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Identification of miR-146a and miR-196a-2 single nucleotide polymorphisms at patients with high-grade serous ovarian cancer

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Highlights

- Determination of miR-146a and miR-196a-2 single nucleotide polymorphisms (SNP) in Hungarian female population at first time.
- This is the first comparative analysis of miR-146a and miR-196a-2 SNPs between Hungarian controls and patients with ovarian cancer.
- We performed network analysis related to both of miR-146a and miR-196a-2.

Abstract

MicroRNAs play an essential role in the regulation of gene expression and tumor development. Single nucleotide polymorphism (SNP) can be observed in miRNAs and could influence gene expression.

We aimed to identify miR-146a rs2910164 and miR-196a-2 rs11614913 polymorphisms in ovarian cancer patients and controls.

75 patients and 75 controls were involved. DNA was isolated from blood samples. MiR-146a rs2910164 and miR-196a-2 rs11614913 were determined by LightSnip kit. We used melting curve analysis for allele classification. Network analysis was made to find common target genes.

We detected 72.67% G allele frequency of miR-146a rs2910164 in controls and 82.00% in patients group (p=0,053). GG, GC and CC genotypes occurred with 53.33%, 38.67% and 8.00% among controls, with 65.33%, 33.33% and 1.33% among patients, (p=0.0917). Allele C of miR-196a-2 rs11614913 occurred in 59.33% of controls and in 67.33% of patients (p=0.15). CC, CT and TT genotypes occurred with 37.33%, 44.00%, and 18.67% frequency in controls, with 46.67%; 41.33% and 12.00% in patients (p=0.3815). Network analysis found ATG9A, LBR, MBD4 and RUFY2 genes to be targets for both miRNAs.

SNPs of miR-146a and miR-196a-2 showed no significant differences between patients and controls. More investigations are required to clarify the exact role of these SNPs in ovarian cancer.

1. Introduction

In the last decade, the functions of microRNAs (miRNAs) got into the limelight. Micro RNAs are a new group of small non-coding RNAs, which take a role in the degradation of RNA and the inhibition of messenger RNA translation. They affect transcription while binding to the 3'non translated region (3'UTR) of target messenger RNAs. Much microRNAs take a role in carcinogenesis by regulating oncogenes and tumor suppressor genes. Studies about their role in ovarian cancer prove their contribution to development, progression, forming metastases and chemoresistance of cancers. The so-called single nucleotide polymorphisms (SNPs) of miRNAs can determine the features and expression of miRNAs that is why they can create individual susceptibility to cancers. Four genetic types of microRNAs, miR-146a, miR-149, miR-196a-2 and miR-499 show association with susceptibility to many diseases, including gastrointestinal cancers, esophageal squamous cell carcinoma, colorectal cancer, and prostate cancer. The miRNAs are non-coding small RNAs consisting of 18-23 nucleotides. Recently, it has been investigated and shown whether these RNAs are associated with or susceptible to ovarian cancer.

Many miRNA polymorphisms show relations with microRNA formation and regulation of microRNA-messenger RNA interaction [1]. So far, more than a thousand different microRNAs have shown association with oncogenic or tumor suppressor function [2,3].

The variation of miR-146a C allele was studied earlier in cases of breast cancer and ovarian cancer, and a higher level of related gene expression was detected [4,5]. The miR-196a-2 plays a role in the development of several tumors, like lung, breast, stomach and epithelial ovarian cancer [6,7]. Making a meta-analysis Kang et al. (2014) collected the polymorphisms of 45816 individuals from 46 studies and looked for correlation with the development of different tumor types [8].

MiR-196a-2 has many polymorphisms. So far, data from the Chinese population emerged only. Hu et al. (2008) have experienced reduced survival in non-small cell lung cancer carriers with homozygous CC genotypes [9]. Peng et al. (2010) showed that the risk of stomach cancer is higher with the same genotype [10], while Dou et al. (2010) showed a reduced risk for glioma [11].

The meta-analysis of Chu et al. (2011) involved 9341 patients and more than 10,000 controls. They found that TT genotype protects against more cancer types, except breast and lung cancer, in which CC genotype was a lower risk factor [12]. Qi et al. (2010) found a connection between the polymorphism and hepatitis B related hepatocellular cancer in cases of men [13]. However, Hu et al. (2009) found a connection between Chinese susceptibility to breast cancer and SNP [14].

Hoffmann et al. (2009) recorded lower risk for breast cancer at TT homozygous people [15], on the other hand, Catucci et al. (2010) did not reveal a correlation between the alleles and breast cancer in German and Italian populations [16]. T variant showed higher risk in a study collecting data in colorectal, esophageal, skin, lung, thyroid and kidney cancer in Egypt [17].

In the Japanese population, the CC variant means a higher risk for gastric cancer [18]. In the North-Indian population higher susceptibility is proved for prostatic cancer [19]. Christensen et al. (2010) found that patients with pharyngeal tumor can expect shorter survival if they have homozygous variant alleles [20].

According to Xu et al. (2013) in the cases of TT genotype lower susceptibility for several types of cancer was recorded based on the data of 21 pieces of research in a meta-analysis [21].

Only a few of those mentioned above studied showed SNPs of rs11614913. The most researched area is non-small cell lung cancer, breast cancer, and gastric cancer. Three studies emerged so far about ovarian cancer patients in this topic, all of them from the Chinese population.

The above also shows how difficult it is to study the effect of miRNAs. As only a few data are available from the European population, we decided to identify one polymorphism of both miR146a and miR-196a-2 in the ovarian cancer group.

2. Material and methods

2.1 Patients and funding

We recruited healthy controls (n=75) and patients with high-grade serous papillary ovarian carcinoma (n=75) for this prospective study. The Hungarian Ministry of Health: ETT TUKEB (30231-2/2016) issues ethical permission for the study. All participants gave signed written informed consent to the study.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

2.2 SNP determination

Blood samples were drawn into 9 ml EDTA tubes from all participants. DNA was isolated from 200 μl blood by silica adsorption method according to the recommendations of the manufacturer (High Pure PCR Template Preparation kit, Roche, Mannheim, Germany) [22]. Performing a quantitative real-time PCR we measured 1 μl DNA, 1 μl LightSnip reagent mix, 3.0 mM MgCl2 and 1 μl LightCycler FastStart DNA Master Hybridization Probes mix (Roche, Penzberg, Germany) as a PCR mixture to 10 μl blood volume. LightSnip primers were planned and produced by TibMolbiol (Berlin, Germany). During PCR after 10 minutes of denaturation the amplification of products was carried out in 36 cycles with 60 C° annealing. Amplification was followed by melting curve analysis. MiR-146a rs2910164 T allele showed 54 C°, C allele 65 C° of Tm (melting temperature); miR-196a-2 rs11614913 SNP analysis showed melting-point at 54 C° for C allele and 62 C° for T allele.

2.3 Bioinformatic analysis (network analysis)

Determining the target genes of has-miR-146a-5p and has-miR-196a-2-5p microRNAs, we used TargetScan (www.targetscan.org), MirBase (www.microrna.sanger.ac.uk) and microRNA-Data-Integration-Portal (http://ophid.utoronto.ca/mirDIP) predictive data basis and offered algorithms. Softwares recognizing the valid target consider not only miRNA seed

sequences and mRNA complementarity but also take into account the environment of seed sequences and the evolutionary conservation.

2.4 Statistical analysis

Chi-square test was used to compare the frequency of categorical variables (alleles and genotypes) (www.socstatistics.com/tests/chisquare2). We used free software to calculate the adequacy to the Hardy-Weinberg equilibrium.

3. Results

We introduced a (new) method to determine the SNPs of miR-146a and miR196a-2 while working in this study. The applied PCR conditions were suitable to distinguish the alleles based on their melting temperature. In our work, we determined the frequency of occurrence in case of the two miRNA SNPs using the samples of 75 healthy controls and 75 patients with high-grade serous papillary ovarian cancer. The mean age was not significantly different in the two groups. Tables 1 and 2 show the frequency of alleles and genotypes of the two SNPs. Based on melting curve analysis of miR-146a rs2910164 we found 72.67% G allele frequency in the control group, and 82.00% in the patient group (p=0,053). GG, GC and CC genotypes occurred with 53.33%, 38.67% and 8.00% frequency among controls, while they were detected with 65.33%, 33.33% and 1.33% frequency among patients, respectively (p=0.0917). On analysis of miR-196a-2 rs11614913 allele C occurred in 59.33% of controls and 67.33% of patients (p=0.15). CC, CT and TT genotypes occurred with 37.33%, 44.00%, and 18.67% frequency in control group, while with 46.67%; 41.33% and 12.00% in patients' group, respectively (p=0.3815). During the network analysis, we found Lamin B receptor (LBR), Rab4 interacting protein (RUFY2), Autophagy-related 9a (ATG9A), and Methyl CpG binding domain 4 (MBD4) genes to be targets for both miRNAs (Figure 1).

4. Discussion

The primary transcript of miR146a is converted by the Drosha ribonuclease III to a precursor miRNA built by 70 bases, cytoplasmic Dicer ribonuclease produces mature miRNA of it. [23]. The mature miRNA develops in the RNA induced silencing complex (RISC), which inhibits and destabilizes the target RNAs by imperfect base-pair binding. The targets RNAs of miR-146a include tumor necrosis factor, interleukin-1 receptor-associated kinase 1, interleukin 1-beta, tumor necrosis factor receptor-associated factor 6 and the complement factor H genes. Earlier Sun et al. (2016) performed experiments with miR-146a expression in ovarian cancer, and they found that in ovarian cancer patient compared to healthy controls GG genotype compared to CC genotype 3.73 times, the CG+GG genotype compared to CC genotype 1.68 times, the GG genotype compared to CG+CC genotype 3.02 times more frequently occur. The difference was significant in all the three comparisons [24]. In our present study, the same comparison resulted in 7.35 (GG to CC), 6.4 (CG+GG to CC), 1.65 (GG to CC+GG) ratios, but

none of them was statistically significant. Considering the high number of cases, we suppose that the role of miR-146a polymorphism is not exclusive in the development of ovarian cancer.

Already 25 years ago ovarian cancer was associated with the loss of alleles of chromosome 17 long arm genes [25]. It is not surprising that miR196a-1 also derives from the gene clusters of the homeobox of chromosome 17 [25]. While examining the genes influenced by miR-196a, researchers identified HMGA2-gene as the primary target. The protein product of that is a non-histone protein (high mobility AT-hook 2), and its function is permissive in those effects (some types of radiation, chemotherapy) causing double-stranded DNA fractures. Earlier studies analyzed the expression of miR-196-a in ovarian cancer cell lines [26]. Based on their results miR-196a was identified as a tumor promoter with the target HOXA 10 gene in downstream localization. Increased expression of miR-196a means poor prognosis according to a study by Fan et al. (2015) in patients with ovarian cancer [27]. Song et al. (2016) found that CC genotypes are 1.34 times more likely to occur compared to CG + TT genotype in ovarian cancer patient than among healthy controls [28]. The odds ratio in our material was 1.5, but it was not statistically significant. The results of Chinese studies might not be generalized because less than half of the patients had serous papillary tumors, less than one-third of the patients had high-grade tumors. As against European and American statistics, most of them were mucinous and endometrioid tumors. The Chinese test population is not considered to be representative in the classical serous papillary ovarian cancer because of the frequency of the genetically different types.

 In our network analysis, we found four genes which are influenced by both tested miRNAs (miR-146a and miR-196a-2). These two miRNAs play an essential role in the development of the tumor and chemoresistance.

Dai et al. (2014) showed the overexpression of ATG9a-gene in a group of ovarian tumor patients [29]. In their opinion, the level of protein is an important biomarker and correlates with the prognosis, as well as with chemosensitivity.

Helleman et al. (2006) found nine genes in the molecular analysis of platinum-resistant ovarian cancer. The expression of these genes indicated resistance, LMB was one of it [30]. LMB is a cell membrane protein that binds to chromatin, but further studies are necessary to understand the exact mechanism.

Howard et al. (2009) revealed the reduced expression of MED1/MBD4 gene in the cases of colorectal and ovarian cancer [31]. MBD4 is connected to methylated DNA together with DNA mismatch repair protein (MMR). MBD4 plays an essential role in repairing mutations in DNA.

 Shin et al. (2011) were investigating microsatellite instability, and RUFY2 gene was found to be one of the frequently mutated genes [32].

Based on melting curve analysis of miR-146a rs2910164 we found 72.67% G allele frequency in control group, and 82.00% in patients group (p=0,053). GG, GC and CC genotypes occurred with 53.33%, 38.67% and 8.00% frequency among controls, while they were detected with 65.33%, 33.33% and 1.33% frequency among patients, respectively (p=0.0917). On analysis of miR-196a-2 rs11614913 allele C occurred in 59.33% of controls and in 67.33% of patients (p=0.15). CC, CT and TT genotypes occurred with 37.33%, 44.00%, and 18.67% frequency in control group, while with 46.67%; 41.33% and 12.00% in patients' group, respectively (p=0.3815).

During the network analysis, we found Lamin B receptor (LBR), Rab4 interacting protein (RUFY2), Autophagy-related 9a (ATG9A), and Methyl CpG binding domain 4 (MBD4) genes to be targets for both miRNAs. Based on our study we concluded that in high-grade serous papillary ovarian cancer cases rs2910164 polymorphism of miR-146a and rs11614913 polymorphism of miR-196a-2 did not show statistically significant differences in comparison to healthy controls. Recently published studies by Ni and Huang (2016) concluded the same, but there was a significant difference in rs3746444 polymorphism of the miR-499 which miRNA was not studied by us [33]. The GG genotype occurred in 12 % of the ovarian cancer patients while in 6% in healthy controls, and this difference was statistically significant.

We have to perform further studies with higher number of patients to clarify that the revealed discrepancies in what extent connectable to the susceptibility to ovarian cancer and the genetic changes during tumor progression.

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Tables

Table 1

Distribution of alleles and genotypes of miR-146a rs 2910164 in ovarian cancer patients and healthy controls

alleles

miR-146a rs 2910164	G	C
Control (n=75)	109 (72.67%)	41 (27.33%)
Patient (n=75)	123 (82%)	27 (18%)

p= 0,053

genotypes

miR-146a rs 2910164	GG	GC	CC
Control (n=75)	40 (53.33%)	29 (38.67%)	6 (8%)
Patient (n=75)	49 (65.33%)	25 (33.33%)	1 (1.33%)

p=0,0917

Table 2

Distribution of alleles and genotypes of miR-196a-2 rs 11614913 in ovarian cancer patients and healthy controls

alleles

miR-196a-2 rs 11614913	T	C
Control (n=75)	61 (40.67%)	89 (59.33%)
Patient (n=75)	49 (32.67%)	101 (67.33%)

p= 0,15

genotypes

miR-196a-2; rs 11614913	TT	TC	CC
Control (n=75)	14 (18.67%)	33 (44%)	28 (37.33%)
Patient (n=75)	9 (12%)	31 (41.33%)	35 (46.67%)

p= 0,3815

Figures

Figure 1

The collective target genes of miR-146a and miR-196a-2 detected by network analyzing. Made by using of miRTargetLink Human website
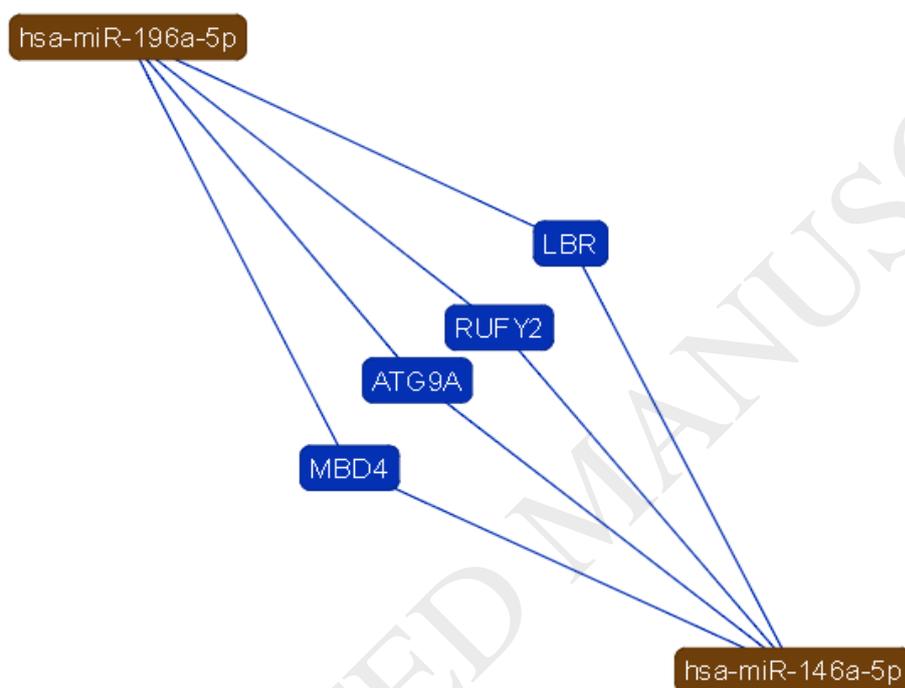
miRNA	ccNS n	proved
hsa-miR-146a-5p	ATG9A	+
hsa-miR-146a-5p	MBD4	+
hsa-miR-146a-5p	RUFY2	+
hsa-miR-146a-5p	LBR	+
hsa-miR-196a-5p	LBR	+
hsa-miR-196a-5p	RUFY2	+
hsa-miR-196a-5p	ATG9A	+
hsa-miR-196a-5p	MBD4	+