAs expressions may responsible for individual differences in response to the radiotherapy.

It is known that bone marrow system and blood cells are highly susceptible to ionizing radiation damage. Many studies have shown that ionizing radiation causes a reduction in the number of blood cells and degradation of the clotting mechanism. In our findings, leukocyte, neutrophil, lymphocyte counts were found to be decreased. Also, ionizing radition induces chemical changes in biomolecules, such as proteins. These changes are cross-linking, aggregations, structures, conformation and concentration. It is well known that these physicochemical changes in protein molecules closely influence the rheological properties of the blood. In the present study, we found that the changes in protein levels were no statistical differences. The increase in plasma viscosity observed after radiotherapy in our study may be due to other physicochemical changes in protein structures rather than protein concentrations, and other factors that play a role in the clotting mechanism.

Our findings showed that expression levels of some miRNAs such as miR-20a and miR-106b and hemorheological parameters were changed in prostate cancer patients after radiotherapy. These changes might be an important factor for cancer treatment and metastasis. In determining the efficacy of radiotherapy and the survival of the patient, the mechanisms of these microRNA levels need to be supported by more detailed further studies.

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Conflict of Interest
The authors declare that they have no conflicts of interest related to the publication of this manuscript.

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