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Original Article

Expression levels of microRNA machinery components Drosha, Dicer and DGCR8 in human (AGS, HepG2, and KEYSE-30) cancer cell lines

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Abstract: MicroRNAs (miRNAs) have recently been shown to play fundamental roles in diverse cellular processes and linked to variety of cancers. Dicer and Drosha are two major enzymes in the miRNA maturation process. DGCR8 is the assistant of Drosha in the microprocessor complex. In this study, we evaluated the mRNA expression profiles of major miRNA processing machinery Drosha, Dicer, and DGCR8 in human gastrointestinal (AGS, KYSE30 and HepG2) cancer cell lines. Materials and Methods: The cells were cultured and harvested, and total cellular RNA was isolated from cells. Then, first-strand cDNA was synthesized from the RNA of cells. Afterward, Quantitative analysis was performed by real-time RT-PCR using the *Power*SYBR Green PCR Master Mix. Results: Expression levels of Drosha in AGS and HepG2 cells were higher than the controls, whereas, Drosha's expression level in KYSE-30 cell line was lower. The Dicer expression levels in AGS and HepG2 cells were higher, while, its expression level in KYSE-30 cell was lower. The DGCR8 expression levels in all three cell lines were significantly higher than the control samples. Conclusion: Expression levels of the two most important enzymes of the miRNA machinery, Drosha and Dicer, and microprocessor complex component, DGCR8 were noticeably dysregulated when compared to healthy controls.

Keywords: Drosha, Dicer, DGCR8, microRNA machinery, cancer cell line

Introduction

MicroRNAs (miRNA) are 17 to 24 nucleotide (nt) RNA molecules that are considered as a group of non-protein-coding RNAs; miRNAs are capable of post-transcriptional gene regulation by binding to target mRNAs [1]. The miRNA base-pairs with target mRNA to direct gene silencing through mRNA cleavage or translation repression based on the level of complementarily between the miRNA and the mRNA target [2]. miRNAs have recently been shown to play fundamental roles in diverse developmental and cellular processes and linked to variety of cancers [3].

miRNA maturation begins in the nucleus, where the primary miRNA (pri-miRNA) transcript is transcribed by RNA polymerase II [4]. The RNA III endonuclease Drosha inside the nucleus, beside with DiGeorge syndrome critical region gene 8 (DGCR8 or Pasha) construct the microprocessor complex. This complex cuts the pri-miRNA transcript within the nucleus into several precursor miRNAs (pre-miRNAs) [5]. Drosha is the catalytic subunit of the pri-miRNA processing microprocessor complex, while DGCR8 stabilizes Drosha and recognizes the RNA substrate [6, 7]. The 70-90 nt long pre-miRNAs are then transported to the cytoplasm for further processing, where Dicer, another RNase III enzyme cleaves it to produce mature miRNA [8]

Although several studies have revealed dysregulation of microRNA (miRNA) expression profiles in various cancers, there has been little

research on the miRNA machinery itself. In this study, we evaluated the mRNA expression profiles of major miRNA processing machinery Drosha and Dicer, and DGCR8 in human gastrointestinal (AGS, KYSE30 and HepG2) cancer cell lines.

Materials and methods

Cell lines

The human cancer cell lines including, human gastric carcinoma (AGS), human esophageal squamous cell carcinoma (KYSE-30), and human hepatocellular carcinoma (HepG2) were purchased from Pasteur Institute of Iran Cell Bank (Tehran, Iran). All reagents and medium were prepared just before use. Normal cells were obtained from healthy people.

Cell culture

The cells were cultured in RPMI-1640 (Cat. No: 51800–035, GIBCO, UK) medium supplemented with 10% fetal bovine serum (FBS; Cat. No: 10270–106, GIBCO, UK), penicillin 100 unit/ml and streptomycin 100 µg/ml. The cells were incubated at 37°C in a water saturated atmosphere of 5% CO2 and 95% air until confluence. One week later, the cells were removed with a solution containing 0.25 (w/v) trypsin and 0.02 (w/v) ethylenediaminetetraacetic acid.

RNA isolation

Total cellular RNA was isolated from cells using Trizol reagent (Cat. No: 15596-026, Invitrogen, CA, USA), following this procedure; cultured cells were harvested and centrifuged (1000 rpm, 10 min) for removing supernatant media; afterward 0.8ml of Trizol reagent were added per $5x10^6$ cell, after pipetting, 0.2ml of chloroform were added and tubes were shaken vigorously, after that samples were centrifuged at 12000g for 15min at 4°C, then upper aqueous phase was transferred to a new tube by angling the tube at 45° and pipetting the solution out, subsequently 0.5ml of isopropanol were added and incubated at room temperature for 10min, next samples were centrifuged at 12000g for 10min at 4°C, after that supernatants were removed and pellet of the tubes were washed and vortexed with 1ml of 75% ethanol and were centrifuged at 7500g for 5min at 4°C, finally supernatants were discarded and RNA pellets were air dried for 10min; and were re-suspended in 40µl of RNAase free water. Prepared RNAs were stored at -80°C refrigerator until downstream application.

Reverse transcription and cDNA synthesis

First-strand cDNA was synthesized from the RNA of cells by RevertAid First Strand *cDNA* Synthesis *Kit*, Fermentas (Cat No: #K1621, Maryland, USA) according to the manufacturer's protocol. Briefly, 4µl of isolated RNA from cells were mixed with 1µl of random hexamer primer and 7µl RNAase-free water and incubated at 65°C for 5min. then micro-tubes were chilled on ice, afterward, mixture of (reaction buffer 4µl + RNAase inhibitor 1µl + dNTP mix 2µl + reverse transcriptase 1µl) was added on each sample. Immediately samples were incubated at 25°C for 5min followed by 42°C for 60min; the reaction was terminated by heating at 70°C for 5min. Reverse transcription was performed in the final volume of 20µL.

Real-time quantitative RT-PCR

Primers were used from previous studies [9, 10] that Sand, et al, evaluated Drosha, Dicer, DGCR8 and RISC components in their studies. Primers (Drosha, Dicer, DGCR8 and the RPL38 as a housekeeping gene) were designed using Primer Express 3.0 (PE Applied Biosystems, Foster City, CA, USA). See Supplementary **Table 1** for the details of primers used in quantitative real-time PCR. For accuracy and specificity all primers were blasted in NCBI website: http://www.ncbi.nlm.nih.gov/tools/primer-blast/. Primers were produced by the custom oligonucleotide synthesis service, Metabion (Martinsried, Germany).

Quantitative analysis was performed by real-time RT-PCR using the *Power*SYBR Green PCR Master Mix (Cat. No: 4309155, Applied Biosystems, Foster City, CA, USA) and StepOne Real-Time PCR System (Applied Biosystems 7500, Foster City, CA, USA). Each reaction mixture contained a total volume of 25µl (master mix 12.5µl, cDNA 3µl, primer 3µl, and H2O 6.5µl).

The quantitative RT-PCR conditions were: 50°C for 2 minutes, 95°C for 10 minutes, then 60 cycles of 95°C for 30 seconds, and 60°C 30 seconds and 72°C for 30 seconds.

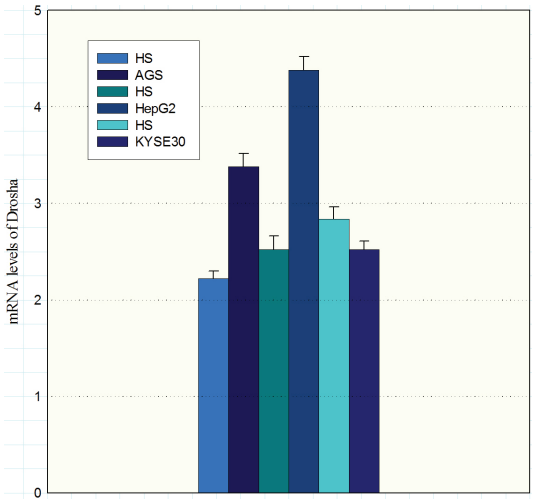

Figure 1. Expression of Drosha mRNA in cell lines AGS, HepG2 and KYSE30 was evaluated by quantitative RT-PCR. Values presented in the log scale. (HS: healthy subjects).

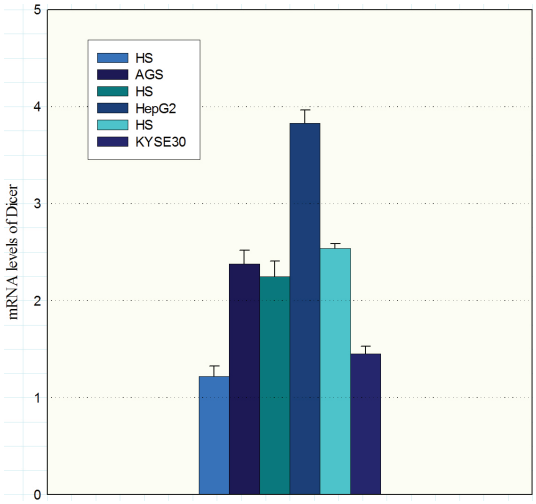

Figure 2. Expression of Dicer mRNA in cell lines AGS, HepG2 and KYSE30 was evaluated by quantitative RT-PCR. Values presented in the log scale. (HS: healthy subjects).

Relative amounts of target mRNA in test sample was calculated and normalized to the corresponding RPL38 mRNA transcript level as a housekeeping gene. The comparative Ct method was used to evaluate expression as previously described by Livak and Schmittgen [11].

Statistical analysis

Data analysis was performed using a graph and data analysis software package (SigmaPlot 12.0, Systat Software, Inc.). Data were analyzed by paired *t*-tests to compare cell lines with healthy controls.

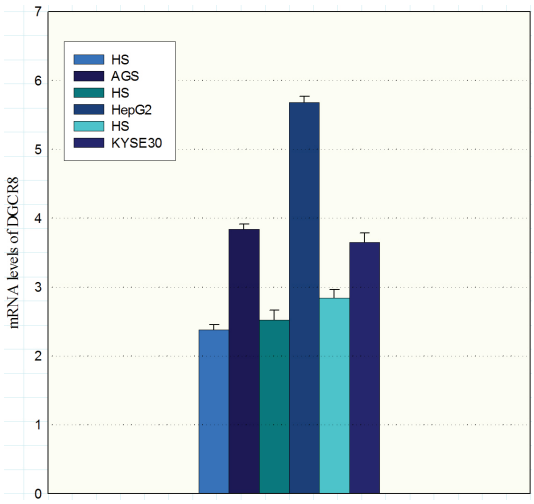

Figure 3. Expression of DGCR8 mRNA in cell lines AGS, HepG2 and KYSE30 was evaluated by quantitative RT-PCR. Values presented in the log scale. (HS: healthy subjects).

Results

Expression levels of Drosha in AGS and HepG2 cells were higher than the controls (P = 0.011 and P = <0.001 respectively). On the contrary, Drosha's expression levels in KYSE-30 cell line were lower in comparison to the control (**Figure 1**, P = 0.038).

The Dicer expression levels in AGS and HepG2 cells were higher than the controls (P = 0.013 and P = 0.009 respectively). In contrast, Dicer expression levels in KYSE-30 cell line were lower in comparison to the control (**Figure 2**, P = <0.001).

The DGCR8 expression levels in all three cell lines were significantly higher than the control samples (**Figure 3**). There was a statistically significant difference between AGS and HepG2 and healthy subjects (P = <0.001), and also between KYSE30 and normal controls (P= 0.013).

Discussion

Changes in the microRNA machinery components play crucial roles in the carcinogenesis of a variety of tumors [12]. These alterations are thought to elucidate abnormal miRNA profiles in various cancers. Both down and up-regula-

Table 1. Primers used in quantitative PCR, the amplicon sizes and the melting temperature (Tm) of each reaction

Target	Sequence	Amplicon Size (bp)	Tm (°C)
Dicer *F*	5′-TTAACCTTTTGGTGTTTGATGAGTGT-3′	94	58.5
Dicer *R*	5′-GGACATGATGGACAATTTTCACA-3′		57.1
Drosha *F*	5′-CATGTCACAGAATGTCGTTCCA-3′	115	58.4
Drosha *R*	5′- GGGTGAAGCAGCCTCAGATTT-3′		59.8
DGCR8 *F*	5′-GCAAGATGCACCCACAAAGA-3′	93	57.3
DGCR8 *R*	5′-TTGAGGACACGCTGCATGTAC-3′		59.8
RPL38 *F*	5′-TCACTGACAAAGAGAAGGCAGAGA-3′	88	61
RPL38 *R*	5′-TCAGTGTGTCTGGTTCATTTCAGTT-3′		59.7

F: forward primer, *R:* reverse primer, bp: base pairs, RPL38: ribosomal protein (housekeeping gene).

tion of miRNAs have been reported, however, it is not clear whether the observed up- or down-regulation of miRNA expression simply reflects malignant weakening of the tumor or directly causes tumor triggering and progression [9]. Hence, there are studies that have been proven both down and up-regulation of these machinery components in a variety of cancers [10, 13-17]. Since miRNAs control both oncogenesis and tumor suppressors, it remains doubtful whether miRNAs may control carcinogenesis.

To analyze expression levels of important miRNA machinery components, we evaluated expression levels of two essential enzymes in the miRNA maturation process, Dicer and Drosha. And also DGCR8 which is a double-stranded RNA binding protein (dsRBP) and determines the cleavage sites on the pri-miRNA. Furthermore, there are also other factors that are necessary for appropriate pri-miRNA processing and are the subject of current research [18]. They include RNA-induced silencing complex (RISC) components including, argonaute-1 (AGO1), argonaute-2 (AGO2), as well as double-stranded RNA-binding proteins PACT, TARBP1, and TARBP2 [19]. Nevertheless, in this pilot study, we investigated the expression of Dicer, Drosha and DGCR8, while the other factors could be the subject of further studies.

It has been established that Drosha expression were correlated with poor prognostic factors in prostate cancer and esophageal carcinoma, besides, reduction of Drosha expression by means of siRNA has been reported to reduce cellular proliferation in esophageal cancer cell lines [20]. There are several probable explanations for the divergent expression patterns of Drosha in various tumors and how they correlate with other pathological parameters. Here, we found expression levels of Drosha in AGS and HepG2 cells were up-regulated (P = 0.011 and P = <0.001); on the other hand, it was down-regulated in KYSE-30 cell line (P = 0.038).

Regardless of growing evidence that Dicer mRNA levels differ in tumors, the regulation of this gene is indefinite. Dicer gene mutations have been found in humans, and alterations of the Dicer gene were detected in some pre-cancerous and invasive lung adenocarcinomas [21]. Additionally, there are other studies that suggest miRNA processing may be hindered in tumors with low Dicer and low Drosha expression, which could lead to a poor clinical outcome [13, 22]. In our study we found that the expression of Dicer in AGS and HepG2 cells were higher than the controls (P = 0.013 and P = 0.009), on the other side, we have seen it was down-regulated in KYSE-30 cell line (P = <0.001).

DGCR8 is an RNA-binding protein that assists Drosha in the processing of miRNAs. Our experiment showed that DGCR8 expression up-regulated in AGS and HepG2 cells (P = <0.001), and also in KYSE30 cell line (P= 0.013).

To sum up all the facts, expression levels of the two most important enzymes of the miRNA machinery, Drosha and Dicer, and microprocessor complex component, DGCR8 were clearly dysregulated when compared to healthy controls. Our results are consistent with other findings and propose that miRNAs are involved in the carcinogenesis, and emphasize the importance of miRNA expression profiles to further investigate the role of miRNA dysregulation in human cancers.

However, this cannot be assessed based on our data alone. Hence, this was an *in vitro* study; it would be much more informative to design other experiments on animal tumor models and especially human tumors.

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