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How do the effective therapeutics for hepatocellular carcinoma treatment change PIWI Interacting RNA expressions?

Hepatoselüler karsinom tedavisi için etkili terapötikler PIWI Interacting RNA ifadelerini nasıl değiştirir?

Çağrı Öner[1]*, Necdet Altıner[2], Ertuğrul Çolak[3]

1.*Maltepe University, Medical Faculty, Department of Medical Biology and Genetics, 34857, İstanbul, Turkey*
2.*Maltepe University, Medical Faculty, Department of Histology and Embryology, 34857, İstanbul,Turkey*
3.*Eskişehir Osmangazi University, Medical Faculty, Department of Biostatistics, 26040, Eskişehir, Turkey*

ABSTRACT

Aim: PIWI interacting RNAs (piRNAs) are novel members of small non-coding RNAs that cannot produce proteins but are effective on transcription and post-transcriptional mechanisms of cells. Nowadays, the application of both natural compounds and vitamins is essential for treatment of cancer cells instead of chemical compounds. In this study, we aimed to detect possible expression changes of piRNAs in order to compare 4- Hydroxycoumarin to the active form of vitamin D (1.25-Dihydroxyvitamin D) in hepatocellular carcinoma.

Methods: According to our previous study, HePG2 cells were treated with 4- Hydroxycoumarin, 1.25-Dihydroxyvitamin D and drug form of vitamin D at the optimal time and concentration. After treatment, the total RNA was isolated and expressions of piR-Hep-1 and piR-651 were determined by using Real Time Polymerase Chain Reactions.
Results: According to our obtained data, statistically significant upregulation of piR-651 expression was observed in 4-Hydroxycoumarin-treated HePG2 cells compared to control ($p<0.001$). However, the expression of piR-Hep-1 statistically was not affected from 4- Hydroxycoumarin treatment ($p>0.05$). In contrast, 1.25-dihydroxyvitamin treatment downregulated the expression of piR-Hep-1 statistically significant in HePG2 cells ($p<0.001$). piR-Hep-1 was not statistically significant effected from drug form of vitamin D treatment ($p>0.05$).
Conclusion: Our results indicated that some of the piRNAs might have special expression patterns in hepatocellular carcinoma and these expression patterns can be regulated by treated natural compounds. We suggest that substances that are observed to be effective in hepatocellular carcinoma individually may result in different piRNA expression changes contrary to the expectations.

Keywords: 1.25-Dihydroxyvitamin D, 4-Hydroxycoumarin, Hepatocellular Carcinoma, piR- 651, piR-Hep-1

ÖZ

Amaç: PIWI interacting RNA'lar (piRNA'lar) herhangi bir protein üretemeyen ancak hücrelerin transkripsiyon ve transkripsiyon sonrası mekanizmalarında etkili olan küçük kodlayıcı olmayan RNA'ların yeni üyeleridir. Günümüzde, kanser hücrelerinin tedavisinde kimyasal bileşikler yerine, hem doğal bileşikler hem de vitaminler uygulanabilirliği araştırılmaktadır. Bu çalışmadaki amacımız, 4-Hidroksikoumarinin ve aktif D vitamini formunun (1.25-dihidroksivitamin D) hepatoselüler karsinomada piRNA'ların olası ekspresyonları üzerindeki değişiklikleri belirlemektir.
Yöntemler: Önceki çalışmamızdan elde edilen verilere göre, optimal zaman ve konsantrasyonu belirlenen 4-Hidroksikoumarin, 1.25-dihidroksivitamin D ve D vitamininin ilaç formu HePG2 hücrelerine uygulandı. Uygulamadan sonra total RNA izole edildi. piR-Hep-1 ve piR-651'in ekspresyonları Gerçek Zamanlı Polimeraz Zincir Reaksiyonları kullanılarak belirlendi.
Bulgular: Elde edilen verilere göre, 4-Hidroksikoumarin uygulanan HePG2 hücrelerinde kontrole göre piR-651 ekspresyonunda istatistiksel olarak anlamlı bir artış gözlenmiştir ($p< 0.001$). Bununla birlikte, 4-Hidroksikoumarin uygulamasından sonra piR-Hep-1 ekspresyonundaki değişim istatistiksel olarak anlamlı değildir ($p> 0.05$). Buna karşılık, 1,25- dihidroksivitamin uygulaması HePG2 hücrelerinde piR-Hep-1 ekspresyonunu istatistiksel olarak anlamlı şekilde azaltmıştır ($p <0.001$). D vitamininin ilaç formunun uygulamasından sonra piR-Hep-1 ekspresyonundaki azalma istatistiksel olarak anlamlı değildir ($p> 0.05$).
Sonuç: Tüm bu veriler, piRNA'ların bazılarının hepatoselüler karsinomda özel ekspresyon paternlerine sahip olabileceğini ve bu ekspresyon paternlerinin, uygulanan doğal bileşikler tarafından düzenlenebileceğini göstermektedir. Hepatoselüler karsinomda tek tek etkili olduğu gözlenen maddelerin, beklentilerin aksine farklı piRNA ekspresyon değişikliklerine neden olabileceğini savunmaktayız.

Anahtar Kelimeler: 1.25-Dihidroksivitamin D, 4-Hidroksikoumarin, Hepatoselüler Karsinom, piR-651, piR-Hep-1

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*Corresponding Author: Çağrı ÖNER. Maltepe University, Medical Faculty, Department of Medical Biology and Genetics, İstanbul, Turkey, +905057460721, cagri.oner@maltepe.edu.tr

ORCİD: 0000-0003-3771-3277

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INTRODUCTION

PIWI Interacting RNAs (piRNAs) are the novel member of a small non-coding RNA family. piRNAs are short (26-31 nucleotide in length) and single stranded RNA sequences that work with Argonate proteins, which are called PIWI proteins. piRNAs are functional on transposon silencing, gene and protein regulation, genome rearrangement, spermatogenesis and survival of germ cells. piRNAs can be tumor suppressing or oncogenic according to the characteristics of the target regions they affect, such as miRNAs. Studies shows that these features may differ according to the cancer types [1-3]. piR-Hep-1 expression has been investigated especially in hepatocellular carcinoma and liver cancers. Furthermore, high piR- Hep-1 expression was detected in hepatic cancerous tumors and cell lines [4]. piR-651 is another piRNA which especially has oncogenic characteristics in gastric, colon, lung and breast cancer [3, 5]. Furthermore, there are some clinical evidence indicating that piR-651 and PIWI proteins might have an essential role on the development of pancreas, gastric and esophagus cancer [6, 7]. In mesothelioma and hepatocellular carcinoma, high piR-651 expressions were observed in tumors compared to healthy tissues. It is thought that piRNAs and PIWIs have a potential role in tumor formation [8].

Hepatocellular carcinoma (HCC) is the fourth most common cancer worldwide. The main causes of this type of cancer are Type II diabetes, obesity and alcohol [9]. Since HepG2 cells have properties similar to normal hepatocytes, they are used more in the study of liver toxicity and the metabolism of xenobiotics. Moreover, HepG2 cells can hydroxylate compounds to the active form of vitamin D, which is also known as 1.25-dihydroxyvitamin [1.25(OH)2D3] [10].

Vitamin D is a type of fat-soluble vitamin that is metabolized in the liver. It is used as a treatment or a preventive measure in various liver diseases. Excessive vitamin D deficiency is observed in liver diseases such as cirrhosis and hepatic hemangioma [11]. Vitamin D, obtained both from nutrients and directly from sunlight, is transformed into an active form in the liver, 1.25-Dihydroxyvitamin D, and then it is used throughout the body. The hormonal form of vitamin D (1.25-Dihydroxyvitamin D) is a transcription factor, which is the stimulating molecule of the vitamin D receptor (VDR), and which binds to Vitamin D responsive elements (VDRE) in DNA. 1.25-Dihydroxyvitamin D prevents cancer from occurring; it might also suppress tumor development [12].

The other therapeutic, which is used to detect the piR-Hep-1 and piR-651, is 4- Hydroxycoumarin. 4-Hydroxycoumaarin is a phenolic natural compound which is extracted from vanilla and cinnamon [13]. Coumarins are heterocyclic substances and are used in treatment of various diseases, especially cardiovascular diseases. Moreover, long ter

Coumarin usage causes an increase of coroner arterial calcification via inhibition of carboxylation Gla Protein through vitamin K. Coumarins (1,2-benzopyrone) and their hydroxylated forms (Hydroxycoumarins) triggers the formation of free radicals which leads cells to have oxidative stress [14]. Some clinical trials indicate the effect of Coumarins derivatives on the treatment of prostate cancer, malign melanoma and metastatic kidney carcinoma [15, 16].

Each therapeutic was used to treat HePG2 hepatocellular carcinoma cells individually and it was observed that each of them previously had a negative impact on the survival and the proliferation of HePG2 cells. In this study, we aimed to observe the effect of different types of therapeutics (4-Hydroxycoumarin and 1.25-Dihydroxyvitamin D), which are individually useful to treat hepatocellular carcinoma, on piRNA expressions.

MATERIALS AND METHODS

Cell Culture, 4-Hydroxycoumarin, 1.25-Dihydroxyvitamin D and Drug Form of Vitamin D Treatment: Hepatocellular carcinoma cell line HePG2 (ATCC, Washington D.C., USA) was maintained in a humidified atmosphere with 5% CO2 at 37°C. Dulbecco's Modified Eagle's Medium (DMEM; Wisent, Canada) with 10% fetal bovine serum (FBS; Capricorn, Germany) and 1% Penicillin/Streptomycin (FBS; Capricorn, Germany) were used to culture HePG2 cells.

Before treatment of 4-Hydroxycoumarin (Sigma, USA),1.25-Dihydroxyvitamin D (Cayman, USA) and drug form of vitamin D (1.25-Dihydroxyvitamin D, Butylhydroxyanisole and Sunflower oil) to HePG2 cells, 5x105 cells were seeded to each well of 6-well plate (Greiner, Germany). According to our previous study, the optimal concentration of 4-Hydroxycoumarin is 5 µM at the 48th hour, so 5 µM 4-hydroxycoumarin was treated to HePG2 cells for 48 hours. The optimal concentration of 1.25-Dihydroxyvitamin D is 250 nM at the 48th hour and

250 nM 1.25-Dihydroxyvitamin D was treated to HePG2 cells for 48 hours. To observe the drug form of vitamin D, 250 nM was treated to HePG2 cells for 96 hours [17].

Total RNA Isolation and Real Time Polymerase Chain Reaction (RT-PCR): Total RNA was isolated using a Nucleospin RNA Kit (Macherey-Nagel, Germany) in accordance with the manufacturer protocols. The total RNAs were converted to cDNA through a reverse transcription (Genaxxon, Germany). SYBR Green based primer sets for the amplification of piR-Hep-1, piR–651 and Glyseraldehide-3-phosphate dehydrogenase (GAPDH) were designed and supplied by Oligomer (Ankara, Turkey). The primer sequences are shown in Table 1. RT-PCR was carried out inside a Roche Lightcycler96 (Vedbaek, Denmark).

Table 1. The primer sequences used in RT-PCR

Primer Name	Forward Sequence	Reverse Sequence
piR-Hep-1	5'-TCCCTGGTGGT CTAGTGGTTAGAGAA-3'	3'-CCAGTCTCAGGG TCCGAGGTATTC-'5
piR-651	5' -AGAGAGGG GCCCGTGCCTTG-'3	3'-CCAGTCTCAGG GTCCGAGGTATTC-'5
GAPDH	5'-CGAGGGGG GAGCCAAAAGGG-'3	3'-GAAACTGC GACCCCGACCGT-'5

GAPDH was used as an internal control, and the expression of related genes was normalized in line with the expression of GAPDH. The SYBR Green RT-PCR was conducted at following conditions: pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 30 s. Gene expression changes were quantified using the delta-delta CT (ΔΔCT) method. *Table 1

Statistical Analysis: The normal distribution of the continuous variables was enabled using the Kolmogorov-Smirnov suitability test. Multiple comparisons of gene expressions were compared using the Student t test. All analyses were carried out using the IBM SPSS Statistics 21.0 software package. The obtained data were indicated as mean ± standard deviation (sd). In the figures, only mean values have been shown.

RESULTS

According to our obtained data, treatment of 4-Hydroxycoumarin caused an increase in piR-Hep-1 expression in treated group (-5.72±0.379) compared to the control (-5.99±0.071). But we cannot detect statistically significant changes between groups (p>0.05). The piR-651 expression of 4-Hydroxycoumarin treated group (3.66±0.282) was upregulated compared to control (-5.87±0.071) and was statistically significant (p<0.001; Fig. 1).

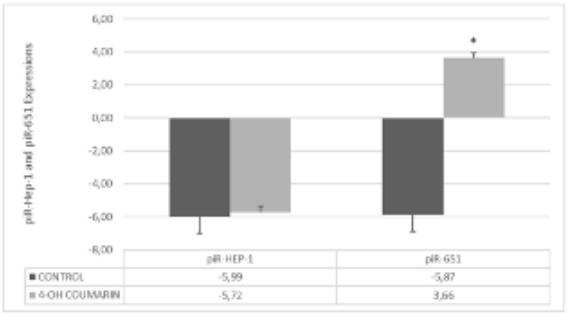

Fig 1. The effect of 4-Hydroxycoumarin treatment on piR-Hep-1 (p>0.05) and piR-651 expressions (p<0.001).

In treatment of Vitamin D active form (5.379±0.035), the piR-Hep-1 expression was downregulated compared to control group (12.223±0.072) and was statistically significant (p<0.001). Furthermore, we wanted to observe the drug form of vitamin D on piR-Hep-1 expression. The drug form of the vitamin D treated group (3.193±0.103) was also downregulated compared to control group (3.233±0.104). However, statistically significant differences cannot be detected between the group treated with the drug form of vitamin D and the control group (p>0.05; Fig. 2).

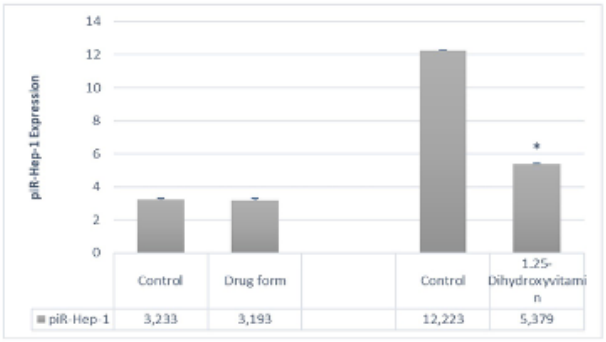

Fig 2. The effect of drug form of vitamin D ($p>0.05$) and 1.25-Dihydroxyvitamin D ($p<0.001$) treatment on piR-Hep-1 expression.

DISCUSSION

PIWI Interacting RNAs are the new perspective of small non-coding RNAs which are important transposon silencing. As a result of this epigenetic regulation, some gene regions become active while some of them become inactive [3, 18]. piR-Hep1 expression is high in HCC tumors and promotes viability and invasive characteristics. Law et al. determined that upregulation of piR-Hep1 by 46.6% in HCC tumors compared to healthy liver tissues causes an increase in viability, motility, and invasiveness depending on the amount of AKT phosphorylation in HCC [4]. In gastric, colon, lung and breast cancer tumors, piR-651 expression is aberrantly high [5]. Furthermore, inhibition of piR-651 in gastric cancer promotes cellular development. piR-651 expression, which were detected in the peripheral blood mononuclear cells from gastric cancer patients, downregulated significantly [2]. piRNA are also complicated and same piRNA might be tumor suppressing or have oncogenic characteristics in various cancer types. Our findings showed that 4-hydroxycoumarin treatment caused an increase in piR-651 expression. It is the first report that indicates that piR-651 might be tumor suppressive piRNA in HCC.

One of the main objectives of our research is to scientifically reveal that natural components applied in cancer treatment have a genetically significant effect in some regions while reducing the survival of cancer cells; while in some regions this natural compound treatment is ineffective. We wanted to show this situation by determining the expression changes of piR-Hep-1. piR-Hep-1 is an important piRNA region for hepatocellular carcinoma. piRNA studies in hepatocellular carcinoma especially showed that piR-Hep-1 is upregulated. Furthermore, high piR-Hep-1 expression was also detected in hepatocellular tumors [4]. Natural compounds extracted from plants have been regarded as a source of potential therapeutic agents, and are also are well known to play essential roles in a variety of cancer treatments. 4-Hydroxycoumarin is a polyphenolic natural compound. Recent studies about polyphenolic compounds and hepatocellular carcinoma indicate that these compounds represent a wide range of pharmacological properties like antioxidant and anti-carcinogenic activities [19, 20]. In cancerous cells, hydroxycoumarins enhances formation of free radicals and oxidative stress occurs. As a result of this mechanism, oxidative stress is effective in decreasing proliferation of cancer cells. In renal carcinoma, 7-Hydroxycoumarin was used as cytotoxic therapeutic in vitro [21]. Furthermore, hydroxylated Coumarins have anti- proliferative and cytotoxic activity in breast, sarcoma and skin cancer cell lines [22, 23]. We determined that piR-Hep-1 expression cannot be effected while piR-651 expression is upregulated. piR-651 expression is detected to indicate that 4-hydroxycoumarin can be related to another piRNA sequence. These results are the first results showing the link between 4-Hydroxycoumarin, piR-Hep-1 and piR-651 in hepatocellular carcinoma.

Vitamin D is not a natural compound but it is important in the treatment of hepatocellular carcinoma. Active form of vitamin D, 1.25-Dihydroxyvitamin D, and its analogs prevent cancer development or delay the recurrence and metastasis of previously developing cancer. Pourgholami et al., found that 1.25-Dihydroxyvitamin D inhibited the growth of HepG2 and Hep3B hepatocellular carcinoma cell lines [24]. Histone deacetylase 2 (HDAC2) is one of the target genes of piR-Hep-1 [18]. Decreased the proliferation of 1.25-Dihydroxyvitamin D treated HDAC2 inhibited HePG2 cells pointed to the possible link between vitamin D and piR-Hep-1 [25]. According to our data, although the drug form of vitamin D was not effective to downregulate piR-Hep-1 expression, 1.25-Dihydroxyvitamin D treatment caused a decrease in piR-Hep-1 expression in HePG2 cells. In the treatment of different forms of vitamin D, it is important to

emphasize however that the basis of molecule is the same, and that small changes can change the piRNA expressions. From this point of view, the drug form of vitamin D treatment did not cause a statistically significant change on the expression of piR-Hep-1, while active form of vitamin D (1.25-Dihydroxyvitamin D) had a statistically significant change on the piR-Hep-1. This is also the first report which identifies the relationship between vitamin D and piR-Hep-1.

As with the majority of studies, the design of the current study is subject to limitations. For example, the number of piRNAs is an important limitation, especially for evaluation. The results should be examined in large-scale studies. Furthermore, the studies of piRNAs increase day by day, however there is more studies needed to identify the cellular mechanisms of piRNA.

Conclusion

In scientific literature, piRNAs and their roles in cancer cells is identified day by day. However, there is a lack of piRNA cellular mechanisms. We have demonstrated in this study that natural compounds cannot always be effective in all genes and/or epigenetic mechanisms of cancer cells. We even showed that two different forms of the same substance may show different expression changes on the same piRNA region. More cellular and molecular mechanisms of piRNAs should be identified by future studies.

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