LITHUANIAN UNIVERSITY OF HEALTH SCIENCES
MEDICAL ACADEMY

Violeta Šaltenienė

ROLE OF MICRORNA AND THEIR TARGET GENES IN PATHOGENESIS OF GASTRIC CANCER

Doctoral Dissertation
Biomedical Sciences,
Biology (01B)

Kaunas, 2017

Dissertation has been prepared at the Laboratory of Clinical and Molecular Gastroenterology of Institute for Digestive System Research of Medical Academy of the Lithuanian University of Health Sciences during the period of 2012–2017.

Scientific Supervisor:

Prof. Dr. Gediminas Kiudelis (Lithuanian University of Health Sciences, Biomedical Sciences, Biology – 01B)

Dissertation is defended at the Biology Research Council of Medical Academy of the Lithuanian University of Health Sciences

Chairperson

Prof. Dr. Žilvinas Dambrauskas (Lithuanian University of Health Sciences, Medical Academy, Biomedical Sciences, Biology – 01B)

Members

Dr. Diana Žaliaduonytė-Pekšienė (Lithuanian University of Health Sciences, Medical Academy, Biomedical Sciences, Medicine – 06B)
Prof. Habil. Dr. Angelija Valančiūtė (Lithuanian University of Health Sciences, Medical Academy, Biomedical Sciences, Biology – 01B)
Prof. Dr. Edita Sužiedėlienė (Vilnius University, Biomedical Sciences, Biology – 01B)
Assoc. Prof. Dr. Aija Linē (University of Latvia, Biomedical Sciences, Biology – 01B)

Dissertation will be defended at the open session of the Biology Research Council of Lithuanian University of Health Sciences on the August 31st, 2017 at 2 p.m. in 204 auditorium of "Santaka" Valley Centre for the Advanced Pharmaceutical and Health Technologies of Lithuanian University of Health Sciences.

Address: Sukilėlių 13, LT-50162 Kaunas, Lithuania.

LIETUVOS SVEIKATOS MOKLSŲ UNIVERSITETAS
MEDICINOS AKADEMIJA

Violeta Šaltenienė

MIKRO RNR IR JŲ REGULIUOJAMŲ GENŲ VAIDMUO SKRANDŽIO VĖŽIO PATOGENEZĖJE

Daktaro disertacija
Biomedicinos mokslai,
Biologija (01B)

Kaunas, 2017

Disertacija rengta 2012–2017 metais Lietuvos sveikatos mokslų universitete, Medicinos akademijos, Virškinimo sistemos tyrimų instituto, Klinikinės ir molekulinės gastroenterologijos laboratorijoje.

Mokslinis vadovas

prof. dr. Gediminas Kiudelis (Lietuvos sveikatos mokslų universitetas, Medicinos akademija, biologija – 01B)

Disertacija ginama Lietuvos sveikatos mokslų universiteto Medicinos akademijos Biologijos mokslo krypties taryboje:

Pirmininkas

prof. dr. Žilvinas Dambrauskas (Lietuvos sveikatos mokslų universitetas, Medicinos akademija, biomedicinos mokslai, biologija – 01B)

Nariai

dr. Diana Žaliaduonytė-Pekšienė (Lietuvos sveikatos mokslų universitetas, Medicinos akademija, biomedicinos mokslai, medicina – 06B)
prof. habil. dr. Angelija Valančiūtė (Lietuvos sveikatos mokslų universitetas, Medicinos akademija, biomedicinos mokslai, biologija – 01B)
prof. dr. Edita Sužiedėlienė (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B)
doc. dr. Aija Linē (Latvijos universitetas, biomedicinos mokslai, biologija – 01B)

Disertacija bus ginama viešame biologijos mokslo krypties tarybos posėdyje 2017 m. rugpjūčio 31 d. 14 val. Lietuvos sveikatos mokslų universiteto Naujausių farmacijos ir sveikatos technologijų centro 204 auditorijoje.

Adresas: Sukilėlių pr. 13, LT-50162 Kaunas.

CONTENTS

ABBREVIATIONS 7

INTRODUCTION 8

1. AIM AND OBJECTIVES 10
   1.1. Objectives of the study 10
   1.2. The novelty and relevance of the work 10

2. REVIEW OF LITERATURE 11
   2.1. Epidemiology of gastric cancer 11
      2.1.1. Classification and histology of gastric cancer 11
      2.1.2. Risk factors of gastric cancer 13
      2.1.3. Environmental Risk Factors 13
      2.1.5. Hereditary factors 15
      2.1.6. Acquired genetic factors 15
      2.1.7. Atrophic gastritis 15
      2.1.8. Diagnosis of gastric cancer 16
   2.2. MicroRNA 17
      2.2.1. MiRNA biogenesis 17
      2.2.2. MiRNA–mRNA interactions 19
      2.2.3. Single nucleotide polymorphisms influence miRNA function 20
      2.2.4. Role of miRNAs in atrophic gastritis 21
      2.2.5. Role of miRNAs in gastric cancer 22
      2.2.6. Circulating miRNAs 23
      2.2.7. Circulating miRNAs in gastric cancer 25

3. MATERIALS AND METHODS 26
   3.1. Ethics statement 26
   3.2. Major reagents 26
   3.3. Study I 27
      3.3.1. Study design and patients samples 27
      3.3.2. DNA extraction, selection of putative miRNA target-gene single nucleotide polymorphisms and genotyping 29
   3.4. Study II 28
      3.4.1. Study design 28
      3.4.2. Study population of part I of study II 29
      3.4.3. Study population of part II of study II 29
      3.4.4. Tissue sample preparation and RNA extraction 32
      3.4.5. Plasma sample preparation and RNA extraction 32
      3.4.6. MicroRNA profiling using the TaqMan Low Density Array card A 32
      3.4.7. MicroRNA validation using the custom TaqMan Low Density Array 33
      3.4.8. Quantitative real-time polymerase chain reaction with TaqMan assay 33
      3.4.9. Preparation of small RNA libraries and next generation sequencing 33
      3.4.10. Small RNA-seq data and statistical analysis 34
   3.5. Study III 36
      3.5.1. Study design 36
      3.5.2. Evaluation of miRNAs target-genes 36
      3.5.3. Cell culture conditions 36

3.5.4. MiRNA genes expression analysis in gastric cancer cells 36
3.5.5. Gastric cancer cells transfection 37
3.5.6. MiRNA putative gene expression analysis in gastric cancer cells 37
3.5.7. Statistics 37

4. RESULTS 38

4.1. Study I 38
4.2. Study II, Part I 38
4.2.1. MiRNA expression profiling of GC and healthy gastric mucosa tissue by Taq-Man low-density array 38
4.2.2. MiRNA validation in gastric cancer and healthy gastric mucosa tissue with quantitative real-time polymerase chain reaction 39
4.2.3. Plasma miRNAs as potential biomarkers for gastric cancer 39
4.3. Study II, part II 39
4.3.1. Global overview of miRNA transcriptome 39
4.3.2. MiRNA expression profiling of gastric cancer, atrophic gastritis and healthy gastric mucosa by small RNA-seq 40
4.3.3. Verification of specific miRNA expression profile in gastric cancerous and precancerous tissues using TaqMan Low Density Array 41
4.4. Study III 42
4.4.1. The expression analysis of hsa-miR-20b-5p, hsa-miR-451-5p and hsa-miR-1468-5p in GC cell lines AGS and MKN-28 42
4.4.2. The prediction analysis of hsa-miR-20b, hsa-miR-451a, hsa-miR-1468 target-genes 43
4.4.3. The expression analysis of miRNA mimic and inhibitor positive controls target-genes 44
4.4.4. The expression analysis of putative target-genes of hsa-miR-20b-5p in gastric cancer cells AGS and MKN-28 44
4.4.5. The expression analysis of putative target-genes of hsa-miR-451a-5p in gastric cancer cells AGS and MKN-28. 46
4.4.6. The expression analysis of putative target-genes of hsa-miR-1468-5p in gastric cancer cells AGS and MKN-28. 47

5. DISCUSSION 48

CONCLUSIONS 54

REFERENCES 55

PUBLICATIONS 64

SANTRAUKA 97

SUPPLEMENTS 112

CURRICULUM VITAE 125

ACKNOWLEDGEMENTS 126

ABBREVIATIONS

AG	–	Atrophic gastritis
Ath-miR	–	*Arabidopsis thaliana* micro ribonucleic acid
CagA	–	Cytotoxin-Associated Gene A (of *Helicobacter pylori*)
CAV1	–	Caveolin 1
CDH1	–	E-cadherin gene
CIN	–	Chromosomal instability
DNA	–	Deoxyribonucleic acid
EBV	–	Epstein-Barr Virus
EREG	–	Epiregulin
FAT4	–	FAT Atypical Cadherin 4
FDR	–	False Discovery Rate
GC	–	Gastric cancer
GCadj	–	Gastric cancer adjacent
GNCA	–	Gastric non-cardia adenocarcinoma
GTP	–	Guanosine-5'-triphosphate
H. pylori	–	*Helicobacter pylori*
Hsa-miR	–	*Homo sapiens* micro ribonucleic acid
IRF1	–	Interferon Regulatory Factor 1
JAK2	–	Janus kinase 2
microRNA, miRNA	–	Micro ribonucleic acid
MLH1	–	MutL Homolog 1 is a Protein Coding gene
mRNA	–	Messenger ribonucleic acid
OR	–	Odds ratio
PDCD1LG2	–	Programmed Cell Death 1 Ligand 2
PD-L1	–	Programmed death-ligand 1
PIK3CA	–	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PTEN	–	Phosphatase and Tensin Homolog
qRT-PCR	–	Quantitative real-time polymerase chain reaction
RHO	–	(Rhodopsin) is a Protein Coding gene
RHOA	–	(Ras Homolog Family Member A) is a Protein Coding gene
RNA	–	Ribonucleic acid
Small RNA-seq	–	Small RNA sequencing
SNP	–	Single-nucleotide polymorphism
TLDA	–	TaqMan low density array
TNM	–	TNM Classification of Malignant Tumours
TP53	–	Tumor protein p53
TXNIP	–	Thioredoxin Interacting Protein
vacA	–	Vacuolating cytotoxin A (of *Helicobacter pylori*)
WHO	–	World Health Organization

INTRODUCTION

Gastric cancer (GC) is a common malignancy and a leading cause of cancer related death in the world [1]. According to GLOBOCAN 2012, 952,000 new cases of GC were estimated to have occurred in 2012 (6.8% of the total cancer burden), making it the fifth most common malignancy in the world after lung, breast, colorectal and prostate cancers. Whereas, according to Lithuanian Cancer Registry 2012, 947 new cases of GC were diagnosed in 2012 (5% of the total) and it was the sixth most common cancer by morbidity, but it was the second leading cause of death in both sexes in Lithuania (8% of total), after lung cancer. Mortality rates are notably high because, in most cases, the disease is diagnosed at advanced stages when the treatment is likely to fail. The overall 5-year survival rate of patients with GC ranges from 10% to 30% [2]. Therefore, one of the major focuses in GC research is the evaluation of potential molecular biomarkers that could be used for early non-invasive diagnostics of this malignancy.

Both environmental and genetic factors play a role in etiology of GC; however, as in most cancers, pathogenetic mechanisms in GC are still not fully understood. Demographic and environmental risk factors for GC include older age, male sex, family history, tobacco smoking, *H. pylori* infection and obesity [3]. Nevertheless, the exact mechanisms of malignant transformation from *H. pylori* infection to chronic atrophic gastritis (AG), intestinal metaplasia and GC is still poorly understood [4]. Important studies have shown that miRNAs are already deregulated in early stages of gastric carcinogenesis including H. pylori gastritis and premalignant stages of gastric atrophy and intestinal metaplasia [5, 6].

In recent years, different studies, including genome-wide association studies (GWAS), examined genetic risk factors for GC. The discovery of microRNAs (miRNAs) has opened new opportunities for understanding of pathophysiology and molecular biology of GC [7]. Small non-coding miRNA molecules (approximately 18–25 nucleotides) regulate gene expression through sequence-specific pairing with the target messenger RNA (mRNA) and inhibition of its translation [8] and influence the development of diseases [9], especially cancer [10, 11]. Furthermore, miRNAs have been shown to have a diagnostic or prognostic role and even potential clinical implications for targeted gene therapy in cancer patients [12, 13]. Volinia et al. have provided one of the first miRNA expression profiles in GC tissue showing a specific deregulation pattern [14]. Further studies have also reported significant deregulation of miRNAs belonging to hsa-miR-17, hsa-miR-21, hsa-miR-223, hsa-miR-135 and many other families. Among reported studies

in GC tissues, hsa-miR-21, hsa-miR-25, hsa-miR-92, hsa-miR-223 were the most consistently up-regulated miRNAs, while hsa-miR-375 and hsa-miR-148 were the most consistently down-regulated [15–17].

Other studies have revealed that certain single-nucleotide polymorphisms (SNPs) of miRNA encoding genes may alter miRNA expression and influence cancer development [9, 18]. Moreover, genetic variations within miRNA binding sites affect the miRNA–mRNA interaction. SNPs within a miRNA target can reinforce, weaken or disrupt the binding with miRNAs and change the expression of mRNA targets [19–21].

Target-gene identification may help to understand the function of miRNAs. This process is challenging because miRNAs bind to their target mRNAs over a short sequence and the effectivity of targeting are not completely understood. Computational and experimental approaches to the identification of miRNA-regulated genes are applied [20].

MiRNAs are considered to be a promising candidate for clinical diagnosis of many malignant diseases, but this requires more research [23, 24].

1. AIM AND OBJECTIVES

The aim of this study was to investigate microRNA profile and the role of their target gene in gastric cancer pathogenesis and to assess the relevance of these biomarkers for non-invasive diagnosis of the malignant disease.

1.1. Objectives of the study

1. To determine association between single nucleotide polymorphisms in the predicted microRNA target genes *IL12B (rs1368439)*, *INSR (rs1051690)*, *CCND1* (rs7177) and *IL10* (rs3024498) and the presence of gastric cancer in European population.
2. To establish microRNA expression profile in tissue of patients with gastric cancer and atrophic gastritis.
3. To assess the expression of selected microRNAs in plasma of patients with gastric cancer and to evaluate their suitability for non-invasive diagnostic.
4. To evaluate expression differences of the predicted microRNA target genes by experimentally inhibiting hsa-miR-20b and increasing amount of hsa-miR-451a and hsa-miR-1468 in gastric cancer cell lines.

1.2. The novelty and relevance of the work

Study provides novel evidence on: (i) SNPs in miRNA binding sites of target-gene and GC, (ii) miRNA profile in gastric cancerous and precancerous conditions, as well as their suitability for non-invasive diagnostic, (iii) potential target-genes for miRNA. These findings provide a more detailed molecular understanding of GC and tumorgenesis. Moreover, this data might serve not only for the future scientific works, but are highly significant for diagnostics and further identification of new targets and development of novel therapeutics.

2. REVIEW OF LITERATURE

2.1. Epidemiology of gastric cancer

Gastric cancer (GC) is a common malignancy and a leading cause of cancer related death in the world [1]. According to GLOBOCAN 2012, 952,000 new cases of GC were estimated to have occurred in 2012 (6.8% of the total cancer burden), making it the fifth most common malignancy in the world, after lung, breast, colorectal and prostate cancers. Whereas, according to Lithuanian Cancer Registry 2012, 947 new cases of GC were diagnosed in 2012 (5% of the total) and it was the sixth most common cancer by morbidity, but it was the second leading cause of death in both sexes in Lithuania (8% of total), after lung cancer. Mortality rates are notably high because, in most cases, the disease is diagnosed at advanced stages when the treatment is likely to fail. The overall 5-year survival rate of patients with GC ranges from 10% to 30% [2].

2.1.1. Classification and histology of gastric cancer

According World Health Organization (WHO), gastric tumors are histologicaly classified into epithelial and non-epithelial tumors, as shown in Table 2.1 [23].

About 90% of cancers of the stomach are adenocarcinomas and they develops from the stomach mucosa, usually maintaining glandular differentiation [24]. Other less common tumors of the stomach are lymphomas (4% of total), gastrointestinal tumors, carcinoid tumor and others types of cancer, such as squamous cell carcinoma, small cell carcinoma, and leiomyosarcoma, can also start in the stomach, but these cancers are very rare.

The most frequent site of sub-cardial GC is the distal stomach, i.e. the antro-pyloric region. Carcinomas in the body or the corpus of the stomach are typically located along the greater or lesser curvature [23].

Table 2.1. *Classification of gastric tumors according WHO* [23].

Epitelial tumors	**Non-epithelial tumors**
• Intraepithelial neoplasia – Adenoma • Carcinoma – Adenocarcinoma ▪ intestinal type ▪ diffuse type – Papillary adenocarcinoma – Tubular adenocarcinoma – Mucinous adenocarcinoma – Signet-ring cell carcinoma – Adenosquamous carcinoma – Squamous cell carcinoma – Small cell carcinoma – Undifferentiated carcinoma – Others • Carcinoid (well differentiated endocrine neoplasm)	• Leiomyoma • Schwannoma • Granular cell tumor • Glomus tumor • Leiomyosarcoma • GI stromal tumor – benign – uncertain malignant potential – malignant • Kaposi sarcoma • Malignant lymphomas – Marginal zone B-cell lymphoma of MALT-type – Mantle cell lymphoma – Diffuse large B-cell lymphoma – Others • Secondary tumors

Adenocarcinomas are classified according to histology and location. In the traditional Laurén classification, GC is divided into two types: intestinal and diffuse types. Intestinal type is more common than diffuse [27–29]. These two types seem to follow different precancerous processes and show clinical and epidemiologic differences, *H. pylori* infection is the strongest risk factor for the development of most tumors of both types [27]. The intestinal type is more common in males, blacks, and older age [28]. Diffuse carcinoma tends to affect younger individuals, mainly females; it frequently has hereditary characterristics, perhaps modulated by environmental influences [32, 33].

The consortium of The Cancer Genome Atlas (TCGA) described molecular classification of GC and suggested four subtypes:

- chromosomal instability (CIN) type;
- genomically stable (GS) tumors (near-diploid type);
- EBV-positive tumors;
- microsatellite instability (MSI)-positive tumors;

And every of them is described:

The CIN tumors have been associated with marked aneuploidy and focal amplification of receptor tyrosine kinases, as well as mutations of TP53.

The GS type has been associated with diffuse tumors, mutations of *CDH1* and *RHOA*, or fusions involving RHO family GTPase-activating proteins.

The EBV-positive tumors have been correlated with *PIK3CA* mutations; high levels of DNA hypermethylation; and amplification of *JAK2, PD-L1*, and *PDCD1LG2*.

The MSI tumors display characteristic hypermutation phenotype and downregulation of *MLH1* gene (Fig. 2.1) [31].

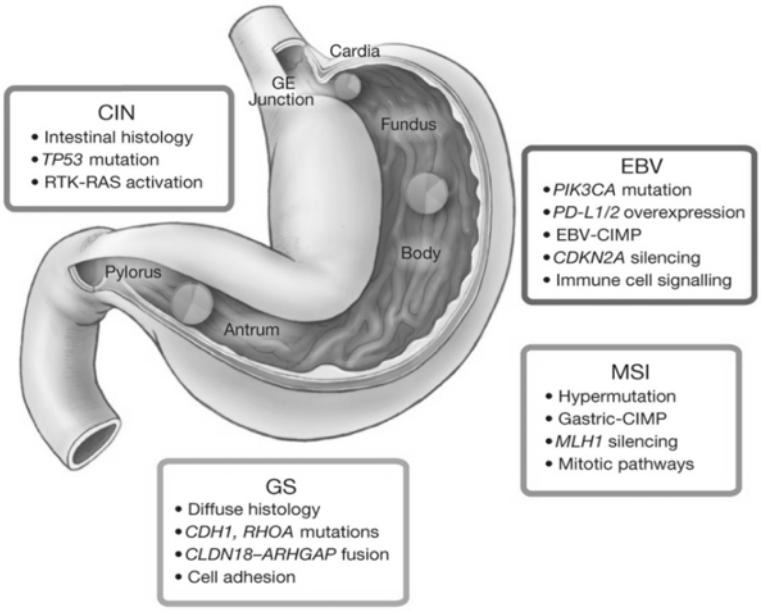

Fig. 2.1. *The four molecular subtypes of gastric cancer. Distribution of molecular subtypes in tumors obtained from distinct regions of the stomach is represented by inset charts* [31].

2.1.2. Risk factors of gastric cancer

GC is a genetically heterogeneous tumor with multifactorial etiologies, associated with environmental and genetic factors.

2.1.3. Environmental Risk Factors

- Gender and ethnicity. GC is more common in men than in woman [28] .
- Age: Risk increases with age, GC is extremely rare below the age of 30 [23].
- Where a person lives: Worldwide, stomach cancer is more common in Eastern Asia, Central and Eastern Europe, Central and South

America. Meanwhile GC is less common in North America, Australia, Africa, Micronesia (GLOBOCAN, 2012).

- Diet: Being overweight or obese increasing the risk of GC. The risk of GC increase when using smoked foods, salted fish and meats, and pickled vegetables. An adequate intake of fresh fruits and vegetables lowers the risk [32], but one new meta-analysis have shown no significant association between GC risk and flavonoid intake (OR = 0.88; 95% CI = 0.74–1.04, I^2 = 63.6%) for a comparison of the highest to the lowest category of intake [33].
- Earlier stomach surgery: The risk of gastric carcinoma increases 5–10 years after gastric surgery, especially when the Bilroth II operation, which increases bile reflux, was performed [23].
- Family history: In most studies, the familial relative risk is approximately three-fold, which is larger than that observed for most other adult forms of solid cancer, with the exception of ovarian cancer. In India, Korea and Turkey, much higher relative risks have been reported [34].
- Physical activity: the risk of GC is lower among the most physically active people as compared with the least physically active people [35].
- *Helicobacter pylori*. The most important development in the epidemiology of adenocarcinoma is the recognition of its association with *H. pylori* infection. Prolonged precancerous process, such as chronic gastritis, multifocal atrophy, intestinal metaplasia, and intraepithelial neoplasia, however, *H. pylori* is genetically heterogeneous, and all strains may not play the same role in the development of malignancy. There are two main virulence factors which are implicated in the progression and the severity of GC, these are Cytotoxin-Associated Gene A (CagA) and Vacuolating Cytotoxin A (VacA) which are injected and secreted by H. Pylori, respectively [39, 40].

2.1.4. Genetic Risk factors

Less than 3% of GC cases arise as a result of inherited syndromes and acquired genetic factors contributing to sporadic GC, which have been systematized and described in a review of Mairi H. McLean and Emad M. El-Omar [38].

2.1.5. Hereditary factors

- Hereditary diffuse GC – autosomal dominant condition is associated most frequently with a heterozygous germline mutation in *CDH1* gene.; *CDH1* germline mutation and potential catenin family genes implicated;
- Gastric adenocarcinoma and proximal polyposis of the stomach – implicated genes are unknown;
- Hereditary nonpolyposis colorectal cancer – mismatch repair genes such as *MSH2* or *MLH1* are implicated;
- Li-Fraumeni syndrome (rare) – *TP53* implicated;
- Peutz-Jeghers syndrome (rare) – *STK11* implicated;
- Familial adenomatous polyposis (rare) – *APC* implicated Acquired genetic factors contributing to sporadic GC.

2.1.6. Acquired genetic factors

- Chromosomal instability, such as aneuploidy, chromosomal translocation, amplification, deletion and loss of heterozygosity;
- Fusion genes, for example, *SLCIA2* (SLCIA2–CD44 fusion protein) and *ROS1* (SLC34A2–ROS1);
- Microsatellite instability, for example, hyper-methylation of promoter of mismatch repair genes, primarily *MSH1*;
- SNPs identified using the candidate gene approach (IL1B-31*C, IL1RN*2/*2, *IL-10*, *TNF*, IL-17A-187*A);
- SNPs identified using genome wide association studies (PSCA rs2976392*A, *MUC1* rs4072037*A);
- Exome sequencing (high-throughput studies) identified driver genes such as *FAT4*, *ARID1A* and *RHOA*;
- Copy number profile sequencing (high-throughput studies) revealed associations with components from RTK/RAS signaling pathway, e.g., *FGFR2*;
- Changes in miRNA profile; e.g., overexpression of hsa-miR-181a influencing target-gene *KLF6* or loss of hsa-miR-449 resulting in reduced expression of tumor suppressor genes such as *TP53*.

2.1.7. Atrophic gastritis

Invasive gastric carcinoma is preceded by a cascade of precancerous lesions. The distal GC can be of two types, intestinal and diffuse, each following different developmental pathways. For intestinal type GC starts from uncontrolled gastric inflammation, which may lead to mucosal atrophy and

hypochlorhydria and increase the risk for intestinal metaplasia, dysplasia and finally cancer [39]. Little is known about the development of diffuse type GC, however it is recognized that *H. pylori* and inflammation may also play a signifycant role. The first step causing histologic changes in gastric tissue is acute inflammation leading to (i) non-atrophic chronic gastritis or (ii) advance to multifocal atrophic gastritis (MAG) and further proceeding to precancerous cascade: (iii) intestinal metaplasia (first "complete" and then "incomplete"), (iv) dysplasia (first low grade and then high grade (equivalent to "carcinoma *in situ*")) and finally development of GC, which is thought to be associated with degradation of the intercellular matrix [40] (Fig. 2.2).

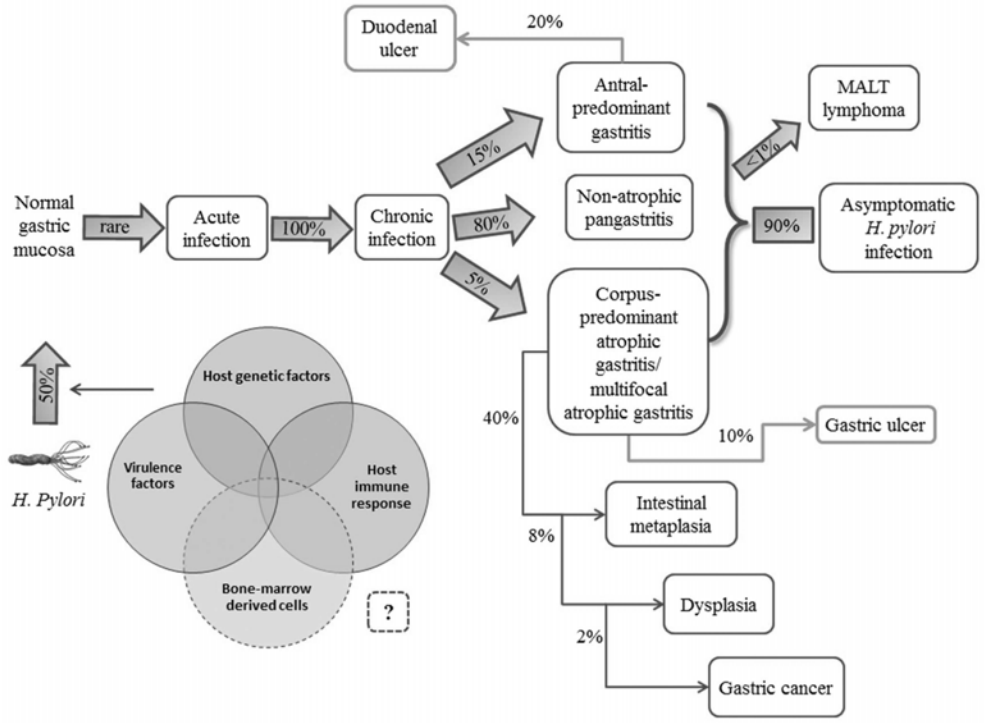

Fig. 2.2. *Precancerous cascade of gastric cancer (MALT-mucosa-associated lymphoid tissue)* [41].

2.1.8. Diagnosis of gastric cancer

Screening is testing for cancer in people without symptoms. In countries such as Japan, where GC is very common, mass screening of the population has helped to find many cases at an early, curable stage [42]. Studies in the United States have not found that routine screening in people at average risk of GC is useful, because this disease is not that common.

The following tests can help diagnose GC: *complete blood count* (CBC) to check for anaemia or *Stool test* to check for blood in the stools. The main test for GC is upper endoscopy, but it is usually performed when people have some signs or symptoms of stomach diseases. Unfortunately most patients are asymptomatic in the early stage of GC [43]. If an abnormal-looking area is seen on endoscopy, the only one way to tell for sure if it is really cancer is by doing a biopsy. The biopsy sample may be tested in 2 different ways:

Immunohistochemistry (IHC): in this test, special antibodies that stick to the HER2/neu protein are applied to the sample, which cause cells to change color if many copies are present. This color change can be seen under a microscope. The test results are reported as 0, 1+, 2+, or 3+.

Fluorescent in situ hybridization (FISH): This test uses fluorescent pieces of DNA that specifically stick to copies of the *HER2/neu* gene in cells, which can then be counted under a fluorescent microscope.

Other imaging tests could be used, such us computed tomography scan, magnetic resonance imaging scan, positron emission tomogramphy scan, sound waves scan and X-ray. But not all methods are readily available, some of them are expensive or invasive.

2.2. MicroRNA

More and more data suggest that small non-coding RNAs such as microRNAs (miRNAs) can be utilized as potential biomarkers for the diagnosis and prognosis of a variety of diseases such as Type-II diabetes, cardiovascular disease, neurological disorders, cancer and other.

2.2.1. MiRNA biogenesis

MiRNAs are ~ 22 nucleotides in length, small non-coding RNAs that function as guide molecules in RNA silencing [44]. Since 1993 a huge number of small RNA classes have been identified, including miRNAs, small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs). These classes differ in their biogenesis, modes of target regulation and in the biological pathways they regulate [45].

The human genome encodes for over 1,800 miRNAs and they regulate about 60% of the human protein-coding genes. Moreover, single miRNA may target hundreds of mRNA [49, 50]. They play a role on the organ development, differentiation and function, as well as tumor formation, progression, invasion and metastasis.

MiRNA genes are scattered among chromosomes in humans, except for the Y chromosome and they are located within different genomic regions (intragenic or intergenic) (Fig. 2.3). The primary miRNA transcripts are transcribed as precursor molecules called pri-miRNAs by RNAPII. Pri-miRNAs are derived either from annotated transcripts (as the introns of protein coding genes, the exons of noncoding genes, or the introns of noncoding genes) or from intergenic regions within the genome and can encode a single or multiple miRNAs [48].

RNase Drosha further processes pri-miRNA into 70- to 100-nt hairpin-shaped precursors, called pre-miRNA, which are exported from the nucleus by exportin 5. In the cytoplasm, the pre-miRNA is cleaved by Dicer into a miRNA:miRNA* duplex. Both arms of the mature product from a hairpin have the potential to function as miRNAs, however which of them will be subsequently loaded into the RISC complex depends on tissue or developmental stage of the cell [49]. MiRNA arm switching is likely to be associated with a change in miRNA function, i.e. a single miRNA precursor encodes two mature miRNAs, with distinct targeting properties [49].

Assembled into RISC, mature miRNA negatively regulates gene expression by either translational repression or mRNA degradation, which is dependent on sequence complementarity between the miRNA and the target mRNA. A role of miRNA depends on its target-genes, i.e. miRNA could be an oncogene or a tumor suppressor gene. A recent computational analysis showed that miRNAs from the same precursor not only target different genes, but also generally target genes involved in different cellular processes.

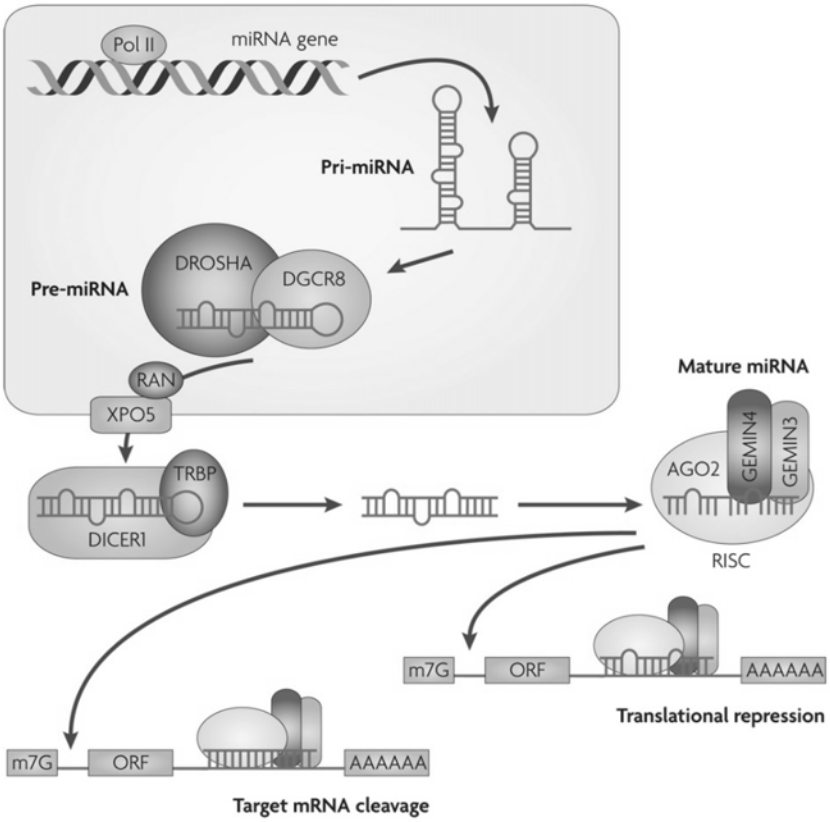

Fig. 2.3. *MiRNA biogenesis* [50].

2.2.2. MiRNA–mRNA interactions

MiRNAs function by targeting complementary sequences in mRNA transcripts, usually in the 3′ untranslated region (3′ UTR), and prevent protein synthesis by inhibiting translation or inducing target-gene degradation. The seed sequence of miRNA (the first 2–8 nucleotides starting at the 5′- end and counting toward the 3′-end) has to be perfectly complementary to target mRNA sequence in order to form Watson-Crick pairs with the cognate target-gene (Fig. 2.4). A Watson-Crick match between a miRNA and mRNA nucleotide occurs when adenosine (A) pairs with uracil (U) and guanine (G) pairs with cytosine (C). G:U wobble in the seed match refers to the allowance of a G pairing with a U instead of a C. Furthermore, mRNA can be repressed by more than one miRNA species at the same time. The level of repression achieved is dependent on both the amount of mRNA and the amount of available miRNA complexes [51].

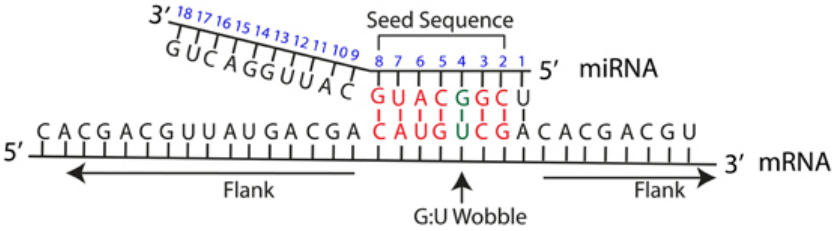

Fig. 2.4. *Schematic miRNA:mRNA interaction. MiRNA position number is shown in blue. The seed sequence refers to nucleotides in miRNA position number 2–8. Flank refers to the mRNA sequence on either side of the region corresponding to the miRNA seed sequence. Watson-Crick matches in the seed sequence are shown in red, and an example of G-U wobble in the seed sequence is shown in green* [47].

Discovering miRNA expression patterns that are unique to a particular cancer and their target-genes is an important step in identifying biomarker signatures that will be effective in cancer detection [52].

2.2.3. Single nucleotide polymorphisms influence miRNA function

Since the discovery of miRNAs, a great number of studies had characterrized genetic polymorphisms in miRNA genes and genes, encoding proteins important in miRNA processing, that affect miRNA regulation [53], affect the miRNA–mRNA interaction and influence the development of diseases [9], especially cancer. However, sequencing has shown that single nucleotide polymorphisms (SNPs) in miRNA coding genes and specifically in miRNA seed regions are rare [57, 58].

SNPs in miRNA genes are thought to affect miRNA processing and function in three ways [53, 59]: (i) through the transcription of the primary transcript (SNPs in ***DROSHA, DGCR8*** **and etc.).** For example, Dicer was found to be reduced in GC tissues in both mRNA and protein levels and down-regulation of Dicer was correlated with tumor differentiation and lymph node invasion in GC tissues, which suggested an essential role of Dicer in cancer invasion [57]. **(ii)** through pri-miRNA and pre-miRNA processing (SNPs in pri-, pre-miRNA). SNP in hsa-miR-27a gene (rs11671784) was shown to be associated with GC [9] and SNP in pri-miR-365b (rs121224) was shown to be associated with intestinal-type GC [58]. SNPs can occur in the pri-miRNA and pre-miRNA strands and are likely to affect miRNA processing and subsequent mature miRNA levels. Such SNPs can lead to either an increase or decrease in processing [50]. (iii) through effects on miRNA–mRNA interactions (**SNPs in miRNA seed and in mRNA regulatory regions)** [8, 53] (Fig. 2.5). SNPs in mature miRNAs and miRNA target binding sites function analogously to

modulate the miRNA–mRNA interaction and create or destroy miRNA binding sites [19–21, 53].Target site polymorphisms still remain poorly explored in different cancers including GC. The importance of miRNA related SNPs in gene regulation and the mechanism by which these SNPs can induce alteration in molecular pathways is largely unknown.

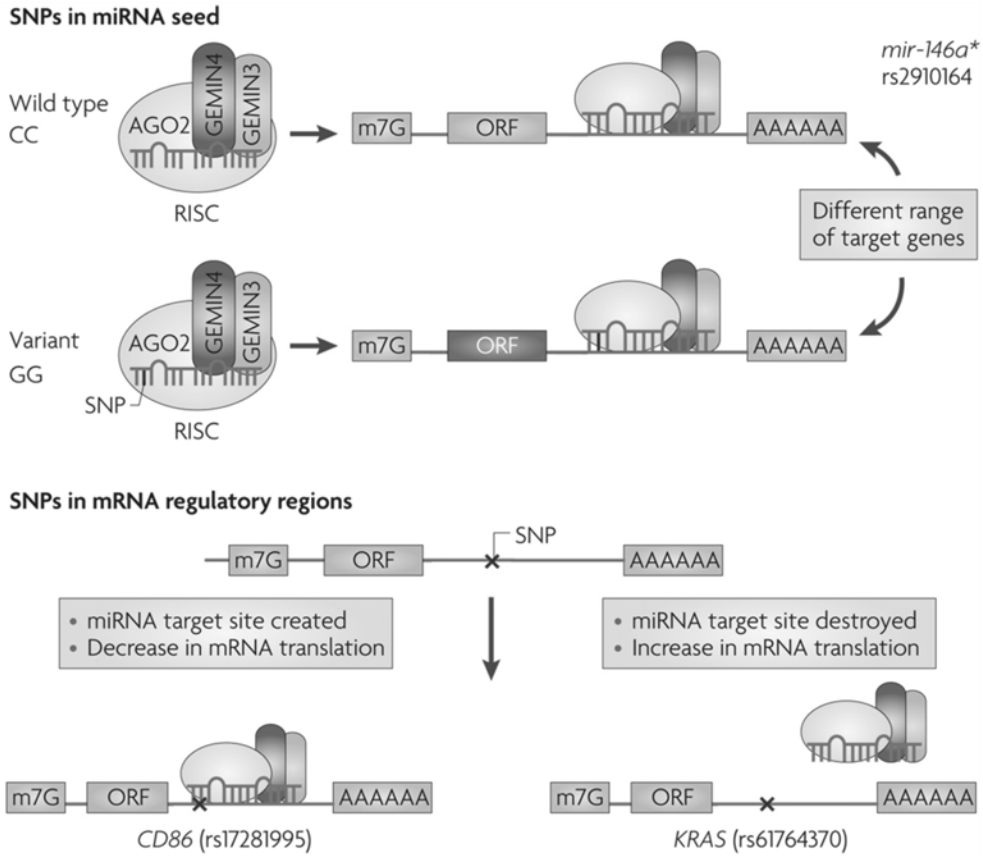

Fig. 2.5. *Example of SNPs in miRNA seed and mRNA regulatory regions.*

2.2.4. Role of miRNAs in atrophic gastritis

Gastric carcinogenesis is a multifactorial *H. pylori*-triggered dynamic process that goes through a cascade of preneoplastic conditions [59]. Important studies have shown that miRNAs are already deregulated in early stages of gastric carcinogenesis, including *H. pylori* induced gastritis and premalignant stages of gastric atrophy and intestinal metaplasia. Study by Petrocca et al. showed that chronic inflammation of the gastric mucosa was associated with alteration of seven miRNAs, specifically hsa-miR-155 [60].

In the recent study by Link et al. the expression of hsa-miR-155, as well as hsa-miR-223 was shown to be increased in chronic non-atrophic gastritis (CNAG) and atrophic gastritis (AG) compared with normal *H. pylori*-negative gastric mucosa [6]. Matsushima et al. profiled tissue samples from *H. pylori* positive and negative subjects and also described an upregulation of hsa-miR-223, as well as down-regulation of other 30 miRNAs (such as has-let-7, hsa-miR-101, hsa-miR-103, hsa-miR-204, hsa-miR-223, hsa-miR-377, etc.). Moreover, eight of the miRNAs (hsa-miR-223, hsa-miR-200a, hsa-miR-31, has-let-7e, hsa-miR-141, hsa-miR-203, hsa-miR-204 and hsa-miR-455) enabled discrimination of *H. pylori* status with acceptable accuracy [61]. These miRNAs were shown to be related with cancer: Kang W et al. confirmed oncogenic role of hsa-miR-223 by targeting *STMN1* gene in GC [62], whereas Li Bo-sheng et al., validated hsa-miR-223 expression in plasma of GC patients [63]. MiRNAs previously shown to be associated with GC, were also shown to be deregulated in precancerous conditions, e.g. hsa-miR-200a was shown to be upregulated in the GC tissue [64]. Other study summarized that hsa-miR-200 family, consisting of 5 members (hsa-miR-200a, -200b, -200c, -141, -429), was associated with cancer initiation and metastasis [65]. This confirms that a number of miRNAs, which are associated with GC are abnormal in precancerous stages as well, so those miRNAs could be used for early GC diagnostic.

2.2.5. Role of miRNAs in gastric cancer

An increasing number of studies have identified miRNAs as potential biomarkers for GC diagnosis, prognosis and therapeutic targets [7, 69, 70]. Volinia et al. have provided one of the first miRNA expression profiles in GC tissue showing a specific deregulation pattern [14]. In tumors up-regulated miRNAs inhibit tumor suppressors, leading to cell proliferation, invasion and reduced apoptosis. These miRNAs are named oncoMiRs. In contrast, down-regulated miRNAs target oncogenes and facilitate the activity of their target oncogenes. Many studies have reported significant deregulation of miRNAs belonging to hsa-miR-17, hsa-miR-19, hsa-miR-21, hsa-miR-223, hsa-miR-135 and other families. Rewiev by Wadhwa [68] indicated the members of hsa-miR-17-92 cluster (hsa-miR-17, hsa-miR-18a, hsa-miR-19a, hsa-miR-20a, hsa-miR-19b-1 and hsa-miR-92) to play an important role in the oncogenesis of GC by inhibiting tumor suppressor genes. Hsa-miR-17 may function as oncogene and promote cancer development by negatively regulating tumor suppressor genes or genes that control cell proliferation [68]. Hsa-miR-19 consists of three subclasses in humans: hsa-miR-19a, hsa-miR-19b1 and hsa-miR-19b2. hsa-miR-19 has been shown to downregulate phosphatase and

tensin homolog *(PTEN)* and effectively increase activity of the cellular survival-promoting signal pathway PI3K-Akt [69]. Another oncomiR - hsa-miR-21 has been found to be deregulated in a wide variety of human cancers, including breast, colorectal, lung, pancreas, skin, liver, gastric, cervical, thyroid, various lymphatic and hematopoietic cancers. Interestingly, high levels of hsa-miR-21 may not only characterize cancer cells but also represent a common feature of pathological cell growth or cell stress [70]. Hsa-miR-155 is a commonly up-regulated oncomiR in human cancers and is suggested as a novel non-invasive biomarker for human cancer detection [71]. Among reported studies in GC tissues, hsa-miR-21, hsa-miR-25, hsa-miR-92, hsa-miR-223 were the most consistently up-regulated miRNAs, while hsa-miR-375 and hsa-miR-148 were the most consistently down-regulated [15–17].

Interestingly, some of miRNAs have opposite deregulation directions in the presence of GC, as separate profiling studies show significant inconsistency among deregulated miRNAs [72]. Hsa-miR-9 was found to be up-regulated in two studies [16, 76], while two other papers showed signifycant down-regulation of this miRNA in GC tissues [63, 77]. These discrepancies regarding opposite direction of deregulation are most likely linked to differrences in anatomical location of the GC, histological subtype, disease stage, profiling platforms, statistical analysis and many other potential confounding factors. Besides, the majority of currently published studies on miRNA profile in GC tissues cover subjects from Asian countries; meanwhile, data on European GC patients is still scarce [76].

Based on the literature survey one could point out a huge number of miRNAs or miRNA families to be associated with GC. However, the acquired results in GC miRNA research are conflicting, therefore further studies are needed in order to validate them.

2.2.6. Circulating miRNAs

MiRNA presence in blood was discovered in 2008. They can be detected in plasma and blood cells (platelets, erythrocytes and nucleated blood cells) [55, 79, 80]. Their occurrence in plasma or serum maybe the result of cell death inside a tissue, or secretion by certain cells in response to a stimulus [81, 82]. However, the origin of these miRNAs is not completely determined. In addition, they can also be secreted in a free form by some other mechanisms [82, 79]. For example, food-derived plant miRNAs may pass through the gastrointestinal (GI) tract, enter into the plasma and serum and interact with mammalian endogenous RNAs and regulate their expression [83, 84]. Furthermore, miRNAs are stable and resistant to pH variations, long-term storage at room temperature, and repeated freeze–thaw cycles both in serum

and plasma samples [55, 85, 81]. It is assumed that the stability of circulating miRNAs and their high resistance to degradation by blood nucleases is due to miRNA complexes with various proteins, such as Ago2, lipoproteins, exosomes and other microparticles in blood (Fig. 2.6).

Since the discovery of circulating miRNAs in body fluids, an increasing number of studies have focused on their potential as non-invasive biomarkers for many diseases, particularly for cancers [55, 86]. Review by Schwarzenbach et al. summarizes clinical relevance of circulating miRNAs and provides lists of cell-free miRNAs that can be used as prognostic and predictive biomarkers in cancer and discusses their utility in personalized medicine. For example, hsa-miR-20a, hsa-miR-24, hsa-miR-25, hsa-miR-145, hsa-miR-152, hsa-miR-199-5p, hsa-miR-221, hsa-miR-222, hsa-miR-223, hsa-miR-320 circulating in serum or plasma may be used as non-invasive biomarkers for the detection of lung cancer, hsa-miR-200b for breast cancer,hsa-miR-375, hsa-miR-141, hsa-miR-378*, hsa-miR-409-3p for prostate cancer and so on [85].

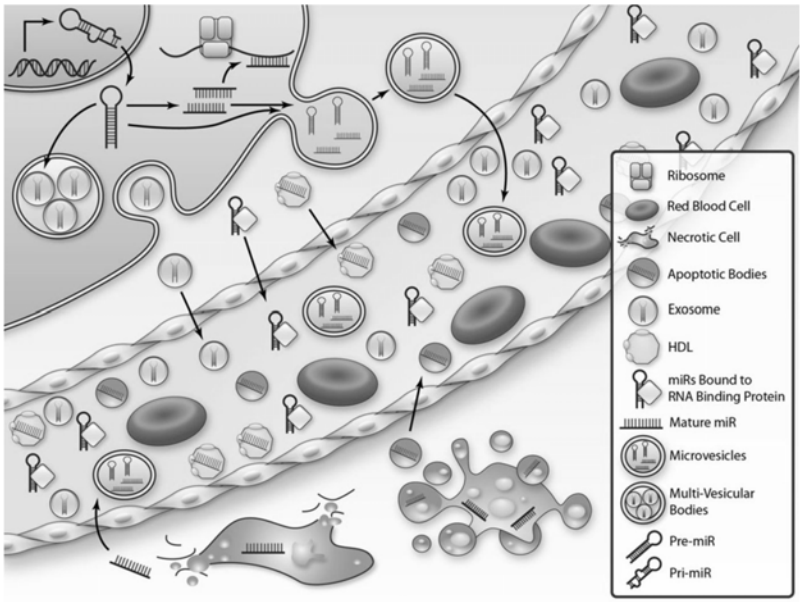

Fig. 2.6. *Putative sources of circulating miRNAs. HDL – high-density lipoprotein [82].*

2.2.7. Circulating miRNAs in gastric cancer

Studies indicate that there are less prognostic miRNA biomarkers in blood compared to tissue of GC patients [86]. Two possible reasons have been proposed: first, there are more studies investigating miRNAs in gastric tissues; second, miRNAs are selectively released into blood. Furthermore, studies indicate that circulating miRNAs are not GC phenotype specific, i.e. miRNAs in blood significantly correlate with no more than two clinicopathological features, whereas miRNAs in tissues are associated with more features. For example, Liu et at al. identified five miRNAs (hsa-miR-1, hsa-miR-20a, hsa-miR-27a, hsa-miR-34, hsa-miR-423-5p) as biomarkers for GC detection and the expression level of these miRNAs correlated with tumor stage [87]. However, miRNAs in blood and tissues of GC patients are associated with survival, i.e. most of the miRNAs with high expression level were detected in the poor survival groups of GC patients. For example, study by Zhu et al. identified and validated five differentially expressed miRNAs (hsa-miR-16, hsa-miR-25, hsa-miR-92a, hsa-miR-451 and hsa-miR-486-5p) in plasma of patients diagnosed with gastric non-cardia adenocarcinoma (GNCA). More importantly, these five miRNAs showed high diagnostic performance in the group of patients with early-stage of GNCA [88].

3. MATERIALS AND METHODS

3.1. Ethics statement

The study was approved by the Kaunas Regional Biomedical Research Ethics Committee (Protocol No BE-2-10). Written informed consent was obtained from all study participants.

For Study I, patients and controls were recruited during the years 2005–2013 at three gastroenterology centers in Germany (Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg), Lithuania (Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas) and Latvia (Riga East University Hospital and Digestive Diseases Centre GASTRO, Riga). Controls were patients from the out-patient departments, who were referred for upper endoscopy because of dyspeptic symptoms and had no history of previous malignancy. GC patients had histological verification of gastric adenocarcinoma and were recruited from out-patient and stationary departments.

For study II patients and controls were included during the years 2007–2015 at Department of Gastroenterology and Department of Surgery, Lithuanian University of Health Sciences (Kaunas, Lithuania). Control group consisted of patients who had no history of previous malignancy. All patients had histological verification of diagnosis. All patients signed an informed consent form to participate in the study.

3.2. Major reagents

TaqMan MiRNA Reverse Transcription Kit and miRNA-specific RT stem-loop primers, High Capacity Reverse Transcription Kit, TaqMan MiRNAs Assay, TaqMan® Low Density Array Card, TaqMan Universal PCR Master Mix, inhibitor hsa-miR-20b-5p and mimics hsa-miR-451a-5p and hsa-miR-1468-5p and nonspecific control (NC) were purchased from Thermo Fisher Scientific (former Applied Biosystems).

The miRNeasy Micro Kit, RNease Mini Kit, RNease Micro Kit, miRNeasy Serum/ Plasma Kit were purchased from Qiagen (USA).

ELISA kit to detect the serum-specific *IgG* antigen was purchased from Virion/Serion GmbH (Germany).

RNAlater, mirVana miRNA Isolation Kit was purchased from Thermo Fisher Scientific (former Ambion, Austin (USA)).

Human gastric carcinoma cell line AGS (CRL-1739™) was obtained from American Cell Bank (ATCCTM), GC cell line MKN-28 was provided by

Alexander Link (from Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University (Magdeburg, Germany).

Fetal bovine serum, Hams's F-12K (Kaighn's) medium, penicillin and streptomycin (5,000 U/ml) were purchased from Gibco™ by Life Technologies (USA). Lipofectamine 3000 were purchased from Thermo Fisher Scientific (former Invitrogen (USA)). Trypsin and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (USA).

3.3. Study I

3.3.1. Study design and patients samples

The aim of Study I was to evaluate associations between miRNA target-genes *IL12B, INSR, CCND1* and *IL10* polymorphisms and GC in European population. In total, 982 individuals were included in this study (508 controls and 474 GC). There were 206 subjects from Germany (104 controls and 102 GC), 285 subjects from Latvia (146 controls and 139 GC) and 491 subjects from Lithuania (258 controls and 233 GC). All patients were of European descent. Clinical characteristic and detailed description of patients is presented in the article *"Polymorphisms of microRNA target-genes IL12B, INSR, CCND1 and IL10 in gastric cancer"*.

3.3.2. DNA extraction, selection of putative miRNA target-gene single nucleotide polymorphisms and genotyping

Genomic DNA was extracted using salting out method from peripheral blood mononuclear cells [89]. In order to select the candidate SNPs falling within 3'-UTR of genes, which are putative targets of frequently deregulated miRNAs in GC, the mirsnpscore database was used (http://www.bigr.medisin.ntnu.no/mirsnpscore). Using this bioinformatic approach we selected four genes: *IL12B* (rs1368439), *INSR* (rs1051690), *CCND1* (rs7177) and *IL10* (rs3024498) that have potential target sites of hsa-miR-27, hsa-miR-146a, hsa-miR-223 and hsa-miR-107. *IL12B* T>G (rs1368439), *INSR* T>C (rs1051690), *CCND1* A>C (rs7177) and *IL10* T>C (rs3024498) SNPs were genotyped using TaqMan® genotyping method. A detailed description is presented in the publication *"Polymorphisms of microRNA target-genes IL12B, INSR, CCND1 and IL10 in gastric cancer"*.

3.4. Study II

3.4.1. Study design

First (Part I), miRNA profile was determined in GC tissues compared to the normal healthy gastric mucosa. Later, expression level of selected miRNAs was validated in GC tissue and plasma samples of GC patients and healthy controls.

Second (Part II), miRNA profile was determined in AG and GC tissue compared to the normal healthy gastric mucosa. In replication analysis we validated profiling results, see Table 3.1.

Table 3.1. *Design of study II*

Design of Study II		**Type of tissue**	**Number of samples**	**Number of miRNA review**	**Used method**
Part I	Profiling cohort	Mucosa of stomach	GC, n = 13 HC, n = 12	377	qRT-PCR with TLDA card A
	Validation cohort	Mucosa of stomach	GC, n = 38 HC, n = 39	6	qRT-PCR with TaqMan assay
		Plasma		4	
Part II	Profiling cohort	Mucosa of stomach	GC, n = 20 AG, n = 18 HC, n = 26	2,180	Small RNA-seq
	Validation cohort	Mucosa of stomach	GC, n = 39 AG, n = 40 HC, n = 40	21	qRT-PCR with custom TLDA

GC – gastric cancer, AG – atrophic gastritis HC – healthy control, TLDA – TaqMan low density array.

3.4.2. Study population of part I of study II

The study included a total of 51 control subjects and 51 GC patients, who were divided into the profiling (GC, n = 13; controls, n = 12) and validation cohorts (GC, n = 38; controls, n = 39). Gastric tissues (obtained during endoscopic or surgical procedures) and plasma samples were collected from healthy patients and patients with GC. Detailed description of study population used for this part of the study is presented in the article *"Analysis of Deregulated microRNAs and Their Target-genes in Gastric Cancer"*.

3.4.3. Study population of part II of study II

The study included a total of 59 control subjects, 58 AG patients and 66 GC patients, which were divided into the profiling (GC, n = 20; AG, n = 18 and controls, n = 26) and validation (GC, n = 39; AG, n = 40 and controls, n = 40) cohorts. Clinical and pathological characteristics of the patient cohorts including age, gender and disease stage are summarized in Table 3.2. Profiling analysis was performed using next-generation sequencing and validation analysis was carried out using custom RT-qPCR with TLDA custom card of selected miRNAs in the larger cohort.

Table 3.2. *Clinical characteristics of the gastric cancer and atrophic gastritis patients and healthy controls.*

		Profiling cobort (n = 64)			**Validation cohort (n = 119)**			**p-value**
		Gastric cancer (n = 20)	**Atrophic gastritis (n = 18)**	**Healthy control (n = 26)**	**Gastric cancer (n = 39)**	**Atrophic gastritis (n=40)**	**Healthy control (n=40)**	
Age	Mean±	65 ± 10.9	68.9 ± 8.9	60.9 ± 15	70.5 ± 10.8	63.3 ± 11.7	57 ± 16.5	
Gender	Male, n (%)	15 (75)	3 (16.7)	7 (26.9)	24 (61.5)	16 (40)	10 (25)	> 0.05*
	Female, n (%)	5 (25)	15 (83.3)	19(73.1)	15 (38.5)	24 (60)	30 (75)	
Lauren classification	Diffuse, n (%)	10 (50)			15 (38.5)			> 0.05*
	Intestinal, n (%)	10 (50)			24 (61.5)			
	Mixed, n (%)	0			0			
	Unknown, n (%)	0			0			
H. pylori infection	Positive, n (%)	9 (45)	7 (38.9)	11 (42.3)	24 (61.5)	29 (72.5)	20 (50)	>0.05 [GC] [Con] 0.02 [AG]*
	Negative, n (%)	6 (30)	11 (61.1)	15 (57.7)	9 (23)	11 (27.5)	18 (45)	
	Unknown, n (%)	5 (25)	0	0	6 (15.4)	0	2 (5)	
Tumor localization	Cardia, n (%)	2 (10)			6 (15.4)			>0.05*
	Corpus, n (%)	9 (45)			22 (56.4)			
	Antrum, n (%)	9 (45)			8 (20.5)			
	Linitis plastica, n (%)	0			3 (7.7)			
TNM staging	I, n (%)	6 (30)			7 (18.0)			0.02*
	II, n (%)	2 (10)			11 (28.2)			
	III, n (%)	10 (50)			7 (17.9)			
	IV, n (%)	2 (10)			12 (30.8)			
	Unknown, n (%)				2 (5.1)			

Table 3.2. *Continued*

		Profiling cobort (n = 64)			Validation cohort (n = 119)			p-value
		Gastric cancer (n = 20)	**Atrophic gastritis (n = 18)**	**Healthy control (n = 26)**	**Gastric cancer (n = 39)**	**Atrophic gastritis (n=40)**	**Healthy control (n=40)**	
T	1/2	8 (40)			12 (30.8)			> 0.05*
	3	7 (35)			12 (30.8)			
	4	5 (25)			13 (33.3)			
	Unknown, n (%)	0			2 (5.1)			
N	0	9 (945)			14 (35.9)			> 0.05*
	1	3 (15)			12 (30.8)			
	2	3 (15)			6 (15.4)			
	3	5 (25)			5 (12.8)			
	Unknown, n (%)	0			2 (5.1)			
M	0	14 (70)			14 (35.9)			0.05 *
	1	2 (10)			12 (30.8)			
	Unknown, n (%)	4 (20)			13 (33.3)			
Differentiation grade	1/2	10 (50)			12 (30.8)			> 0.05*
	3	10 (50)			21 (53.8)			
	Unknown, n (%)	0			6 (15.4)			

* – p value meaning is calculated compared profiling and validation cohort in each group (GC to GC; AG to AG and HC to HC groups). p > 0.05 means that groups are not statistically different, (Mann-Whitney test).

3.4.4. Tissue sample preparation and RNA extraction

Gastric biopsy samples were obtained from antral part of the stomach from control subjects who were referred for upper GI endoscopy due to dyspeptic symptoms and had no previous history of malignancy. GC tissue specimens were obtained from surgical specimens immediately after resection from patients undergoing primary surgery for GC with no preoperative irradiation and chemotherapy. Gastric adenocarcinoma in GC patients was verified by histology and classified according to Lauren into diffuse and intestinal types [25]. *H. pylori* status was assessed in GC, AG and control subjects using indirect ELISA to detect the serum-specific IgG antigen. Gastric tissue samples were stored in RNAlater for +4°C and 24 hours and later stored at 80°C. 30 mg of tissue was homogenized in sterile conditions before total RNA isolation with mirVana miRNA Isolation Kit for profiling study (part I of study II) and miRNeasy Mini Kit for profiling study (part II of study II) and validation studies (part I of study II and part II of study II), according to the manufacturers' instruction. The quality and quantity of RNA was assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

3.4.5. Plasma sample preparation and RNA extraction

Plasma samples were collected from healthy patients and patients with GC. MiRNA profiling (part I of study II) was carried out using plasma samples from 38 GC patients and 39 healthy volunteers. Venous blood was collected in EDTA anticoagulation vacuum tubes and was centrifuged at 3500 rpm for 15 min at room temperature; separated plasma was transferred into 1.5 ml tubes and placed in a −80°C freezer for short-term storage. Small RNAs were extracted from 200 µl of plasma using the miRNeasy Serum/ Plasma Kit according to the manufacturers' instruction.

3.4.6. MicroRNA profiling using the TaqMan Low Density Array card A

MiRNA profiling (part I of study II) in GC tissue compared to normal gastric tissue was carried out using TLDA card A which enabled to quantify 374 miRNAs. Complementary DNA (cDNA) was reverse transcribed from total RNA samples using Megaplex^{TM} pool. More details are presented in publication *"Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer"*.

3.4.7. MicroRNA validation using the custom TaqMan Low Density Array

Validation of miRNA expression profiles (part II of study II) was performed in GC, AG and normal tissue using TLDA custom card which enabled to quantify 24 selected miRNAs. In short, 600 ng of total RNA was initially reverse transcribed using Custom RT pool (G1406). 0.88 µl of cDNA, 49.9 µl of TaqMan® Universal Master Mix II, No AmpErase UNG (2X) and 49.1 µl nuclease free water was loaded per port on 8-port TLDA card and run on ViiA 7 Real-time PCR System (Applied Biosystems) following the standard protocol. All GC and AG tissue samples were randomized and placed with healthy tissue controls on the same TLDA card. The expression level of miRNAs in tissue was normalized to the mean expression values of hsa-miR-16-5p.

3.4.8. Quantitative real-time polymerase chain reaction with TaqMan assay

TaqMan® miRNA assays were used to quantify tissue-validated miRNAs in tissue and plasma samples (part I of study II). Reverse transcription was performed using TaqMan® MiRNA Reverse Transcription Kit and miRNA-specific RT stem-loop primers. Singleplex qPCR reactions were scaled down to 7.5 µl and performed according to the manufacturer's instructions. Each sample was run in duplicate. The expression level of miRNAs in tissue and plasma was normalized to the mean expression values of hsa-miR-127 and hsa-miR-16-5p, respectively [90,91].

3.4.9. Preparation of small RNA libraries and next generation sequencing

Small RNA libraries were prepared using TruSeq Small RNA Sample Preparation Kit (Illumina) according to the manufacturer's protocol with 1µg RNA input per sample followed by RNA 3' adapter ligation, RNA 5' adapter ligation, cDNA synthesis, PCR amplification using unique barcode sequences for each sample and gel size-selection of small RNA library. The yield of sequencing libraries was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Small RNA libraries were pooled with 6 samples per lane for plasma and 24 samples per lane for tissue samples and sequenced on HiSeq2500 (Illumina) next-generation sequencing platform.

3.4.10. Small RNA-seq data and statistical analysis

Statistical analysis of study II part I is presented in publication “Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer”. In study II part II in profiling cohort several filtering steps were performed after obtaining and multiplexing the raw reads. At first, cutadapt was used to trim low-quality ends of the reads with Phred quality score value (Q) > 30 and to remove 3' adapter (5’-TGGAATTCTCGGGTGCCAAGG-3’) sequences from the reads. The trimmed sequences shorter than 17 bp were discarded. Second, in order to reduce computational time the reads with exact sequences were collapsed while saving the information about the read counts. Third, the collapsed reads which mapped to viral genome (RefSeq) [92], human tRNAs, rRNAs, snRNAs, sRNAs (Rfam) [93] and non-human miRNA precursors (miRBase v20) [94] were filtered using BLASTN v2.2.30 [95]. The mature miRNA quantification in filtered reads were performed using the quantifier.pl module from miRDeep2 v2.0.0.7 software [96], with default parameters using mature miRNA sequences as reference (miRBase v20). Differential expression analyses of the size factor normalized counts of mature miRNAs between samples were performed by use of negative binomial generalized linear models implemented DESeq2 package [97]. The p-values resulting from Wald tests [98] were corrected for multiple testing using the false discovery rate (FDR) proposed by Benjamini and Hochberg [99]. The mature miRNAs with a corrected $p < 0.01$, and fold change > 2 were considered to be significantly differentially expressed. Power of the miRNA-seq study was 80% in GC group and 99% in AG group (Fig. 3.1).

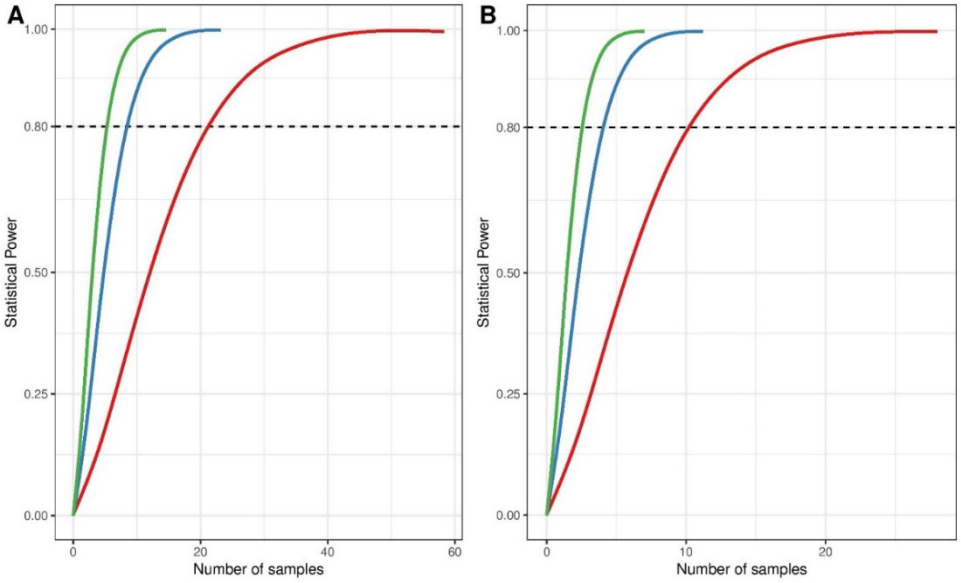

Fig. 3.1. *Power of miRNA-seq study in gastric cancer (A) and in atrophic gastritis (B). Effect size, when: (i) fold change = 2 ($p < 0.01$) showed in red, (ii) fold change = 3 ($p < 0.01$) showed in blue, (iii) fold change = 4 ($p < 0.01$) showed in green.*

The differentially expressed miRNAs were selected for validation study in the following steps: (i) differentially expressed miRNAs in tissue were filtered based on their expression level in plasma (read count sum of miRNAs present in plasma samples > 5); (ii) in order to show similarities in miRNA expression between GC and AG conditions, overlapping differentially expressed miRNAs in both conditions were selected when compared to HC; (iii) top differentially expressed miRNAs (lowest FDR adjusted p-value and highest fold change value) were chosen for both GC and AG conditions in order to select the most informative and condition-specific miRNAs.

In order to evaluate the prediction performance of GC and AG miRNA expression profiles in plasma, radial support vector machine (SVM) and logistic regression models were built. The samples containing normalized miRNA expression data were used to construct the model. The tuning parameters thereby were trained using 10-fold cross-validation. For model testing the samples were randomly split into two sets at 5:3 ratio and the smaller partition was used to evaluate models prediction performances. The classifiers' performances were measured by the area under the receiver operating characteristic (ROC) curve, sensitivity (SN) and specificity (SP). The statistical prediction analysis was performed using caret [100] and ROCR [101] packages.

3.5. Study III

3.5.1. Study design

First, we performed hsa-miR-20b-5p, hsa-miR-451a-5p and hsa-miR-1468-5p expression analysis in GC cells (AGS and MKN-28) compared to normal tissue. Second, putative target-genes of these miRNAs were selected using mathematics algorisms in "R" package. Third, GC cells transfection with inhibitor of hsa-miR-20b-5p and mimics hsa-miR-451a-5p and hsa-miR-1468-5p was performed using Lipofectamine 3000. Finally, putative target-gene expression was performed 24 h and 48 h after transfection using qRT-PCR.

3.5.2. Evaluation of miRNAs target-genes

MiRNAs target-genes were evaluated using mathematical algorithms in "R" package (RStudio Desktop v. 1.0.143) software. First, DisGenet data base was used to determine GC associated genes. Second, DIANA-microT-CDS, ElMMo, MicroCosm, miRanda, miRDB, PicTar, PITA and TargetScan mathematical prediction tools were used for hsa-miR-20b-5p, hsa-miR-451a-5p, hsa-miR-1468-5p potential target-genes analysis. Next, depending on information in miRecords, miRTarBase and TarBase target-genes, which were already validated in other studies, were rejected. Finally, target-genes were filtrated according their function in oncogenesis (oncosupressor or protooncogene) and literature analysis was performed.

3.5.3. Cell culture conditions

The AGS and MKN-28 cells were cultured in Ham's F-12K medium containing 10% fetal bovine serum, 1% penicillin-streptomycin solution and incubated at 37 °C, 5% CO2 and saturated humidity. Cell growth was observed under an inverted microscope. When cell growth reached 70% to 80% confluence, the cells were digested with 0.25% trypsin and passaged. The culture medium was changed every other day, and the cells were passaged every 3 to 4 days. Cells in the logarithmic growth phase were collected for experiments.

3.5.4. MiRNA genes expression analysis in gastric cancer cells

Total RNA from gastric cells AGS (n = 7) and MKN-28 (n = 7) and normal gastric tissue (n = 11) was extracted using miRNeasy Micro Kit in accordance with manufacturer's recommendations. Complementary DNA was reverse transcribed from total RNA samples using TaqMan miRNA assays hsa-miR-20b (001014), hsa-miR-451a (001141), hsa-miR-1468 (121107_mat) and hsa-miR-16 (000391) as endogenous control. The resulting cDNA was subsquently

amplified by PCR using TaqMan assay and Universal Master Mix according to the manufacturer's recommendations using a qRT-PCR System ABI 7500 Fast (Applied biosystem, USA).

3.5.5. Gastric cancer cells transfection

GC cells (AGS and MKN-28) were seeded into twenty-four-well culture plates at a concentration of 40,000 cells/well. The volume in each well was 500 µl. Once the cells adhered, they were transfected with the inhibitor of hsa-miR-20b-5p, mimics of hsa-miR-451a-5p and hsa-miR-1468-5p, and nonspecific control (NC) according to the Lipofectamine 3000 transfection manual. Mimic positive control (PC) (hsa-miR-1) and inhibitor PC (hsa-anti-let-7c) were used to optimize transfection experiments. Transfection procedure was performed three times with different passages of GC cells.

3.5.6. MiRNA putative gene expression analysis in gastric cancer cells

Total RNA was extracted from transfected cells (AGS and MKN-28) using the RNease Micro Kit and reverse transcription was performed from 1 µg of total RNA in 20 µl reaction using High-Capacity Reverse Transcription Kit in accordance to manufacturer's recommendations. The mRNA expression levels of the putative target-genes *EREG, FAT4, IRF1, TXNIP, PTEN* for hsa-miR-20b; *CAV1* and *ADAM28* for hsa-miR-451a; *TNFα, DNMT1* and *CITED2* for hsa-miR-1468 were assessed by quantitative RT-PCR 24 h and 48 h after transfection, using TaqMan Gene Expression assays. Two technical replicates were made for each gene assay and reaction was performed on ABI 7500 Fast Real-Time PCR system (Applied Biosystems, USA). Relative fold mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method, with *ACTB* as an internal control.

3.5.7. Statistics

The data are expressed as the mean dCt of three independent experiments and processed by GraphPad Prism software (GraphPad Software Inc., San Diego, USA). The Mann Whitney U-test were used to distinguish differences between groups. A P value of < 0.05 was considered statistically significant.

4. RESULTS

4.1. Study I

Similar distribution of genotype and allele frequencies was observed between GC patients and controls for all polymorphisms under study (*IL12B* (rs1368439), *CCND1* (rs7177) *and IL10* (rs3024498)) except for *INSR* SNP (rs1051690). The frequency of the rare allele T of *INSR* gene was significantly higher in GC patients than in controls (23.26% and 19.19% respectively, P=0.028). CT genotype was also more prevalent in patients compared to control group (38.48% and 30.12% respectively, $p < 0.021$). Logistic regression analysis revealed that only one polymorphism (rs1051690 in *INSR* gene) was associated with increased risk of GC. Carriers of CT genotype had higher odds of GC when compared to CC genotype (OR – 1.45, 95% PI 1.08 – 1.95, P = 0.01). Similar association was observed in a dominant model for *INSR* gene, where combination of TT+CT genotypes showed an increased risk for GC (OR – 1.44, 95% PI 1.08 – 1.90, P = 0.01). Other analyzed SNPs were not associated with the presence of GC. *A detailed description of the study results is presented in the article "Polymorphisms of microRNA target genes IL12B, INSR, CCND1 and IL10 in gastric cancer"*

4.2. Study II, Part I

4.2.1. MiRNA expression profiling of GC and healthy gastric mucosa tissue by Taq-Man low-density array

Tissue miRNA profiles from GC patients were compared with profiles of gastric mucosa from healthy individuals and 15 differentially expressed miRNAs were identified, 7 of which were up-regulated and 8 were down-regulated with FDR adjusted p-value < 0.01 and fold change > 2. Among these, 6 miRNAs (hsa-miR-135a-5p, hsa-miR-148a-3p, hsa-miR- 204-5p, hsa-miR-375, hsa-miR-223-3p and hsa-miR-155-5p), showing the highest significance and fold change values, were selected for the validation study. *A detailed description of the study results is presented in the article "Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer"*.

4.2.2. MiRNA validation in gastric cancer and healthy gastric mucosa tissue with quantitative real-time polymerase chain reaction

The six candidate miRNAs selected for verification were quantified using qRT-PCR. The expression levels of hsa-miR-148a-3p, hsa-miR-204-5p, hsa-miR-223-3p and hsa-miR-375 in GC tissue showed significant differential expression in the same direction as in the discovery study (FDR adjusted $p < 0.05$). A detailed description is presented in the article *"Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer"*.

4.2.3. Plasma miRNAs as potential biomarkers for gastric cancer

In GC tissue validated hsa-miR-148a-3p, hsa-miR-204-5p, hsa-miR-223-3p and hsa-miR-375 were further analyzed in plasma samples. The relative levels of the candidate miRNAs in individual samples were determined using qRT-PCR. The hsa-miR-16-5p was used as the endogenous control to normalize the expression levels of candidate miRNAs. The expression levels of hsa-miR-148a-3p and hsa-miR-375 showed significant down-regulation in GC plasma; while hsa-miR-223-3p was significantly up-regulated (FDR adjusted $p < 0.05$). No significant differences were detected in the expression levels of hsa-miR-204-5p. *A detailed description is presented in the article "Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer"*.

4.3. Study II, part II

4.3.1. Global overview of miRNA transcriptome

Small RNA-seq of normal stomach tissue, AG and GC tissue samples yielded ~ 271M raw sequencing reads (from ~417K to ~29.5M reads/sample). Pre-filtering and filtering steps retained 50.4% (~137M) of initial raw reads. The majority of filtered reads were of 20–23 nt length which corresponds to the range of mature miRNA sequences. Quantification of filtered reads and identification of known miRNAs have yielded ~56.5M sequences to be mapped to 1,661 known miRNAs from miRBase v20.

4.3.2. MiRNA expression profiling of gastric cancer, atrophic gastritis and healthy gastric mucosa by small RNA-seq

The similarity structure of the tissue miRNA transcriptomes among GC, GCadj, AG and HC was identified by a multidimensional scaling (MDS) analysis using Spearman's correlation distance (1-correlation coefficient). In the picture we can see four different groups, and it is interesting that GCadj and HC show miRNA expression profile similarity (Fig. 4.1).

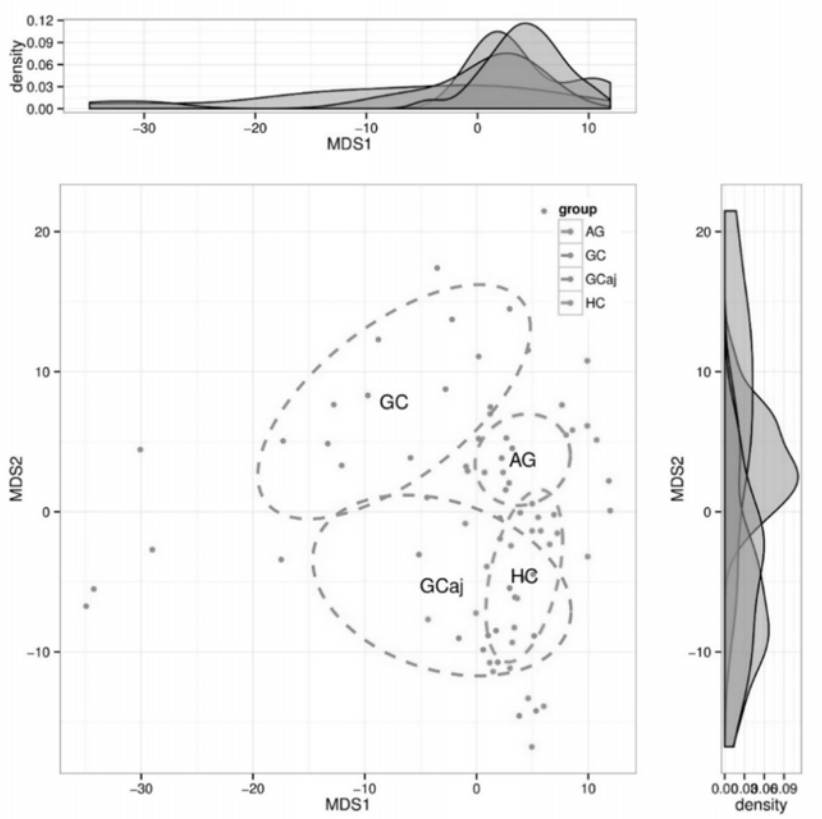

Fig. 4.1. *Similarity structure of miRNA transcriptome generated by next generation sequencing. Multidimensional scaling plot showing four clusters corresponding to tissue of gastric cancer (GC), gastric cancer adjacent (GC adj), atrophic gastritis (AG) and normal mucosa (healthy control (HC)). The analysis was performed on normalized miRNA count data using Spearman's correlation distance (1-correlation coefficient).*

For identification of differentially expressed miRNAs among cancerous, precancerous and healthy gastric mucosa tissue specimens, the comparative analysis of normalized small RNA-seq expression data was performed.

110 differentially expressed miRNAs were identified in GC group compared to HC group, 72 of which were up-regulated and 38 were down-regulated. Whereas, 32 differentially expressed miRNAs were identified in GCadj group compared to HC group, 25 of which were up-regulated and 7 were down-regulated. It confirms similarity among GC adjacent tissue and healthy tissue. Only 17 differentially expressed miRNAs were identified in AG compared to HC, 11 of which were down-regulated and 6 were up-regulated. Comparison of the miRNA expression profiles between GC and AG identified 76 differentially expressed miRNAs, 54 of which were up-regulated and 22 were down-regulated.

To further determine miRNAs that could be used as a signature for GC and AG prediction, 21 miRNAs present in plasma (sum of read counts > 5) were selected for validation study, see Table 5 in supplement. Hsa-miR-16, U6 and ath-miR-159a were selected as endogenous controls.

4.3.3. Verification of specific miRNA expression profile in gastric cancerous and precancerous tissues using TaqMan Low Density Array

Taq-Man low-density array (TLDA) custom card analysis was used to measure expression levels of the 21 selected miRNAs in gastric tissue of independent cohort, which consisted of 119 samples (GC = 39; AG = 40; HC = 40). Two-class differential expression analyses were applied on continuous expression data which was normalized to the expression value of hsa-miR-16, U6 and ath-miR-159a.

The expression levels of 8 out of 21 miRNAs in GC and 2 out of 21 miRNA in AG compared to normal tissue showed significant differential expression (with FDR adjusted $p < 0.01$ and fold change > 2.0) to the same direction as in profiling study, as shown in Table 4.1. It shows only slight change of miRNAs expression level in precancerous condition of stomach. The expression level of 5 out of 21 miRNAs was statistically different in GC compared to AG, as showed in Table 4.1.

Table 4.1. *List of deregulated miRNAs determined by custom TLDA between gastric cancer (GC) (n = 39), atrophic gastritis (AG) (n = 40) and healthy control (HC) (n = 40) tissues (FDR adjusted (adj.) p < 0.01 and fold change (FC) > 2).*

Genes	Direction of expression	GC vs HC		AG vs HC		GC vs AG	
		Fold change	Adj. p value	Fold change	Adj. p value	Fold change	Adj. p-value
hsa-miR-204-5p	down	8.29	6.6×10^{-12}	2.23	1.5×10^{-6}	3.71	1×10^{-4}
hsa-miR-148a-5p	down	3.24	3.1×10^{-7}			2.92	7.1×10^{-6}
hsa-miR-375	down	3.07	2.9×10^{-8}				
hsa-miR-142-3p	up	2.94	4.6×10^{-12}				
hsa-miR-223-3p	up	3.58	1.1×10^{-12}			2.54	2×10^{-9}
hsa-miR-215-5p	up	4.41	3.3×10^{-7}	7.19	2.9×10^{-9}		
hsa-miR-224-5p	up	5.04	5.7×10^{-8}			3.10	4.4×10^{-4}
hsa-miR-335-3p	up	5.77	4.6×10^{-12}			5.12	1.8×10^{-10}

4.4. Study III

4.4.1. The expression analysis of hsa-miR-20b-5p, hsa-miR-451-5p and hsa-miR-1468-5p in GC cell lines AGS and MKN-28

Expression of hsa-miR-20b-5p was upregulated ($p < 0.01$) in GC cells AGS and MKN-28, compared to normal gastric tissue, while expression of hsa-miR-451-5p and hsa-miR-1468-5p was downregulated ($p < 0.01$), compared to normal gastric tissue, Fig. 4.3.

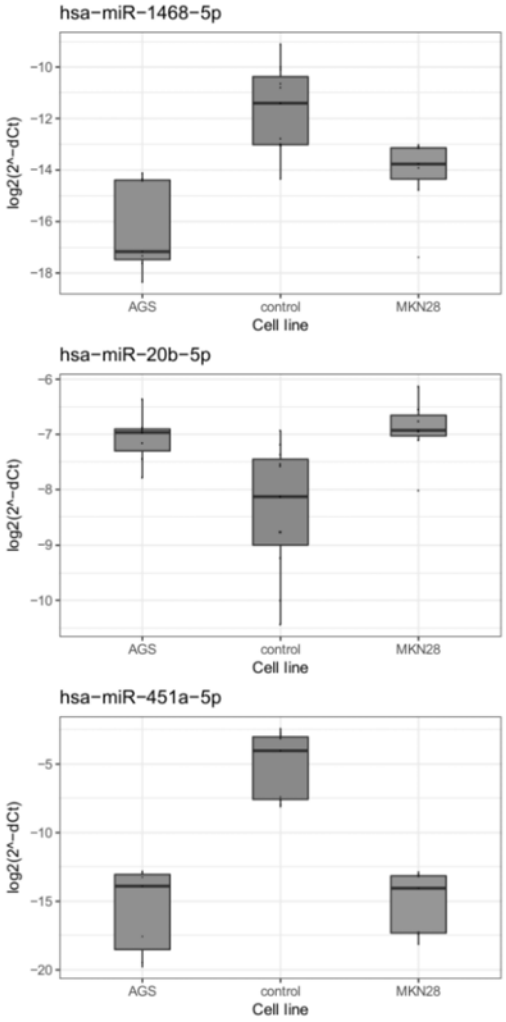

Fig. 4.3. *The expression analysis of hsa-miR-20b-5p, hsa-miR-451-5p and hsa-miR-1468-5p in GC cells (AGS and MKN-28) (normalized delta Ct (dCt) value are showed in logarithmic scale).*

4.4.2. The prediction analysis of hsa-miR-20b, hsa-miR-451a, hsa-miR-1468 target-genes

After the analysis of the most commonly used miRNA gene-target prediction databases and literature, five putative target-genes were selected for hsa-miR-20b, two for hsa-miR-451a and three for hsa-miR-1468, see Table 4.2.

Table 4.2. *Putative target-genes of hsa-miR-20b, hsa-miR-451a and hsa-miR-1468.*

Mature_mirna_ID	Genes function	Putative target-genes
hsa-miR-20b-5p	Proto-oncogen	*EREG, FAT4, IRF1, TXNIP, PTEN*
hsa-miR-451a-5p	Onco-suppressor	*CAV1, ADAM28*
hsa-miR-1468-5p	Onco-suppressor	*TNFα, DNMT1, CITED2*

4.4.3. The expression analysis of miRNA mimic and inhibitor positive controls target-genes

MiRNA mimic and inhibitor positive controls were used for optimization of transfection (for functionally effective miRNA concentration and incubation time after transfection). 24 h after transfection with mimic positive control (hsa-miR-1), *TWF1* gene expression level decreased on average by 1.47 times ($p < 0.01$) and by 1.82 times ($P = 0.034$) in AGS and MKN-28 cell lines, respectively. 48 h after transfection *TWF1* gene expression decreased on average by 1.81 times ($P = 0.021$) and by 2.41 times ($p < 0.01$) in AGS and MKN-28 cells, respectively (Fig. 4.4.1, 4.4.2).

24 h after transfection with inhibitor positive control (hsa-anti-let-7c), *HMGA2* gene expression level increased on average by 6.27 times ($p < 0.01$) and by 5.80 times ($p < 0.01$) in AGS and MKN-28 cell lines, respectively. 48 h after transfection, *HMGA2* gene expression increased on average by 5.19 times ($p < 0.01$) and by 4.46 times ($p < 0.01$) in AGS and MKN-28 cells, respectively.

4.4.4. The expression analysis of putative target-genes of hsa-miR-20b-5p in gastric cancer cells AGS and MKN-28

The results of our study indicated that the expression of hsa-miR-20b-5p putative target-gene *IRF1* increased on average by 1.38 times ($p < 0.01$) and 1.43 times ($p < 0.01$) in AGS and MKN-28 cell lines, respectively, 24 h after transfection with inhibitor hsa-miR-20b-5p. 48 h after transfection the expression of target-gene *IRF1* was significantly increased on average by 1.4 times ($p < 0.01$) only in AGS compared to cells transfected with inhibitor negative control.

The expression of putative target-gene *PTEN* increased on average by 1.25 times ($P = 0.021$) and by 1.87 times ($p < 0.01$) in gastric cell lines AGS and MKN-28, respectively, 24 h after transfection with inhibitor of hsa-miR-20b-5p, compared to cells transfected with negative control of inhibitor (let-7c). There were no significant differences in expression levels of putative target-gene *PTEN* 48 h after transfection in GC cells AGS and MKN-28.

Expression of *TXNIP* increased on average by 1.65 times (P = 0.046) and by 1.7 times ($p < 0.01$) in AGS and MKN-28 cell lines, respectively, 24 h after transfection with inhibitor of hsa-miR-20b-5p, while 48 h after transfection expression of *TXNIP* remained increased by 1.63 times ($p < 0.01$) and by 1.80 ($p < 0.01$) in AGS and MKN-28 cell lines, respectively. Differences in expression of putative target-genes *EREG* and *FAT4* after transfection with inhibitor of hsa-miR-20b-5p were not significant (Fig. 4.4.1, 4.4.2).

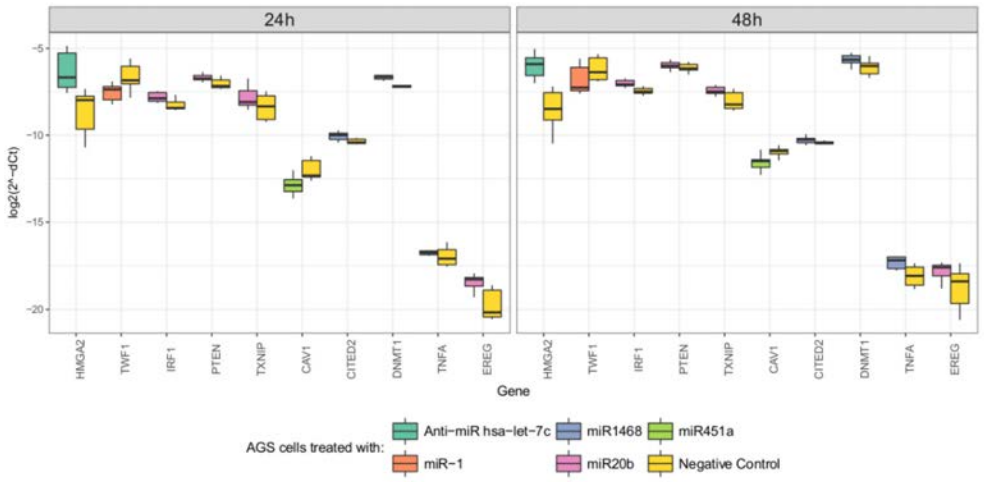

Fig. 4.4.1. *The expression levels of putative target-genes 24 h and 48 h after transfection with inhibitor and mimics in GC cell line AGS. The boxplots show the gene expression levels of IRF1, PTEN, TXNIP, EREG, FAT4 after transfection with inhibitor hsa-miR-20b-5p; Expression levels of putative target-genes CAV1 and ADAM28 after transfection with mimic hsa-miR-451a-5p; Expression levels of putative target-genes TNFα, DNMT1 and CITED2 after transfection with mimic hsa-miR-1468-5p. HMGA2 was used as positive control for gene expression and as target of hsa-let-7c. TWF1 was used as positive control for gene expression and as target of hsa-miR-1-3p.*

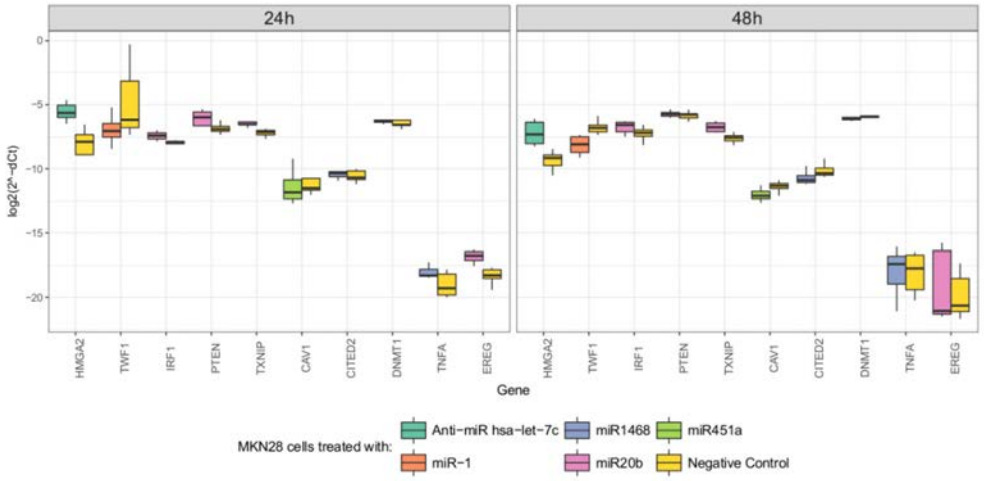

Fig. 4.4.2. *The expression levels of putative target-genes 24 h and 48 h after transfection with inhibitor and mimics in GC cell line MKN-28. The boxplots show the gene expression levels of IRF1, PTEN, TXNIP, EREG, FAT4 after transfection with inhibitor hsa-miR-20b-5p; Expression levels of putative target-genes CAV1 and ADAM28 after transfection with mimic hsa-miR-451a-5p; Expression levels of putative target-genes TNFα, DNMT1 and CITED2 after transfection with mimic hsa-miR-1468-5p. HMGA2 was used as positive control for gene expression and as target of hsa-let-7c. TWF1 was used as positive control for gene expression and as target of hsa-miR-1-3p.*

4.4.5. The expression analysis of putative target-genes of hsa-miR-451a-5p in gastric cancer cells AGS and MKN-28.

Genes *CAV1* and *ADAM28* were identified as putative target-genes of hsa-miR-451a. Transfection of GC cell lines AGS and MKN-28 was performed with mimic of hsa-miR-451-5p.

The results of our study indicated that after 24 h and 48 h the expression of putative target-gene *CAV1* decreased by 1.76 times ($P = 0.008$) and by 1.40 ($P = 0.03$), respectively in GC cell line AGS. The expression levels of putative target-gene *CAV1* decreased by 1.46 times ($p = 0.009$) only 24 h after transfection in GC cell line MKN-28. No significant differences were observed in expression levels of gene *ADAM28* in GC cell lines compared to GC cells transfected with negative control of mimic.

4.4.6. The expression analysis of putative target-genes of hsa-miR-1468-5p in gastric cancer cells AGS and MKN-28.

No significant differences were observed in expression levels of putative target-genes *DNMT1* and *CITED2* both 24 h and 48 h after transfection with mimic hsa-miR-1468-5p in both cell lines AGS and MKN-28. Meanwhile, gene *TNFα* was unexpressed in both cell lines.

5. DISCUSSION

GC with a very poor prognosis accounts for considerable amount of morbidity and mortality in the world. Gastric carcinogenesis is the result of interactions between *Helicobacter pylori*, genetic predisposition, epigenetic alterations, eating habits and environmental factors, which cause inactivation of tumor suppressor genes and activation of oncogenes. [1]. It has been shown, that miRNAs can function as tumor suppressors or oncogenes and repress the expression of important cancer-related genes and might prove to be useful biomarkers in the diagnosis of cancer [52]. The aim of this thesis was to investigate miRNA profile and their target-genes in pathology of GC and to assess the relevance of miRNAs as non-invasive biomarkers for the diagnosis of the malignant disease.

Generally, miRNAs regulate gene expression of a target-genes by binding to its 3′-UTR. Therefore, polymorphisms in the miRNA binding site of the gene affect miRNA–mRNA interaction and influence the development of diseases (Yang et al., 2014) [53, 59]. In study I of this thesis, four SNPs (*IL12B* (rs1368439), *INSR* (rs1051690), *CCND1* (rs7177), and *IL10* (rs3024498) were shown to be associated with GC and present in the putative binding sites of miRNA (hsa-miR-27, hsa-miR-146a, hsa-miR-223 and hsa-miR-107, respectively) was performed. We found that *INSR* (rs1051690) SNP was associated with increased risk of GC; whereas no link was found for the polymorphisms in *IL12B, CCND1* and *IL10* genes and GC risk. It is the first study evaluating the association of these SNPs with GC. Several studies have described the role of miRNAs in the regulation of *INSR* gene in different cancers, such as prostate, colorectal and etc. [104–106]. *INSR* gene codes for the insulin receptor. Insulin may regulate cell growth and apoptosis by binding to its receptor (INSR) or to the structurally related insulin-like growth factor-I receptor (IGFIR) [107, 108]. De Freitas-Junior JC et al. demonstrated that changes in the *INSR* gene can affect the insulin signaling pathway by modulating E-cadherin glycosylation and destabilization of cellular membranes that may have detrimental effects in gastric carcinogenesis [106]. One recent study also identified *INSR* as new candidate gene for diffuse GC susceptibility [107]. In our study we selected rs1051690 of *INSR* gene which is a potential binding site for hsa-miR-146a [108]. Previous case-control studies carried out in Czech Republic, Spain and Israel revealed an association between rs1051690 and colorectal cancer [20, 112, 113]. The findings of our study are partly in line with the latter studies, suggesting that this SNP might mediate not only colorectal but also GC risks, pointing to a potential joint mechanism of gastrointestinal cancers.

Our study did not find significant association between polymorphisms in *IL12B, IL10* and *CCND1* genes with GC risk. Several of previous studies in other populations analyzing associations between these SNPs and GC also did not find any association [114–117]. However, our study carries certain limitations that have to be taken into account. The selection of putative miRNA target-genes and corresponding gene polymorphism is based on bioinformatical databases that may over- or under- estimate real interaction effects. Future studies are needed to validate our findings in other cohorts and to investigate whether the gene variant affecting the insulin receptor (*INSR* gene) leads to changes in the expression level of the receptor. Since this is the first study on these SNPs in GC, direct comparison with the results of other studies is not possible, yet. Overall our data provide important novel aspects on SNPs in miRNA binding sites of target-gene and GC. Detailed discussion of the results of Study I is described in the publication *"Polymorphisms of microRNA target-genes IL12B, INSR, CCND1 and IL10 in gastric cancer"*.

MiRNA profiling in GC and AG patients has been performed using two different approaches, i.e. TLDA qRT-PCR analysis (study II part I) and small RNA-seq (study II part II). Study II part I not only evaluated the miRNA profile in GC tissue, but also enabled to evaluate the suitability of the GC tissue associated miRNAs for non-invasive diagnosis, i.e. expression was evaluated in plasma of the patients; whereas study II part II provided detailed miRNA profile for the tissue of GC and AG patients, which enabled to evaluate differences of miRNA's expression in early stage of GC. Profiling study II part I performed using pre-designed TLDA qRT-PCR analysis, showed 15 miRNAs, of total 377, to be differentially expressed. Validation analysis showed four miRNAs to be differentially expressed in GC tissue, of total 6 and 3 miRNAs - in plasma of patients with GC, of total 4. Profiling study II part II, performed using small RNA-seq analysis, showed different expression of 110 and 17 miRNAs, of total 2180, in GC and AG tissue, respectively. 76 differentially expressed miRNAs were detected in GC tissue compared to AG tissue. To further determine miRNAs which could be used as a signature for GC and AG prediction, 21 miRNAs which were present in plasma were selected for validation study. In validation study eight and two miRNAs showed different expression in GC and AG tissue, respectively. Five differently expressed miRNAs were detected in GC tissue compared to AG tissue. In summary, the results of both studies revealed 9 deregulated miRNAs in GC tissue (down-regulated: hsa-miR-148a-3p, hsa-miR-148a-5p, hsa-miR-204-5p, hsa-miR-375; up-regulated: hsa-miR-142-3p, hsa-miR-215-5p, hsa-miR-223-3p, hsa-miR-224-5p and hsa-miR-335-3p) and two deregulated miRNAs in AG tissue: hsa-miR-204-5p and hsa-miR-215-5p. Evaluation of miRNAs as early diagnostic biomarkers in GC and AG tissue

revealed deregulation of hsa-miR-148a-5p, hsa-miR-204-5p, hsa-miR-223-3p, hsa-miR-224-5p and hsa-miR-335-3p. Evaluation of miRNAs as non-invasive biomarkers in GC plasma revealed different expression of hsa-miR-148a-3p, hsa-miR-375 and hsa-miR-223-3p, however, they showed relatively weak diagnostic performance as sole biomarkers. In the recent review, which summarizes 14 currently published miRNA profiling studies, more than 300 miRNAs have been shown to be differentially expressed in GC tissue compared to normal tissue (Shrestha et al., 2014). The GC miRNA profile defined in our study coincide with other GC miRNA profiling studies. All of the miRNAs in the profile are involved in biological processes important in gastric cancerogenesis. Shui-Long Guo et al. showed that the knockdown of hsa-miR-148a inhibited cell proliferation in GC cell lines, whereas overexpression promoted cell proliferation and cell cycle progression [114]. Silencing of hsa-miR-204-5p expression promoted proliferation of GC cells *in vitro* [115]. Hsa-miR-215 expression level has been shown to correlate with tumor differentiation, TNM stage and lymph-node metastasis and promotion of GC [67, 120]. Hsa-miR-223 was shown to have an impact on different cellular processes, ranging from cell cycle regulation and invasiveness to hematopoietic differentiation and immune cell function [117]. Inhibition of hsa-miR-224 suppressed cell growth, migration and invasion, while hsa-miR-224 overexpression resulted in opposite effects. Hsa-miR-224 inhibition also suppressed tumor growth *in vivo* [118]. MiR-375 has been shown to affect gastric cell proliferation [119].

It is worth noting, that it is the first study that has identified hsa-miR-204-5p and hsa-miR-215-5p to be associated with AG. These miRNAs have been shown to be involved in cancer related processes, but there is no previous study showing their involvement in any kind of inflammatory processes. Moreover, it is the first study that has shown that hsa-miR-148a-5p, hsa-miR-204-5p, hsa-miR-224-5p and hsa-miR-335-3p are deregulated in AG and that they are differentially expressed compared with GC, therefore they have a potential to become early diagnostic biomarkers. Hsa-miR-223-3p has already been shown to be deregulated in AG and GC [120]. One of the major focuses in GC research is the evaluation of potential molecular biomarkers that could be used for non-invasive diagnostics of this malignancy. Meta-analysis by Wang R. analyzed circulating miRNA data in GC and summarized, that circulating miRNAs had a relatively good diagnostic performance in gastrointestinal cancers, with a sensitivity of 0.75, a specificity of 0.81. Another interesting finding revealed in the study was that plasma-based miRNA assays reach a higher accuracy compared to serum-based ones for GC [76]. Altered miRNA expression profiles have been reported in GC tissue and blood samples [125, 66], but the results are inconsistent and controversial. Study by Li et al. identified hsa-miR-223,

hsa-miR-21 and hsa-miR-218 to be deregulated in the GC plasma and revealed that combined ROC analysis had the highest area under the curve (AUC) value of 0.9531 in discriminating GC patients from healthy controls [63]. Study by Zhu et al. showed five miRNAs (hsa-miR-16, hsa-miR-25, hsa-miR-92a, hsa-miR-451 and hsa-miR-486-5p) to be consistently elevated in plasma of the GC patients and were identified to be potential biomarkers [88]. Large-scale analysis by Tsujiura et al. identified four plasma miRNAs (hsa-miR-17-5p, hsa-miR-21, hsa-miR-106a, hsa-miR-106b) to be significantly higher in GC patients compared to healthy controls [121]. Our profiling results revealed three hsa-miR-148a-3p, hsa-miR-375 and hsa-miR-223-3p to be deregulated in plasma of patients with GC compared to healthy control. Moreover, in our study hsa-miR-148a-5p and hsa-miR-223-3p were shown to be differentially expressed in AG tissue compared to GC tissue. However, these circulating miRNAs showed relatively weak diagnostic performance as sole biomarkers. Single-miRNA assay have been shown to display a relatively low diagnostic performance compared to multiple-miRNAs assay in meta-analysis of Wang R. [76]. Therefore, large scale sequencing study of GC and AG plasma samples should be performed in order to determine the possible biomarkers for early and non-invasive diagnosis.

Discussion of the results of Study II part I is described in detail in the article *"Analysis of deregulated miRNAs and their target-genes in gastric cancer"*.

In the study III of this thesis the experimental analysis of potential miRNA target-genes using inhibitor or mimics of miRNAs in GC cell lines (AGS and MKN-28) was performed. Transfection of hsa-miR-20b inhibitor in GC cell lines AGS and MKN-28 increased the expression level of *IRF1*, *PTEN* and *TXNIP* mRNA. Transfection of hsa-miR-451a mimic decreased the expression level of *CAV1* mRNA in GC cell lines AGS and MKN-28.

Hsa-miR-20b belongs to the hsa-miR-106a-363 cluster, which together with hsa-miR-17-92 (known as "oncomiR-1") and hsa-miR-106b-25 clusters form a large family of highly similar miRNAs called the hsa-miR-17 family [122]. In our study we found the hsa-miR-20b-5p expression to be upregulated in GC cells (AGS and MKN-28). We identified that putative target-genes of hsa-miR-20b-5p *IRF1, PTEN* and *TXNIP* increased after transfection with inhibitor in GC cells AGS and MKN-28 24 h after transfection, whereas expression of *IRF1* increased only in GC cell line AGS and expression of *TXNIP* increased in both GC cell lines 48 h after transfection. Expression level of *EREG, FAT4* did not change after transfection with hsa-miR-20b-5p inhibitor. Previous studies have identified, that high expression of hsa-miR-20b is significantly associated with tumor progression and decreased overall survival in patients of hepatocellular carcinoma [123]. In other study hsa-miR-20b was shown to be up-regulated in esophageal tumor tissues compared to

normal [124]. A number of studies have identified potential target-genes of hsa-miR-20b (*MMP-2* [125], *HIF1A* [126], *NFAT5* and *CAMTA1* [127], including *PTEN* [124]). This is the first study that evaluated hsa-miR-20b expression changes in GC cell lines and identified novel potential target-genes.

PTEN is a confirmed target-gene of hsa-miR-20b in multiple cancers. It was shown that hsa-miR-20b expression changed PTEN protein level, but not mRNA expression in breast cancer cells (ZR-75-30 and MCF-7). Furthermore, up-regulation of hsa-miR-20b significantly promoted the proliferation, colony formation and DNA synthesis of breast cancer cells. Conversely, knockdown of hsa-miR-20b inhibited the growth of breast cancer cells *in vitro* and *in vivo* [128]. *PTEN* was shown to be a target of hsa-miR-20b in esophagus cancer, as well [124]. Zheng et al. analyzed *PTEN* expression in GC samples compared to adjacent normal tissues and found significant down-regulation of this gene [129]. Functional inactivation of the tumor suppressor protein PTEN (Phosphatase and Tensin Homolog) has been detected in multiple cases of GC, and has already been shown to be closely linked to the development, progression and prognosis of the disease. Levels of PTEN can be used as an indicator to diagnose the pathological state of GC [130]. However, our study is the first providing preliminary evidence that hsa-miR-20b might be a potential regulator of *PTEN* in GC as well.

TXNIP gene was shown to be down-regulated in colorectal cancer and GC compared to adjacent normal tissue [131]. In other study *TXNIP* gene was shown as potential biomarker for gastro-esophageal adenocarcinoma [132]. This gene has been shown to be regulated by a number of miRNAs in different pathologies (hsa-miR-33a [133], hsa-miR-20a [134], hsa-miR-17 [135] and etc.). However, this is the first study that has identified *TXNIP* to be a potential target-gene of hsa-miR-20b.

IRF1 encodes interferon regulatory factor 1, a member of the interferon regulatory transcription factor (IRF) family. IRF1 has been shown to play roles in regulating apoptosis and tumor-suppression in GC cells [136]. Previous studies indicated that this gene might be targeted by hsa-miR-383 in testicular embryonal carcinoma [137]. Our study is the first that showed *IRF1* to be a possible target-gene of hsa-miR-20b.

A second miRNA understudy hsa-miR-451a-5p was down-regulated in GC cells (AGS and MKN-28). This has already been shown in study of Riquelme I et al. [138]. Moreover, this study identified a member of PI3K/AKT/mTOR pathway *TSC1* to be down-regulated after targeting with hsa-miR-451a mimic [138]. In our study after transfection with hsa-miR-451a mimic expression of putative target-gene *CAV1* was down-regulated in both cell lines (AGS and MKN-28) after transfection. Caveolin-1 (Cav-1), a family of ubiquitously expressed oligomeric structural proteins in many mammalian

cells, has been shown to be an effective regulator of tumorigenesis [139]. Deregulation of *CAV1* expression was shown in several human tumors: upregulated in prostate cancer cells [140] and pancreas cancer [141] and down-regulated in colon cancer [142], lung cancer [143] and ovarian carcinoma [139]. Kyung Han Nam et al. showed that *CAV1* expression in GC cells plays an important role in disease progression, *via* inducing cancer cell adhesion to endothelial cells, which is more conspicuous in the late stage of GC [144]. In other study the expression of *CAV-1* was shown to correlate with worse outcomes and was shown as essential for pancreatic tumor growth and invasion [141]. Our study is the first showing that hsa-miR-451 might be a possible regulator of *CAV-1* expression.

However, it should be noted that the potential target-genes of miRNAs under-study should be validated using western-blot analysis and luciferase assay.

CONCLUSIONS

1. *INSR* (rs1051690) single-nucleotide polymorphism was associated with gastric cancer; whereas polymorphisms in *IL12B* (rs1368439), *CCND1* (rs7177) and *IL10* (rs3024498) genes were not linked to the disease.

2.1. MicroRNA expression profiling in gastric cancer and control tissue revealed nine deregulated microRNAs: hsa-miR-148a-3p, hsa-miR-148a-5p, hsa-miR-204-5p, hsa-miR-375 were down regulated and hsa-miR-142-3p, hsa-miR-215-5p, hsa-miR-223-3p, hsa-miR-224-5p, hsa-miR-335-3p were up-regulated.

2.2. MicroRNA expression profiling in atrophic gastritis and control tissue revealed down-regulation of hsa-miR-204-5p and up-regulation of hsa-miR-215-5p.

2.3. Evaluation of microRNA expression in gastric cancer and atrophic gastritis tissue revealed down-regulation of hsa-miR-223-3p, hsa-miR-224-5p, hsa-miR-335-3p and up-regulation of hsa-miR-148a-5p, hsa-miR-204-5p.

3. Evaluation of microRNA expression in plasma of gastric cancer and control group revealed down-regulation of hsa-miR-148a-3p, hsa-miR-375 and upregulation of hsa-miR-223-3p. Sensitivity and specificity values for microRNAs were insufficient, therefore they are not suitable for non-invasive diagnostic.

4. Inhibition of hsa-miR-20b-5p up-regulated expression of the possible target-genes *IRF1*, *TXNIP* and *PTEN*, whereas overexpression of hsa-miR-451a-5p decreased expression of target-gene *CAV1* in gastric cancer cells AGS and MKN-28.

REFERENCES

1. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer. 2013 Apr;49(6):1374–403.
2. Green D, Ponce de Leon S, Leon-Rodriguez E, Sosa-Sanchez R. Adenocarcinoma of the stomach: univariate and multivariate analysis of factors associated with survival. Am J Clin Oncol. 2002 Feb;25(1):84–9.
3. Karimi P, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric Cancer: Descriptive Epidemiology, Risk Factors, Screening, and Prevention. Cancer Epidemiol Prev Biomarkers. 2014;23(5).
4. Bornschein J, Rokkas T, Selgrad M, Malfertheiner P. Gastric cancer: clinical aspects, epidemiology and molecular background. Helicobacter. 2011 Sep;16 Suppl 1:45–52.
5. Xiao B, Liu Z, Li B-S, Tang B, Li W, Guo G, et al. Induction of microRNA-155 during Helicobacter pylori infection and its negative regulatory role in the inflammatory response. J Infect Dis. 2009 Sep 15;200(6):916–25.
6. Link A, Schirrmeister W, Langner C, Varbanova M, Bornschein J, Wex T, et al. Differential expression of microRNAs in preneoplastic gastric mucosa. Sci Rep. 2015 Feb 5;5:8270.
7. Link A, Kupcinskas J, Wex T, Malfertheiner P. Macro-role of MicroRNA in gastric cancer. Dig Dis. 2012;30(3):255–67.
8. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008 Feb;2008(2):102–14.
9. Yang Q, Jie Z, Ye S, Li Z, Han Z, Wu J, et al. Genetic variations in miR-27a gene decrease mature miR-27a level and reduce gastric cancer susceptibility. Oncogene. 2014 Jan 9;33(2):193–202.
10. Lujambio A, Lowe SW. The microcosmos of cancer. Nature. 2012 Feb 16;482(7385):347–55.
11. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2002 Dec;99(24):15524–9.
12. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006 Mar;9(3):189–98.
13. Yamakuchi M, Lotterman CD, Bao C, Hruban RH, Karim B, Mendell JT, et al. P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis. Proc Natl Acad Sci U S A. 2010 Apr;107(14):6334–9.
14. Volinia S, Calin G a, Liu C-G, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006 Feb 14;103(7):2257–61.
15. Shrestha S, Hsu S-D, Huang W-Y, Huang H-Y, Chen W, Weng S-L, et al. A systematic review of microRNA expression profiling studies in human gastric cancer. Cancer Med. 2014 Aug;3(4):878–88.
16. Ueda T, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, et al. Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. Lancet Oncol. 2010 Feb;11(2):136–46.

17. Xu Q, Liu J, Yuan Y. Comprehensive assessment of the association between miRNA polymorphisms and gastric cancer risk. Mutat Res Mutat Res. 2015 Jan;763:148–60.
18. Chen K, Song F, Calin GA, Wei Q, Hao X, Zhang W. Polymorphisms in microRNA targets: a gold mine for molecular epidemiology. Carcinogenesis. 2008 May 29;29(7):1306–11.
19. Liu C, Rennie WA, Carmack CS, Kanoria S, Cheng J, Lu J, et al. Effects of genetic variations on microRNA: target interactions. Nucleic Acids Res. 2014 Sep;42(15):9543–52.
20. Thomas M, Lieberman J, Lal A. Desperately seeking microRNA targets. Nat Struct Mol Biol. 2010 Oct;17(10):1169–74.
21. Mishra PJ. MicroRNAs as promising biomarkers in cancer diagnostics. Biomark Res. 2014;2:19.
22. Faruq O, Vecchione A. microRNA: Diagnostic Perspective. Front Med. 2015;2:51.
23. Hamilton SR, Aaltonen LA. Pathology and Genetics of Tumours of the Digestive System. 2006;16(7):8–3.
24. Carl-mcgrath S, Ebert M, Röcken C. Gastric adenocarcinoma : epidemiology , pathology and pathogenesis Review Article. 2007;5:877–94.
25. LAUREN P. THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA: DIFFUSE AND SO-CALLED INTESTINAL-TYPE CARCINOMA. AN ATTEMPT AT A HISTO-CLINICAL CLASSIFICATION. Acta Pathol Microbiol Scand. 1965;64:31–49.
26. Hu B, El Hajj N, Sittler S, Lammert N, Barnes R, Meloni-Ehrig A. Gastric cancer: Classification, histology and application of molecular pathology. J Gastrointest Oncol. 2012 Sep;3(3):251–61.
27. Correa P, Piazuelo MB. Helicobacter pylori Infection and Gastric Adenocarcinoma. US Gastroenterol Hepatol Rev. 2011 Jun;7(1):59–64.
28. Henson DE, Dittus C, Younes M, Nguyen H, Albores-Saavedra J. Differential trends in the intestinal and diffuse types of gastric carcinoma in the United States, 1973-2000: increase in the signet ring cell type. Arch Pathol Lab Med. 2004 Jul;128(7):765–70.
29. Shiao Y-H, Bovo D, Guido M, Capella C, Cassaro M, Busatto G, et al. Microsatellite instability and/or loss of heterozygosity in young gastric cancer patients in Italy. Int J Cancer. 1999 Jul 2;82(1):59–62.
30. Rugge M, Busatto G, Cassaro M, Shiao YH, Russo V, Leandro G, et al. Patients younger than 40 years with gastric carcinoma: Helicobacter pylori genotype and associated gastritis phenotype. Cancer. 1999 Jun 15;85(12):2506–11.
31. Bass AJ, Thorsson V, Shmulevich I, Reynolds SM, Miller M, Bernard B, et al. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014 Jul 23;513(7517):202–9.
32. Key TJ, Schatzkin A, Willett WC, Allen NE, Spencer EA, Travis RC. Diet, nutrition and the prevention of cancer. Public Health Nutr. 2004 Feb;7(1A):187–200.
33. Bo Y, Sun J, Wang M, Ding J, Lu Q, Yuan L, et al. Dietary flavonoid intake and the risk of digestive tract cancers: a systematic review and meta-analysis. Sci Rep. 2016 Apr 26;6:24836.
34. Yaghoobi M, Bijarchi R, Narod SA. Family history and the risk of gastric cancer. Br J Cancer. 2010 Jan 19;102(2):237–42.
35. Singh S, Varayil JE, Devanna S, Murad MH, Iyer PG. Physical Activity Is Associated with Reduced Risk of Gastric Cancer: A Systematic Review and Meta-analysis. Cancer Prev Res. 2014;7(1).

36. Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. Science. 2003 May 30;300(5624):1430–4.
37. Atherton JC, Cover TL, Twells RJ, Morales MR, Hawkey CJ, Blaser MJ. Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of Helicobacter pylori. J Clin Microbiol. 1999 Sep;37(9):2979–82.
38. McLean MH, El-Omar EM. Genetics of gastric cancerart. Nat Rev Gastroenterol Hepatol. 2014 Aug 19;11(11):664–74.
39. Ishiguro H, Kimura M, Takeyama H. Role of microRNAs in gastric cancer. World J Gastroenterol. 2014 May 21;20(19):5694–9.
40. Correa P, Piazuelo MB. The gastric precancerous cascade. J Dig Dis. 2012 Jan;13(1):2–9.
41. Conteduca V. H. pylori infection and gastric cancer: State of the art (Review). Int J Oncol. 2012 Nov 15;
42. Hamashima C, Shibuya D, Yamazaki H, Inoue K, Fukao A, Saito H, et al. The Japanese Guidelines for Gastric Cancer Screening. Jpn J Clin Oncol. 2008 Mar 4;38(4):259–67.
43. Layke JC, Lopez PP. Gastric cancer: diagnosis and treatment options. Am Fam Physician. 2004 Mar 1;69(5):1133–40.
44. Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol. 2014;15(8):509–24.
45. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. Nat Rev Genet. 2009 Feb;10(2):94–108.
46. Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature. 2008 Sep 4;455(7209):58–63.
47. Peterson SM, Thompson JA, Ufkin ML, Sathyanarayana P, Liaw L, Congdon CB. Common features of microRNA target prediction tools. Vol. 5, Frontiers in Genetics. 2014.
48. van Rooij E. The art of microRNA research. Circ Res. 2011 Jan 21;108(2):219–34.
49. Marco A, Ninova M, Griffiths-Jones S. Multiple products from microRNA transcripts. Biochem Soc Trans. 2013;41(4).
50. Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: the implications for cancer research. Nat Rev Cancer. 2010 Jun;10(6):389–402.
51. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. Genes Dev. 2004 Mar 1;18(5):504–11.
52. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008 Jul 29;105(30):10513–8.
53. Sun G, Yan J, Noltner K, Feng J, Li H, Sarkis DA, et al. SNPs in human miRNA genes affect biogenesis and function. RNA. 2009 Sep;15(9):1640–51.
54. Saunders MA, Liang H, Li W-H. Human polymorphism at microRNAs and microRNA target sites. Proc Natl Acad Sci U S A. 2007 Mar 27;104(9):3300–5.
55. Chen K, Rajewsky N. Natural selection on human microRNA binding sites inferred from SNP data. Nat Genet. 2006 Dec 29;38(12):1452–6.
56. Mishra PJ, Bertino JR. MicroRNA polymorphisms: the future of pharmacogenomics, molecular epidemiology and individualized medicine. Pharmacogenomics. 2009 Mar;10(3):399–416.
57. Zhang J, Zhang X-H, Wang C-X, Liu B, Fan X-S, Wen J-J, et al. Dysregulation of

microRNA biosynthesis enzyme Dicer plays an important role in gastric cancer progression. Int J Clin Exp Pathol. 2014;7(4):1702–7.
58. Wu Y, Xu Q, He C, Li Y, Liu J, Deng N, et al. Association of Polymorphisms in three pri-miRNAs that Target Pepsinogen C with the Risk and Prognosis of Gastric Cancer. Sci Rep. 2017 Jan 9;7:39528.
59. Link A, Schirrmeister W, Langner C, Varbanova M, Bornschein J, Wex T, et al. Differential expression of microRNAs in preneoplastic gastric mucosa. Sci Rep. 2015 Jan 5;5:8270.
60. Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, et al. E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell. 2008 Mar;13(3):272–86.
61. Matsushima K, Isomoto H, Inoue N, Nakayama T, Hayashi T, Nakayama M, et al. MicroRNA signatures in Helicobacter pylori-infected gastric mucosa. Int J Cancer. 2011 Jan 15;128(2):361–70.
62. Kang W, Tong JHM, Chan AWH, Lung RWM, Chau SL, Wong QWL, et al. Stathmin1 plays oncogenic role and is a target of microRNA-223 in gastric cancer. El-Rifai W, editor. PLoS One. 2012 Jan;7(3):e33919.
63. Li B sheng, Zhao Y liang, Guo G, Li W, Zhu E dong, Luo X, et al. Plasma microRNAs, miR-223, miR-21 and miR-218, as novel potential biomarkers for gastric cancer detection. PLoS One. 2012 Jan;7(7):e41629.
64. Chen Z, Liu X, Hu Z, Wang Y, Liu M, Liu X, et al. Identification and characterization of tumor suppressor and oncogenic miRNAs in gastric cancer. Oncol Lett. 2015 Jul;10(1):329–36.
65. Humphries B, Yang C. The microRNA-200 family: small molecules with novel roles in cancer development, progression and therapy. Oncotarget. 2015 Mar 30;6(9):6472–98.
66. Zhang Z, Li Z, Li Y, Zang A. MicroRNA and signaling pathways in gastric cancer. Cancer Gene Ther. 2014 Aug 25;21(8):305–16.
67. Jiang C, Chen X, Alattar M, Wei J, Liu H. MicroRNAs in tumorigenesis, metastasis, diagnosis and prognosis of gastric cancer. Cancer Gene Ther. 2015 Jun 22;22(6):291–301.
68. Wadhwa R, Song S, Lee J-S, Yao Y, Wei Q, Ajani JA. Gastric cancer—molecular and clinical dimensions. Nat Rev Clin Oncol. 2013 Sep 24;10(11):643–55.
69. Zhang B, Pan X, Cobb GP, Anderson T a. microRNAs as oncogenes and tumor suppressors. Dev Biol. 2007 Feb 1;302(1):1–12.
70. Krichevsky AM, Gabriely G. miR-21: a small multi-faceted RNA. J Cell Mol Med. 2009 Jan;13(1):39–53.
71. Hou Y, Wang J, Wang X, Shi S, Wang W, Chen Z. Appraising MicroRNA-155 as a Noninvasive Diagnostic Biomarker for Cancer Detection: A Meta-Analysis. Medicine (Baltimore). 2016 Jan;95(2):e2450.
72. Shrestha S, Hsu S-D, Huang W-Y, Huang H-Y, Chen W, Weng S-L, et al. A systematic review of microRNA expression profiling studies in human gastric cancer. Cancer Med. 2014 Aug;3(4):878–88.
73. Kim CH, Kim HK, Rettig RL, Kim J, Lee ET, Aprelikova O, et al. miRNA signature associated with outcome of gastric cancer patients following chemotherapy. BMC Med Genomics. 2011 Nov 23;4:79.
74. Li X, Luo F, Li Q, Xu M, Feng D, Zhang G, et al. Identification of new aberrantly expressed miRNAs in intestinal-type gastric cancer and its clinical significance. Oncol Rep. 2011 Dec 24;26(6):1431–9.

75. Wan H-Y, Guo L-M, Liu T, Liu M, Li X, Tang H. Regulation of the transcription factor NF-kappaB1 by microRNA-9 in human gastric adenocarcinoma. Mol Cancer. 2010 Jan;9:16.
76. Wang R, Wen H, Xu Y, Chen Q, Luo Y, Lin Y, et al. Circulating microRNAs as a novel class of diagnostic biomarkers in gastrointestinal tumors detection: a meta-analysis based on 42 articles. Gorlova OY, editor. PLoS One. 2014 Nov 18;9(11):e113401.
77. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008 Oct;18(10):997–1006.
78. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol. 2008 Jun;141(5):672–5.
79. Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. Cardiovasc Res. 2012 Mar 15;93(4):555–62.
80. Kumarswamy R, Thum T. Non-coding RNAs in Cardiac Remodeling and Heart Failure. Circ Res. 2013;113(6).
81. Zhang H, Li Y, Liu Y, Liu H, Wang H, Jin W, et al. Role of plant MicroRNA in cross-species regulatory networks of humans. BMC Syst Biol. 2016 Dec 8;10(1):60.
82. Pastrello C, Tsay M, McQuaid R, Abovsky M, Pasini E, Shirdel E, et al. Circulating plant miRNAs can regulate human gene expression in vitro. Sci Rep. 2016 Sep 8;6:32773.
83. Chen G, Shen Z-L, Wang L, Lv C-Y, Huang X-E, Zhou R-P. Hsa-miR-181a-5p expression and effects on cell proliferation in gastric cancer. Asian Pac J Cancer Prev. 2013 Jan;14(6):3871–5.
84. Wang W-T, Chen Y-Q. Circulating miRNAs in cancer: from detection to therapy. J Hematol Oncol. 2014 Dec 5;7(1):86.
85. Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol. 2014 Feb 4;11(3):145–56.
86. Yan W, Qian L, Chen J, Chen W, Shen B. Comparison of Prognostic MicroRNA Biomarkers in Blood and Tissues for Gastric Cancer. J Cancer. 2016;7(1):95–106.
87. Liu R, Zhang C, Hu Z, Li G, Wang C, Yang C, et al. A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. Eur J Cancer. 2011 Mar;47(5):784–91.
88. Zhu C, Ren C, Han J, Ding Y, Du J, Dai N, et al. A five-microRNA panel in plasma was identified as potential biomarker for early detection of gastric cancer. Br J Cancer. 2014 Apr 29;110(9):2291–9.
89. Protocol:DNA from blood: salting-out method - Ithapedia [Internet]. [cited 2017 Jun 18].
90. Wang L, Liu Y, Du L, Li J, Jiang X, Zheng G, et al. Identification and validation of reference genes for the detection of serum microRNAs by reverse transcription-quantitative polymerase chain reaction in patients with bladder cancer. Mol Med Rep. 2015 Jul;12(1):615–22.
91. Zheng G, Wang H, Zhang X, Yang Y, Wang L, Du L, et al. Identification and validation of reference genes for qPCR detection of serum microRNAs in colorectal adenocarcinoma patients. PLoS One. 2013 Jan;8(12):e83025.
92. O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, et al. Reference sequence (RefSeq) database at NCBI: current status, taxonomic

expansion, and functional annotation. Nucleic Acids Res. 2016 Jan;44(D1):D733-45.
93. Griffiths-Jones S, Bateman A, Marshall M, Khanna A, Eddy SR. Rfam: an RNA family database. Nucleic Acids Res. 2003 Jan;31(1):439–41.
94. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 2014 Jan;42(Database issue):D68-73.
95. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. BMC Bioinformatics. 2009 Jan;10:421.
96. Friedlander MR, Mackowiak SD, Li N, Chen W, Rajewsky N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Res. 2012 Jan;40(1):37–52.
97. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014 Dec;15(12):550.
98. Wald A. Sequential Tests of Statistical Hypotheses. Ann Math Stat. 1945 Jun;16(2):117–86.
99. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B. 1995;57(1):289–300.
100. Kuhn M. Building Predictive Models in R Using the caret Package. J Stat Softw. 2008;28(5):1–26.
101. Beerenwinkel N, Sing T, Sander O. ROCR - visualizing classifier performance in R, with only 3 commands. Internet. 2011;2003–4.
102. Giovannucci E. Insulin, insulin-like growth factors and colon cancer: a review of the evidence. J Nutr. 2001 Nov;131(11 Suppl):3109S–20S.
103. Pechlivanis S, Pardini B, Bermejo JL, Wagner K, Naccarati A, Vodickova L, et al. Insulin pathway related genes and risk of colorectal cancer: INSR promoter polymorphism shows a protective effect. Endocr Relat Cancer. 2007 Sep;14(3):733–40.
104. LeRoith D, Roberts CT. The insulin-like growth factor system and cancer. Cancer Lett. 2003 Jun;195(2):127–37.
105. Moore SC, Leitzmann MF, Weinstein SJ, Snyder K, Albanes D, Virtamo J, et al. Insulin resistance-related gene polymorphisms and risk of prostate cancer. Cancer Epidemiol Biomarkers Prev. 2007 Jun;16(6):1315–7.
106. de-Freitas-Junior JCM, Carvalho S, Dias AM, Oliveira P, Cabral J, Seruca R, et al. Insulin/IGF-I Signaling Pathways Enhances Tumor Cell Invasion through Bisecting GlcNAc N-glycans Modulation. An Interplay with E-Cadherin. Tang C-H, editor. PLoS One. 2013 Nov 25;8(11):e81579.
107. Donner I, Kiviluoto T, Ristimäki A, Aaltonen LA, Vahteristo P. Exome sequencing reveals three novel candidate predisposition genes for diffuse gastric cancer. Fam Cancer. 2015 Jun 10;14(2):241–6.
108. Thomas LF, Saito T, Saetrom P. Inferring causative variants in microRNA target sites. Nucleic Acids Res. 2011 Sep 1;39(16):e109–e109.
109. Landi D, Moreno V, Guino E, Vodicka P, Pardini B, Naccarati A, et al. Polymorphisms affecting micro-RNA regulation and associated with the risk of dietary-related cancers: a review from the literature and new evidence for a functional role of rs17281995 (CD86) and rs1051690 (INSR), previously associated with colorectal canc. Mutat Res. 2011 Dec 1;717(1–2):109–15.
110. Schmit SL, Gollub J, Shapero MH, Huang S-C, Rennert HS, Finn A, et al. MicroRNA Polymorphisms and Risk of Colorectal Cancer. Cancer Epidemiol Biomarkers Prev. 2015 Jan 1;24(1):65–72.

111. Mahmoudi T, Majidzadeh-A K, Karimi K, Karimi N, Farahani H, Dabiri R, et al. An exon variant in insulin receptor gene is associated with susceptibility to colorectal cancer in women. Tumour Biol. 2015 May 5;36(5):3709–15.
112. Navaglia F, Basso D, Zambon C-F, Ponzano E, Caenazzo L, Gallo N, et al. Interleukin 12 gene polymorphisms enhance gastric cancer risk in H pylori infected individuals. J Med Genet. 2005 Jun 1;42(6):503–10.
113. Park M-J, Hyun M-H, Yang J-P, Yoon J-M, Park S. Effects of the interleukin-1β-511 C/T gene polymorphism on the risk of gastric cancer in the context of the relationship between race and H. pylori infection: a meta-analysis of 20,000 subjects. Mol Biol Rep. 2015 Jan 26;42(1):119–34.
114. Guo S-L, Peng Z, Yang X, Fan K-J, Ye H, Li Z-H, et al. miR-148a promoted cell proliferation by targeting p27 in gastric cancer cells. Int J Biol Sci. 2011 Jan;7(5):567–74.
115. Zhang B, Yin Y, Hu Y, Zhang J, Bian Z, Song M, et al. MicroRNA-204-5p inhibits gastric cancer cell proliferation by downregulating USP47 and RAB22A. Med Oncol. 2015 Jan;32(1):331.
116. Fassan M, Volinia S, Palatini J, Pizzi M, Fernandez-Cymering C, Balistreri M, et al. MicroRNA Expression Profiling in the Histological Subtypes of Barrett's Metaplasia. Clin Transl Gastroenterol. 2013 May;4(5):e34.
117. Haneklaus M, Gerlic M, O'Neill LAJ, Masters SL. miR-223: infection, inflammation and cancer. J Intern Med. 2013 Sep;274(3):215–26.
118. He C, Wang L, Zhang J, Xu H. Hypoxia-inducible microRNA-224 promotes the cell growth, migration and invasion by directly targeting RASSF8 in gastric cancer. Mol Cancer. 2017 Dec 7;16(1):35.
119. Ding L, Xu Y, Zhang W, Deng Y, Si M, Du Y, et al. MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. Cell Res. 2010 Jul;20(7):784–93.
120. Link A, Schirrmeister W, Langner C, Varbanova M, Bornschein J, Wex T, et al. Differential expression of microRNAs in preneoplastic gastric mucosa. Sci Rep. 2015 Feb 5;5:8270.
121. Tsujiura M, Ichikawa D, Komatsu S, Shiozaki a, Takeshita H, Kosuga T, et al. Circulating microRNAs in plasma of patients with gastric cancers. Br J Cancer. 2010 Mar 30;102(7):1174–9.
122. Mogilyansky E, Rigoutsos I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. Cell Death Differ. 2013 Dec;20(12):1603–14.
123. Xue T-M, Tao L, Zhang M, Zhang J, Liu X, Chen G-F, et al. Clinicopathological Significance of MicroRNA-20b Expression in Hepatocellular Carcinoma and Regulation of HIF-1α and VEGF Effect on Cell Biological Behaviour. Dis Markers. 2015;2015:325176.
124. Wang B, Yang J, Xiao B, Bai Y, Chen H, Sun X. MicroRNA-20b (miR-20b) Promotes the Proliferation, Migration, Invasion, and Tumorigenicity in Esophageal Cancer Cells via the Regulation of Phosphatase and Tensin Homologue Expression. Yang C, editor. PLoS One. 2016 Oct 4;11(10):e0164105.
125. Park S, Cho T-M, Won S, Song J-H, Noh D, Kim W-J, et al. MicroRNA-20b inhibits the proliferation, migration and invasion of bladder cancer EJï¿½cells via the targeting of cellï¿½cycle regulation and Sp-1-mediated MMP-2 expression. Oncol Rep. 2015 Jul 9;
126. Danza K, Silvestris N, Simone G, Signorile M, Saragoni L, Brunetti O, et al. Role of

miR-27a, miR-181a and miR-20b in gastric cancer hypoxia-induced chemoresistance. Cancer Biol Ther. 2016 Apr 2;17(4):400–6.
127. Xin Y, Cai H, Lu T, Zhang Y, Yang Y, Cui Y. miR-20b Inhibits T Cell Proliferation and Activation via NFAT Signaling Pathway in Thymoma-Associated Myasthenia Gravis. Biomed Res Int. 2016;2016:9595718.
128. Zhou W, Shi G, Zhang Q, Wu Q, Li B, Zhang Z. MicroRNA-20b promotes cell growth of breast cancer cells partly via targeting phosphatase and tensin homologue (PTEN). Cell Biosci. 2014;4(1):62.
129. Zheng H-C, Sun J-M, Li X-H, Yang X-F, Zhang Y-C, Xin Y. Role of PTEN and MMP-7 expression in growth, invasion, metastasis and angiogenesis of gastric carcinoma. Pathol Int. 2003 Oct;53(10):659–66.
130. Xu W-T, Yang Z, Lu N-H. Roles of PTEN (Phosphatase and Tensin Homolog) in gastric cancer development and progression. Asian Pac J Cancer Prev. 2014;15(1):17–24.
131. Ikarashi M, Takahashi Y, Ishii Y, Nagata T, Asai S, Ishikawa K. Vitamin D3 up-regulated protein 1 (VDUP1) expression in gastrointestinal cancer and its relation to stage of disease. Anticancer Res. 22(6C):4045–8.
132. Woolston CM, Madhusudan S, Soomro IN, Lobo DN, Reece-Smith AM, Parsons SL, et al. Thioredoxin interacting protein and its association with clinical outcome in gastro-oesophageal adenocarcinoma. Redox Biol. 2013;1(1):285–91.
133. Chen J, Young ME, Chatham JC, Crossman DK, Dell'Italia LJ, Shalev A. TXNIP regulates myocardial fatty acid oxidation via miR-33a signaling. Am J Physiol - Hear Circ Physiol. 2016;311(1).
134. Li X-F, Shen W-W, Sun Y-Y, Li W-X, Sun Z-H, Liu Y-H, et al. MicroRNA-20a negatively regulates expression of NLRP3-inflammasome by targeting TXNIP in adjuvant-induced arthritis fibroblast-like synoviocytes. Jt Bone Spine. 2016 Dec;83(6):695–700.
135. Dong D, Fu N, Yang P. MiR-17 Downregulation by High Glucose Stabilizes Thioredoxin-Interacting Protein and Removes Thioredoxin Inhibition on ASK1 Leading to Apoptosis. Toxicol Sci. 2016 Mar;150(1):84–96.
136. Liu X, Ru J, Zhang J, Zhu L, Liu M, Li X, et al. miR-23a targets interferon regulatory factor 1 and modulates cellular proliferation and paclitaxel-induced apoptosis in gastric adenocarcinoma cells. PLoS One. 2013 Jan;8(6):e64707.
137. Lian J, Tian H, Liu L, Zhang X-S, Li W-Q, Deng Y-M, et al. Downregulation of microRNA-383 is associated with male infertility and promotes testicular embryonal carcinoma cell proliferation by targeting IRF1. Cell Death Dis. 2010 Nov;1(11):e94.
138. Riquelme I, Tapia O, Leal P, Sandoval A, Varga MG, Letelier P, et al. miR-101-2, miR-125b-2 and miR-451a act as potential tumor suppressors in gastric cancer through regulation of the PI3K/AKT/mTOR pathway. Cell Oncol. 2016 Feb 12;39(1):23–33.
139. Zou W, Ma X, Hua W, Chen B, Cai G. Caveolin-1 mediates chemoresistance in cisplatin-resistant ovarian cancer cells by targeting apoptosis through the Notch-1/Akt/NF-κB pathway. Oncol Rep. 2015 Oct 1;
140. Thompson TC, Tahir SA, Li L, Watanabe M, Naruishi K, Yang G, et al. The role of caveolin-1 in prostate cancer: clinical implications. Prostate Cancer Prostatic Dis. 2010 Mar;13(1):6–11.
141. Chatterjee M, Ben-Josef E, Thomas DG, Morgan MA, Zalupski MM, Khan G, et al. Caveolin-1 is Associated with Tumor Progression and Confers a Multi-Modality Resistance Phenotype in Pancreatic Cancer. Sci Rep. 2015 Jun 12;5:10867.

142. Basu Roy UK, Henkhaus RS, Loupakis F, Cremolini C, Gerner EW, Ignatenko NA. Caveolin-1 is a novel regulator of K-RAS-dependent migration in colon carcinogenesis. Int J cancer. 2013 Jul;133(1):43–57.
143. Ma J, Fu G, Wu J, Han S, Zhang L, Yang M, et al. 4-cholesten-3-one suppresses lung adenocarcinoma metastasis by regulating translocation of HMGB1, HIF1α and Caveolin-1. Cell Death Dis. 2016 Sep 22;7(9):e2372.
144. Nam KH, Lee BL, Park JH, Kim J, Han N, Lee HE, et al. Caveolin 1 expression correlates with poor prognosis and focal adhesion kinase expression in gastric cancer. Pathobiology. 2013;80(2):87–94.

PUBLICATIONS

Dissertation publication

1. Juzėnas, Simonas; Šaltenienė, Violeta; Kupčinskas, Juozas; Link, Alexander; Kiudelis, Gediminas; Jonaitis, Laimas Virginijus; Jarmalaitė, Sonata; Kupčinskas, Limas; Malfertheiner, Peter; Skiecevičienė, Jurgita. Analysis of deregulated microRNAs and their target genes in gastric cancer // PLoS One. San Francisco: Public Library of Science. (Research article). ISSN 1932-6203. 2015, vol. 10, no. 7, p. 1-20. Prieiga per internetą: https://www.ncbi.nlm.nih.gov/pubmed/26172537.
2. Petkevičius, Vytenis, Šaltenienė, Violeta, Juzėnas, Simonas, Wex, Thomas, Link, Alexander, Leja, Mārcis, Steponaitienė, Rūta, Skiecevičienė, Jurgita, Kupčinskas, Limas, Jonaitis, Laimas Virginijus, Kiudelis, Gediminas, Malfertheiner, Peter, Kupčinskas, Juozas. Polymorphisms of microRNA target genes IL12B, INSR, CCND1 and IL10 in gastric cancer. World journal of gastroenterology: WJG. 2017, vol. 23, no. 19. ISSN 1007-9327 p. 3480-3487.

Other publications

1. Gyvyte U, Juzenas S, Salteniene V, Kupcinskas J, Poskiene L, Kucinskas L, Jarmalaite S, Stuopelyte K, Steponaitiene R, Hemmrich-Stanisak G, Hübenthal M, Link A, Franke S, Franke A, Pangonyte D, Lesauskaite V, Kupcinskas L, Skieceviciene J. MiRNA profiling of gastrointestinal stromal tumors by next-generation sequencing. Oncotarget. 2017 Mar 29. doi: 10.18632/oncotarget.16664.
2. Ramonaitė, Rima; Skiecevičienė, Jurgita; Juzėnas, Simonas; Šaltenienė, Violeta; Kupčinskas, Juozas; Matusevičius, Paulius; Borutaitė, Vilmantė; Kupčinskas, Limas. Protective action of NADPH oxidase inhibitors and role of NADPH oxidase in pathogenesis of colon inflammation in mice // World journal of gastroenterology: WJG. Pleasanton, CA: Baishideng Publishing Group. ISSN 1007-9327. 2014, vol. 20, iss. 35, p. 12533-12541.

Scientific conferences

1. Šaltenienė, Violeta; Juzėnas, Simonas; Kupčinskas, Juozas; Link, Alexander; Jonaitis, Laimas Virginijus; Kiudelis, Gediminas; Jarmalaitė, Sonata; Kupčinskas, Limas; Malfertheiner, Peter; Skiecevičienė, Jurgita. Analysis of deregulated micrornas and their target genes in gastric cancer // United European gastroenterology journal: 23rd United European Gastroenterology Week: Barcelona, Spain October 24-28, 2015: Abstract Issue: [Abstracts book] / The United European Gastroenterology (UEG) ; Editor: Jan Tack. London: Sage Publications. (Monday, October 26, 2015.). ISSN 2050-6406$$2050-6414 (e). 2015, vol. 3, suppl. 5, October, p. A311-A311, no. P0562.
2. Šaltenienė, Violeta; Juzėnas, Simonas; Kupčinskas, Juozas; Jonaitis, Laimas Virginijus; Kiudelis, Gediminas; Jarmalaitė, Sonata; Kupčinskas, Limas; Skiecevičienė, Jurgita. MicroRNA expression profiling in gastric cancer// VI Baltic Genetics Congress: book of abstracts: September 30-October 3, 2015, Tartu, Estonia / University of Tartu. Estonian Society for Microbiology (Eesti Mikrobioloogide Ühendus). Tartu: Estonian Society for Microbiology, 2015. (Abstracts.), ISBN 9789949971909. p. 97-97.
3. Šaltenienė, Violeta; Juzėnas, Simonas; Kupčinskas, Juozas; Link, Alexander; Jonaitis, Laimas Virginijus; Kiudelis, Gediminas; Jarmalaitė, Sonata; Kupčinskas, Limas; Malfertheiner, Peter; Skiecevičienė, Jurgita. MicroRNA expression profiling in gastric cancer / V // Latvijas Universitātes 74. konferences Medicīnas sekcija: 2016. gada 19. februārī, Rīgā, Latvijā: Programma / Latvijas Universitāte. Dabaszinātņu akadēmiskais centrs. Rīgā: Latvijas Universitāte, 2016. (Stenda ziņojumi. Bāzes zinātnes un reģeneratīvā medicīna.). p. 1-1, no. 2.
4. Šaltenienė, Violeta; Juzėnas, Simonas; Kupčinskas, Juozas; Kiudelis, Gediminas; Kupčinskas, Limas; Jarmalaitė, Sonata. MikroRNR profilis skrandžio vėžiniame audinyje // VIII nacionalinė doktorantų mokslinė konferencija „Mokslas – sveikatai“: konferencijos pranešimų tezės [skirta] Pasaulinei sveikatos dienai paminėti, kuri minima balandžio 7-ąją. Šių metų tema – „Maisto sauga“: 2015 m. balandžio 10 d, Kaunas / Lietuvos sveikatos mokslų universitetas. [LSMU Doktorantų taryba. LSMU Mokslo centras. LSMU Mokslo fondas; Org. Vaiva Lesauskaitė]. Kaunas: Lietuvos sveikatos mokslų universiteto Leidybos namai, 2015. (Onkologija ir genetika.), ISBN 9789955153863 (978-9955-15-386-3). p. 92-93.
5. Šaltenienė, Violeta; Kupčinskas, Juozas; Steponaitienė, Rūta; Juzėnas, Simonas; Gyvytė, Ugnė; Ivanauskas, Audrius. MikroRNR koduojančių genų vieno nukleotido polimorfizmų sąsaja su atrofiniu gastritu ir skrandžio

vėžiu // VII nacionalinė doktorantų mokslinė konferencija „Mokslas – sveikatai“: konferencijos pranešimų tezės [skirta Pasaulinei sveikatos dienai paminėti, kuri minima balandžio 7-ąją] : 2014 m. balandžio 09 d, Kaunas / Lietuvos sveikatos mokslų universitetas. [LSMU Doktorantų taryba. LSMU Mokslo centras. LSMU Mokslo fondas; Org. Vaiva Lesauskaitė]. Kaunas: Lietuvos sveikatos mokslų universiteto Leidybos namai, 2014. (Sekcija „Genetika“), ISBN 978-995-515-342-9. p. 38-40, Nr. 6.

6. Šaltenienė, Violeta; Kupčinskas, Juozas; Ramonaitė, Rima; Juzėnas, Simonas; Link, Alexander; Kiudelis, Gediminas; Jonaitis, Laimas Virginijus; Skiecevičienė, Jurgita; Jarmalaitė, Sonata; Kupčinskas, Limas; Malfertheiner, Peter. Profile of Micrornas in Gastric Cancer Tissues and Normal Gastric Mucosa// Anticancer research: Abstracts of the Ninth International Conference of Anticancer Research: October 6-10, 2014 Sithonia, Greece / The Union for International Cancer Control (UICC) [et al.]. Attiki, Greece: International Institute of Anticancer Research. (Session: 78 P.). ISSN 0250-7005. 2014, vol. 34, no. 10, October, p. 6127-6128, no. 529.
7. Šaltenienė, Violeta; Ramonaitė, Rima; Juzėnas, Simonas; Kupčinskas, Juozas; Skiecevičienė, Jurgita; Kupčinskas, Limas. Protective action of NADPH oxidase inhibitors and the role of NADPH oxidase in the pathogenesis of colon inflammation in mice// European journal of human Genetics: ESHG – European Human Genetics Conference 2014, May 31-June 3, 2014 Milan, Italy: Abstracts / European Society of Human Genetics; Scientific Programme Committee: Brunhilde Wirth (Chair), et al. London: Nature Publishing Group. (Abstracts. Posters.). ISSN 1018-4813. 2014, vol. 22, suppl. 1, May, p. 271, no. P13.21-S.
8. Juzėnas, Simonas; Skiecevičienė, Jurgita; Šaltenienė, Violeta; Kupčinskas, Juozas; Hemmrich-Stanisak, Georg; Du, Zhipei Gracie; Hübenthal, Matthias; Kiudelis, Gediminas; Jonaitis, Laimas Virginijus; Franke, Andre; Kupčinskas, Limas; Fumery, Mathurin; Salupere, Riina; Arebi, Naila; Nielsen, Kári Rubek; Giannotta, Martina; Oksanen, Pia; Katsanos, Konstantinos H.; Vegh, Zsuzsanna; Ellul, Pierre; Schwartz, Doron; Čuković-Čavka, Silvija; D’Inca, Renata; Turcan, Svetlana; Magro, Fernando; Goldis, Adrian; Langholz, Ebbe; Lakatos, Péter László; Munkholm, Pia S. MiRNA expression patterns in colon of active and inactive ulcerative colitis // Journal of Crohn's and Colitis: Abstracts of the 12th Congress of ECCO "Inflammatory Bowel Diseases": February 15-18, 2017, Barcelona, Spain / European Crohn’s and Colitis Organisation. Oxford: Oxford University Press. (Issue Section: Genetics.). ISSN 1873-9946. 2017, vol. 11, suppl. 1, p. S469-S469, no. P759.

9. Kupčinskas, Juozas; Šaltenienė, Violeta; Juzėnas, Simonas; Link, Alexander; Kiudelis, Gediminas; Jonaitis, Laimas Virginijus; Kupčinskas, Limas; Malfertheiner, Peter; Skiecevičienė, Jurgita. Analysis of deregulated MicroRNas and their target genes in gastric cancer // Helicobacter: Special Issue: XXVIIIth International Workshop on Helicobacter and Microbiota in Inflammation and Cancer: 24-26 September 2015, Nicosia, Cyprus: Abstracts / European Helicobacter Study Group. Oxford: Wiley-Blackwell. (W5 Gastric Cancer.). ISSN 1083-4389. 2015, vol. 20, suppl. S1, p. 82-82, no: W5.4.
10. Kupčinskas, Juozas; Juzėnas, Simonas; Šaltenienė, Violeta; Jarmalaitė, Sonata; Link, Alexander; Skiecevičienė, Jurgita; Jonaitis, Laimas Virginijus; Malfertheiner, Peter. Analysis of deregulated microRNA and their target genes in gastric cancer // Fit for the Future in Gastroenterology & Postgraduate Course: Berlin, Germany November 20-21, 2015 / European Association for Gastroenterology, Endoscopy and Nutrition (EADEN). Berlin: EADEN, 2015. (Special topic: "Molecular Mechanisms in Colorectal Cancerogenesis".). p. 1-1.
11. Ramonaitė, Rima; Šaltenienė, Violeta; Kupčinskas, Juozas; Kupčinskas, Limas. Epitelio ląstelių NADPH oksidazės svarba kolito patogenezėje // Jaunųjų mokslininkų konferencijos „BIOATEITIS: gamtos ir gyvybės mokslų perspektyvos" pranešimų santraukos: 2014 m. gruodžio 9 d, Vilnius / Lietuvos mokslų akademijos Biologijos, medicinos ir geomokslų skyrius. Vilnius: Lietuvos mokslų akademijos Biologijos, medicinos ir geomokslų skyrius, 2014. (Medicinos sekcija.). p. 10-10.
12. Kupčinskas, Juozas; Šaltenienė, Violeta; Link, Alexander; Kiudelis, Gediminas; Jonaitis, Laimas Virginijus; Skiecevičienė, Jurgita; Jarmalaitė, Sonata; Kupčinskas, Limas; Malfertheiner, Peter. Profile of Micrornas in Gastric Cancer Tissues and Normal Gastric Mucosa // Helicobacter Special Issue: XXVIIth International Workshop on Helicobacter and Microbiota in Chronic Digestive Inflammation and Gastric Cancer: Rome, Italy 11–13 September 2014: Accepted Abstracts / European Helicobacter Study Group; Editor-in-Chief: David Y Graham and Assistant Editor: Francis Megraud. Oxford, England: John Wiley & Sons Ltd. (P02 Molecular Genetics and Genomics.). ISSN 1083-4389. 2014, vol. 19, suppl. 1, p. 94, no.: P02.10.
13. Juzėnas, Simonas; Šaltenienė, Violeta; Kupčinskas, Juozas; Link, Alexander; Kiudelis, Gediminas; Jonaitis, Laimas Virginijus; Skiecevičienė, Jurgita; Jarmalaitė, Sonata; Kupčinskas, Limas; Malfertheiner, Peter. Profile of micro-RNAs in gastric cancer tissues and normal gastric mucosa // European Bridging Meeting. EAGEN Symposium in Gastroenterology & EAGEN Postgraduate Course "Gastroesophageal Reflux Disease",

November 13, 2014, Magdeburg, Germany / European Association for Gastroenterology, Endoscopy and Nutrition (EAGEN) . Magdeburg: EAGEN, 2014. (Parallel Scientific Sessions I, II, III.). p. [b.p.].

14. Ramonaitė, Rima; Skiecevičienė, Jurgita; Juzėnas, Simonas; Šaltenienė, Violeta; Kupčinskas, Juozas; Matusevičius, Paulius; Borutaitė, Vilmantė; Kupčinskas, Limas. The Role of NADPH oxidase in the pathogenesis of colon inflammation in mice// Gastroenterologija ir hepatologija: 1-st Baltic Congress of Gastroenterology Congress of Lithuanian Society of Gastroenterology: January 31–February 1, 2014, Kaunas, Lithuania: Abstracts = 1-ojo Baltijos šalių gastroenterologų suvažiavimo mokslinės tezės / Vyriausiasis redaktorius: Limas Kupčinskas. Kaunas: Medicinos spaudos namai. (Mokslinės tezės/ Abstracts.). ISSN 1822-6086. 2014, Nr. 1(8), p. 6, no. B-11.
15. Ramonaitė, Rima; Šaltenienė, Violeta; Juzėnas, Simonas; Matusevičius, Paulius; Borutaitė, Vilmantė; Kupčinskas, Limas. Pelių natrio dekstrano sulfato opinio kolito modelis uždegiminių žarnų ligų molekuliniuose tyrimuose // VI nacionalinė doktorantų mokslinė konferencija „Mokslas – sveikatai“: konferencijos pranešimų tezės [tema – „Hipertenzija“, skirtos Pasaulinei sveikatos dienai, minimai balandžio 7-ąją]: 2013 m. balandžio 05 d, Kaunas / Lietuvos sveikatos mokslų universitetas. LSMU Doktorantų taryba. LSMU Mokslo centras. Kaunas: Lietuvos sveikatos mokslų universiteto Leidybos namai, 2013. (Sekcija „Mitybos ir virškinimo problemos“), ISBN 978-995-515-267-5. p. 95-96, Nr. 2.

PLOS | ONE

RESEARCH ARTICLE

Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer

Simonas Juzėnas[1☯], Violeta Saltenienė[1☯], Juozas Kupcinskas[1,2]*, Alexander Link[3], Gediminas Kiudelis[1,2], Laimas Jonaitis[1,2], Sonata Jarmalaite[4], Limas Kupcinskas[1,2], Peter Malfertheiner[3], Jurgita Skieceviciene[1]

1 Institute for Digestive Research, Lithuanian University of Health Sciences, Kaunas, Lithuania, **2** Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas, Lithuania, **3** Department of Gastroenterology, Hepatology and Infectious Diseases, Otto von Guericke University, Magdeburg, Germany, **4** Division of Human Genome Research Centre, Faculty of Natural Sciences, Vilnius University, Vilnius, Lithuania

☯ These authors contributed equally to this work.
* j_kupcinskas@yahoo.com

Citation: Juzėnas S, Saltenienė V, Kupcinskas J, Link A, Kiudelis G, Jonaitis L, et al. (2015) Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer. PLoS ONE 10(7): e0132327. doi:10.1371/journal.pone.0132327

Editor: Lin Zhang, University of Pennsylvania School of Medicine, UNITED STATES

Received: April 22, 2015

Accepted: June 13, 2015

Published: July 14, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work of JK, LJ, SiJu, LK, JS was supported by the European Social Fund No. VP1-3.1-ŠMM-07-K-01-156. The work of PM is supported by German Federal Ministry of Education and Research in the frame of ERA-Net PathoGenoMics. Grant No: BMBF-0315905D. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

MicroRNAs (miRNAs) are widely studied non-coding RNAs that modulate gene expression. MiRNAs are deregulated in different tumors including gastric cancer (GC) and have potential diagnostic and prognostic implications. The aim of our study was to determine miRNA profile in GC tissues, followed by evaluation of deregulated miRNAs in plasma of GC patients. Using available databases and bioinformatics methods we also aimed to evaluate potential target genes of confirmed differentially expressed miRNA and validate these findings in GC tissues.

Methods

The study included 51 GC patients and 51 controls. Initially, we screened miRNA expression profile in 13 tissue samples of GC and 12 normal gastric tissues with TaqMan low density array (TLDA). In the second stage, differentially expressed miRNAs were validated in a replication cohort using qRT-PCR in tissue and plasma samples. Subsequently, we analyzed potential target genes of deregulated miRNAs using bioinformatics approach, determined their expression in GC tissues and performed correlation analysis with targeting miRNAs.

Results

Profiling with TLDA revealed 15 deregulated miRNAs in GC tissues compared to normal gastric mucosa. Replication analysis confirmed that miR-148a-3p, miR-204-5p, miR-223-3p and miR-375 were consistently deregulated in GC tissues. Analysis of GC patients' plasma samples showed significant down-regulation of miR-148a-3p, miR-375 and up-regulation of miR-223-3p compared to healthy subjects. Further, using bioinformatic tools we identified targets of replicated miRNAs and performed disease-associated gene enrichment analysis.

Ultimately, we evaluated potential target gene *BCL2* and *DNMT3B* expression by qRT-PCR in GC tissue, which correlated with targeting miRNA expression.

Conclusions

Our study revealed miRNA profile in GC tissues and showed that miR-148a-3p, miR-223-3p and miR-375 are deregulated in GC plasma samples, but these circulating miRNAs showed relatively weak diagnostic performance as sole biomarkers. Target gene analysis demonstrated that *BCL2* and *DNMT3B* expression in GC tissue correlated with their targeting miRNA expression.

Introduction

The discovery of microRNAs (MiRNAs) and establishment of their role in molecular pathways has brought a huge advance in molecular biology [1][2]. These small non-coding RNAs comprised of ~22bp are involved in post-transcriptional regulation of gene expression. MiRNAs are characterized by high stability in biological samples making these molecules an attractive target in biomarker research field [3][4]. Accumulating evidence shows that miRNAs are involved in major carcinogenesis pathways [5]. Previous studies have revealed that deregulation of miRNAs occurs virtually in all major types of cancer [5][6]. Furthermore, miRNAs have been shown to have a diagnostic or prognostic role and even potential clinical implications for targeted gene therapy in cancer patients [7][8].

The incidence of gastric cancer (GC) in Western countries has declined over the last decades; however, this type of cancer still accounts for nearly one million of new disease cases worldwide and carries a very high mortality rate [9]. Recent research on GC has revealed many new insights into the pathogenesis of this malignancy. Nevertheless, the exact mechanisms of malignant transformation from *Helicobacter pylori* (*H. pylori*) infection to chronic atrophic gastritis, intestinal metaplasia and GC is still poorly understood [10]. Current hypothesis of GC development involves combined effects of bacterial, host and nutritional factors; however, to date, this theory suggests very few clinically sound translational implications [11]. The majority of GC cases are diagnosed in late stages of the disease, which is associated with poor patient outcomes. Therefore, one of the major focuses in GC research is the evaluation of potential molecular biomarkers that could be used for early non-invasive diagnostics of this malignancy.

The crucial role of miRNAs in GC has been shown in different studies [12]. Volinia et al. have provided one of the first miRNA expression profiles in GC tissue showing a specific deregulation pattern [13]. Further studies have also reported significant deregulation of miRNAs belonging to miR-17, miR-21, miR-223, miR-135 and many other families. Among reported studies in GC tissues, miR-21, miR-25, miR-92, miR-223 were the most consistently up-regulated miRNAs, while miR-375 and miR-148 were the most consistently down-regulated [14][15][16]. Most of these studies have looked at miRNA profile in patients with GC and paired adjacent normal gastric mucosa [14]. Important studies have shown that miRNAs are already deregulated in early stages of gastric carcinogenesis including *H. pylori* gastritis and premalignant stages of gastric atrophy and intestinal metaplasia [17][18]. Interestingly, some of miRNAs have opposite deregulation directions in the presence of GC, as separate profiling studies show significant inconsistency among deregulated miRNAs [14]. MiR-9 was found to be up-regulated in two studies [15][19], while two other papers showed significant down-

regulation of this miRNA in GC tissues [13][20]. These discrepancies regarding opposite deregulation results for miR-9 and many other miRNAs are most likely linked to differences in anatomical location of the GC, histological subtype, disease stage, profiling platforms, statistical analysis and many other potential confounding factors. Besides, the majority of currently published studies on miRNA profile in GC tissues cover subjects from Asian countries; meanwhile, data on European GC patients is still scarce [21].

The goal of our present study was to determine miRNA profile in GC tissues and compare it to the normal healthy gastric mucosa using wide coverage miRNA TaqMan low density arrays. In further analysis, we aimed to validate our profiling results in tissue and plasma of a larger group of GC patients and healthy controls of European descent. Ultimately, we evaluated the potential target genes of confirmed differentially expressed miRNA and performed disease-association gene enrichment analysis of their target genes.

Materials and Methods

Ethics statement

The study was approved by the Kaunas Regional Biomedical Research Ethics Committee (Protocol No BE-2-10). All patients signed an informed consent form to participate in the study.

Study population

Tissue specimens were prospectively collected between 2011 and 2014 in Departments of Gastroenterology and Surgery, Hospital of Lithuanian University of Health Sciences (Kaunas, Lithuania). The study included a total of 51 control subjects and 51 GC patients, which were divided into the profiling (GC, n = 13; controls, n = 12) and validation cohorts (GC, n = 38; controls, n = 39). All subjects were of European descent. Gastric biopsy samples were obtained from antral part of the stomach from control subjects who were referred for upper GI endoscopy due to dyspeptic symptoms and had no previous history of malignancy. GC tissue specimens were obtained from surgical specimens immediately after resection from patients undergoing primary surgery for GC with no preoperative irradiation and chemotherapy. Gastric adenocarcinoma in GC patients was verified by histology and classified according to Lauren into diffuse and intestinal types [22]. *H. pylori* status was assessed in GC and control subjects using indirect ELISA to detect the serum-specific IgG antigen (Virion/Serion GmbH, Würzburg, Germany). Clinical and pathological characteristics of the patient cohorts including age, gender and disease stage are summarized in **Table 1**.

Tissue sample preparation and RNA extraction

Gastric tissue samples were stored in RNAlater (Ambion, Austin, TX, USA) at +4°C and 24 hours later stored at -80°C. 30 mg of tissue was homogenized in sterile condition before total RNA isolation with mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) for profiling study and miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) for validation study, according to the manufacturers' instruction. The quality of RNA was assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Qualitative examination of RNA integrity was performed by electrophoresis on agarose gel (5%).

Plasma sample preparation and RNA extraction

Plasma samples were collected from health patients and patients with GC. The miRNA expression in plasma cohort consisted of plasma samples from 38 gastric cancer patients and 39 healthy volunteers. Venous blood was collected in EDTA anticoagulation vacuum tubes and

Table 1. Clinical characteristics of the gastric cancer patients and healthy controls.

		Profiling cohort (n = 25)			Validation cohort (n = 77)		
		cancer (n = 13)	normal (n = 12)	p value	cancer (n = 38)	normal (n = 39)	p value
Age	Mean ± SD	71 ± 13.5	59.2 ± 11.5	p = 0.0269	67.36 ± 12.19	55.69 ± 16.1	p = 0.0006
Gender	Male, n (%)	9 (69.23)	5 (41.66)		25 (65.79)	15 (38.46)	
	Female, n (%)	4 (30.77)	7 (58.34)	p = 0.1654	13 (34.21)	24 (61.54)	p = 0.02
Lauren classification	Diffuse, n (%)	3 (23.08)	-	-	14 (36.84)	-	-
	Intestinal, n (%)	10 (76.92)	-	-	20 (52.63)	-	-
	Mixed, n (%)	-	-	-	1 (2.63)	-	-
	Unknown, n (%)	-	-	-	3 (7.89)	-	p = 0.4
H.pylori infection	Positive, n (%)	8 (61.54)	-		17 (44.74)	13 (33.34)	
	Negative, n (%)	4 (30.77)	12 (100)		6 (15.79)	18 (46.15)	
	Unknown, n (%)	1 (7.69)	-	p = 0.0015	15 (39.47)	8 (20.51)	p = 0.0124
Tumor localization	Cardia, n (%)	2 (15.38)	-	-	7 (18.42)	-	-
	Corpus, n (%)	7 (53.85)	-	-	14 (36.84)	-	-
	Antrum, n (%)	3 (23.08)	-	-	13 (34.21)	-	-
	Linitis plastica, n (%)	1 (7.69)	-	-	2 (5.26)	-	p = 0.76
TNM staging	I, n (%)	-		-	2 (5.26)	-	-
	II, n (%)	1 (7.69)	-	-	5 (13.16)	-	-
	III, n (%)	7 (53.85)	-	-	17 (44.74)	-	-
	IV, n (%)	3 (23.08)	-	-	14 (36.84)	-	-
	Unknown, n (%)	2 (15.38)	-	-	-	-	p = 0.11
T	1/2	1 (7.69)	-	-	9 (23.68)	-	
	3	7 (53.85)	-	-	13 (34.21)	-	-
	4	3 (23.08)	-	-	15 (39.47)	-	-
	Unknown, n (%)	2 (15.38)	-	-	1 (2.63)	-	p = 0.13
N	0	2 (15.38)	-	-	13 (34.21)	-	-
	1	7 (53.85)	-	-	6 (15.79)	-	-
	2	1 (7.69)	-	-	7 (18.42)	-	-
	3	1 (7.69)	-	-	9 (23.68)	-	-
	Unknown, n (%)	2 (15.38)	-	-	3 (7.89)	-	p = 0.06-
M	0	10 (76.92)	-	-	10 (26.32)	-	-
	1	3 (23.08)	-	-	14 (36.84)	-	-
	Unknown, n (%)	-	-	-	14 (36.84)	-	p = 0.003
Diferentiation grade	1/2	2 (15.38)	-	-	14 (36.84)	-	-
	3	8 (61.54)	-	-	23 (60.53)	-	-
	Unknown, n (%)	3 (23.08)	-	-	1 (2.63)	-	p = 0.04

H. pylori—Helicobacter pylori; GC–gastric cancer.

doi:10.1371/journal.pone.0132327.t001

was centrifuged at 3500 rpm for 15 min at room temperature; separated plasma was transferred into 1.5 ml tubes and placed in a −80°C freezer for short-term storage. Small RNAs were extracted from 200 μl of plasma using the miRNeasy Serum/ Plasma Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions.

MiRNA profiling using the TaqMan Low-Density Array

MiRNA profiling was performed with the TaqMan Array Human MiRNA Card A v2.1 which enabled to quantify 377 human miRNAs cataloged in miRBase v20 [23]. Briefly, 350 ng of total

RNA was initially reverse transcribed using the Megaplex RT set pool A version 2.1 (Applied Biosystems, Carlsbad, California, USA) and 800 μl of cDNA product was then loaded on the TaqMan Array Human MiRNA Card and run on the ViiA 7 Real-Time PCR System (Applied Biosystems). Primary analysis was done using ViiA 7 Software (Applied Biosystems) to get expression in terms of Ct. Data analysis was performed using Bioconductor HTqPCR package [24]. MiRNAs with a Ct value > 37 were considered unamplified. MiRNAs which were not amplified in more than 25% of samples were considered to be lowly expressed and, therefore, excluded from further analysis. MiRNA expression data was normalized using rank invariant normalization. NormFinder and geNorm algorithms were used to identify a reference gene from TLDA data for validation study [25]. According to the algorithms miR-127-3p showed lowest Ct variance and was chosen as a reference gene for the validation study. Recent studies show that the expression levels of RNU6A and some other small nuclear RNAs might be unstable in certain tissues and conditions and may not serve as best reference genes [26][27][28][29]. One recent publication has also identified miR-127-3p as a good candidate for reference gene selection [30].

Quantitative reverse transcription polymerase chain reactions (qRT-PCR)

Reverse transcription (RT) reactions were performed using TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, California, USA) and miRNA-specific RT stem-loop primers (Applied Biosystems, Carlsbad, California, USA). Singleplex reactions were carried out in a volume of 7.5 μL. Each reaction comprised 2.08 μL nuclease free water, 0.74 μL 10× RT buffer, 0.08 μL dNTPs (100 mM), 1.5 μL 5× miRNA-specific RT primers, 0.1 μL RNase inhibitor, 0.5 μL Multiscribe Reverse Transcriptase and a fixed volume of miRNA template (2,5 μL). RT was carried out in a thermocycler under the following conditions: 16°C for 30 min, 42°C for 45 min and 85°C for 5 min, followed by a hold at 4°C.

TaqMan real-time PCR reactions were performed in duplicate reactions comprising 6.25 μL TaqMan 2× Universal PCR Master Mix with No AmpErase UNG (Applied Biosystems, Carlsbad, California, USA) 0.625 μL 20× miRNA-specific primer and probe mix of the TaqMan MiRNA Assay Kit (Applied Biosystems, Carlsbad, California, USA), 3 μL of the reverse transcription product and 2.625 μL nuclease free water. RT-PCR was carried out by using a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA) under the following conditions: 95°C for 10 min, then 40 cycles of 95°C for 15 s, 60°C for 60 s, followed by a hold at 4°C. Each sample was run in duplicate. The data were analyzed with automatic settings for assigning the baseline, and average Ct and SD values were calculated. The expression level of miRNA in the tissue was normalized to miR-127-3p and the expression level in plasma was normalized to hsa-miR-16-5p. The results were calculated using the ΔΔCT method [31]. The data were analyzed with automatic settings for assigning the baseline, and average Ct and SD values were calculated.

cDNA for *BCL2* and *DNMT3B* gene expression analysis was synthesized from 500 ng of RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, California, USA). Singleplex reactions were carried out in a volume of 7,5 μL. Each reaction comprised 2.1 μL nuclease free water, 1 μL 10× RT buffer, 0,4 μL dNTPs (100 mM), 1 μL 10× RT Random primers, 0.5 μL Multiscribe Reverse Transcriptase and a fixed volume of miRNA template (2,5 μL). RT-PCR was carried out in a thermocycler under the following conditions: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min, followed by a hold at 4°C. Before qPCR cDNA samples were diluted 1:1 with nuclease free water and 2 μl used in 10,5 μl RT-qPCR reactions together with 6,25 μl 2x TaqMan Fast Universal Master mix (Life Technologies, Carlsbad, California, USA), 0.625 μl 20x TaqMan Gene Expression Assay (Life Technologies, Carlsbad, California, USA) and 3.625 μl sterile

water. RT-qPCR assays were run in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA) under the following conditions: 95°C for 10 min, then 40 cycles of 95°C for 15 s, 60°C for 60 s, followed by a hold at 4°C. The expression levels of *BCL2* and *DNMT3B* in the tissue was normalized to *ACTB*.

Target gene network analysis

The lists of validated target genes of the candidate miRNAs were obtained from miRTarbase v4.5 (mirtarbase.mbc.nctu.edu.tw) and miRecords v4.0 (mirecords.biolead.org) databases. Gene-disease association data were retrieved from the DisGeNET database (http://www.disgenet.org/). In order to identify GC-associated genes, the term "gastric adenocarcinoma" (umls: C0278701) was used as query for the database. Biological networks were created using Cytoscape v3.2 open-source software with CyTargetLinker application [32].

Statistical analysis

The clinical characteristics among groups were compared using the $\chi 2$ test and Fisher's exact test for qualitative data, and t-test for quantitative data. The TLDA data was analyzed using t-test and Benjamini Hochberg correction for false discovery rate such that differential expression was considered to be significant with a $p < 0.01$. The data was normalized using rank invariant normalization [33] and analyzed using the HTqPCR package. The validation and plasma qPCR expression data was analyzed using nonparametric Mann–Whitney U-test. A Benjamini Hochberg adjusted $p < 0.05$ was considered to be statistically significant. For qRT-PCR data, the relative expression levels of each target miRNA (Log2 relative level) were calculated according to the difference in CT values between the target miRNAs and miR-127-3p in tissue samples and miR-16-5p in plasma samples (ΔCT) [34]. Hypergeometric test was used for GC-associated target enrichment analysis. A receiver operating characteristic (ROC) curve was generated for each miRNA using ΔCT data. The area under curve (AUC) value and 95% confidence intervals (CI) were calculated to determine the specificity and sensitivity. To analyze diagnostic value of combined changes in plasma miRNA levels, the univariate gene expression average algorithm was used [35]. ROC curves were generated using ROCR package [36]. All data analyses were performed using R version 3.1.1 software.

Results

MiRNA expression profiling of GC and healthy gastric mucosa tissue by TLDA

TaqMan Human miRNA Low-Density Array analysis was performed to identify candidate miRNAs exhibiting altered expression in gastric cancer tissue. Tissue miRNA profiles from GC patients were compared with profiles of gastric mucosa from healthy individuals. After filtering for low abundant miRNAs (Ct value > 37 and non-detectable in over 25% of samples), a set of 193 miRNAs (from total 377) remained for further analysis. A comparison of cancerous versus normal tissue identified 15 differentially expressed miRNAs, 7 of which were up-regulated and 8 were down-regulated with FDR adjusted p-value <0.01 and fold change >2 (Table 2). Results are demonstrated in volcano plot (Fig 1). Among these, 6 miRNAs (miR-135a-5p, miR-148a-3p, miR-204-5p, miR-375, miR-223-3p and miR-155-5p), showing the highest significance and fold change values, were selected for the validation study. Hierarchical clustering under Euclidean distance of selected miRNA normalized expression data revealed two subgroups: one corresponding to the control group and the second corresponding principally to the patient group. (Fig 2).

Table 2. List of deregulated miRNAs determined by TaqMan Human miRNA Low-Density Array between gastric cancer (n = 13) and normal gastric (n = 12) tissues (FDR adjusted p-value <0.01 and fold change >2).

Genes	FoldChange	log2 (Fold Change)	mean of control Ct	mean of case Ct	p-value	FDR adjusted p-value
Down-regulated						
hsa-let-7a-5p	0.362	-1.465	26.969	28.434	9.42E-005	0.002085
hsa-let-7g-5p	0.461	-1.118	26.182	27.300	5.32E-005	0.001844
hsa-miR-135a-5p	0.167	-2.583	29.725	32.307	6.67E-008	0.000013
hsa-miR-148a-3p	0.126	-2.991	25.904	28.894	1.70E-006	0.000164
hsa-miR-204-5p	0.166	-2.589	28.783	31.372	1.64E-005	0.000790
hsa-miR-26b-5p	0.364	-1.457	24.940	26.397	1.25E-005	0.000790
hsa-miR-30b-5p	0.449	-1.155	24.743	25.898	0.000882	0.009462
hsa-miR-375	0.096	-3.376	24.378	27.753	0.000257	0.003821
Up-regulated						
hsa-miR-146b-5p	3.156	1.658	24.302	22.643	0.000327	0.004504
hsa-miR-155-5p	3.959	1.985	28.993	27.008	0.000168	0.002703
hsa-miR-214-3p	2.596	1.376	28.410	27.033	0.000144	0.002525
hsa-miR-223-3p	3.268	1.708	26.777	25.069	5.73E-005	0.001844
hsa-miR-224-5p	2.482	1.311	32.817	31.506	0.000818	0.009392
hsa-miR-331-3p	2.050	1.035	27.299	26.264	0.000138	0.002525
hsa-miR-484	2.160	1.111	25.790	24.678	8.34E-005	0.002085

doi:10.1371/journal.pone.0132327.t002

MiRNA validation in GC and healthy gastric mucosa tissue with qRT-PCR

The six candidate miRNAs selected for verification were quantified using qRT-PCR. For reference gene selection the TLDA data was analyzed using two different algorithms (NormFinder and geNorm) to identify reference genes. MiR-127-3p showed highest expression stability across all of the samples in TLDA data and because of this feature miR-127-3p was chosen as a reference gene to normalize qPCR data. The expression levels of miR-148a-3p, miR-204-5p, miR-223-3p and miR-375 showed significant differential expression in the same direction as in the discovery study (FDR adjusted $p < 0.05$), while no significant differences were detected in the expression levels of miR-135a-5p and miR-155-5p (FDR adjusted $p > 0.05$; Fig 3).

Plasma miRNAs as potential biomarkers for gastric cancer

In GC tissue validated miR-148a-3p, miR-204-5p, miR-223-3p and miR-375 were selected for further analysis in plasma samples. The relative levels of the candidate mRNAs in individual samples were determined using qRT-PCR (Fig 3). The miR-16-5p was used as the endogenous control to normalize the expression levels of candidate miRNAs. To date, the discussion over selection of most appropriate reference genes in miRNA profiling studies is ongoing. Traditionally used small nuclear RNAs (RNU6A, RNU44, RNU48 and others) might be unstable in plasma and can introduce bias in the experiment. We have performed a thorough literature analysis, before choosing miR-16-5p as a reference gene. Previous studies have identified miR-16-5p as an endogenous control miRNA, which might serve as an accurate reference gene due to its relative stable expression level in serum [37][21]. The expression levels of miR-148a-3p and miR-375 showed significant down-regulation in GC plasma; while miR-223-3p was significantly up-regulated (FDR adjusted $p < 0.05$). No significant differences were detected in the expression levels of miR-204-5p (Fig 3). To investigate the characteristics of differentially expressed miRNAs as potential biomarkers of GC, the ROC curve analysis was performed. The

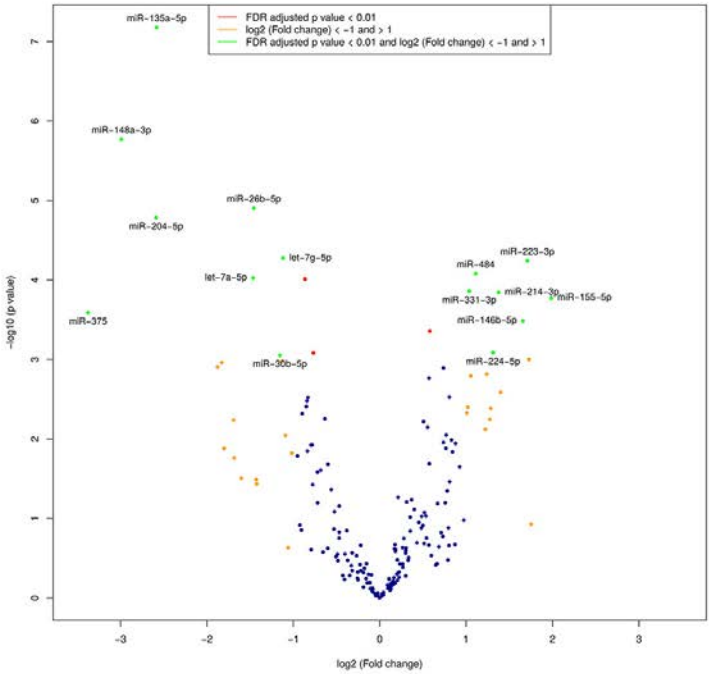

Fig 1. The volcano plot of aberrantly expressed miRNAs detected in TLDA. The green color represents significantly (FDR adjusted p < 0.01) differentially expressed miRNAs with fold change > 2. The red color indicates significantly differentially expressed miRNAs with fold change < 2.

doi:10.1371/journal.pone.0132327.g001

ROC curves of miR-148a-3p, miR-375 and miR-223-3p showed AUC value of 0.349 (95% CI, 0.289–0.408), 0.32 (95% CI, 0.261–0.377) and 0.671 (95% CI, 0.614–0.731), respectively (Fig 4). In the univariate gene expression average analysis of the down-regulated miR-148a-3p and miR-375, the resulting ROC curve had an AUC value of 0.711 (95% CI 0.657–0.769), which corresponds to moderate separation between the GC and control samples (Fig 4). Specificities and sensitivities of candidate miRNAs are shown in Fig 4.

Target gene prediction

To further investigate the possible targets of miR-148a-3p, miR-204-5p, miR-223-3p and miR-375, all of their experimentally validated miRNA-target interactions were obtained from two different databases (miRTarbase and miRecords). The data was visualized in Cytoscape as a biological network containing all of the mentioned miRNAs and their target interactions (Fig 5). Each interaction in the network consisted of two nodes, a regulatory miRNA node

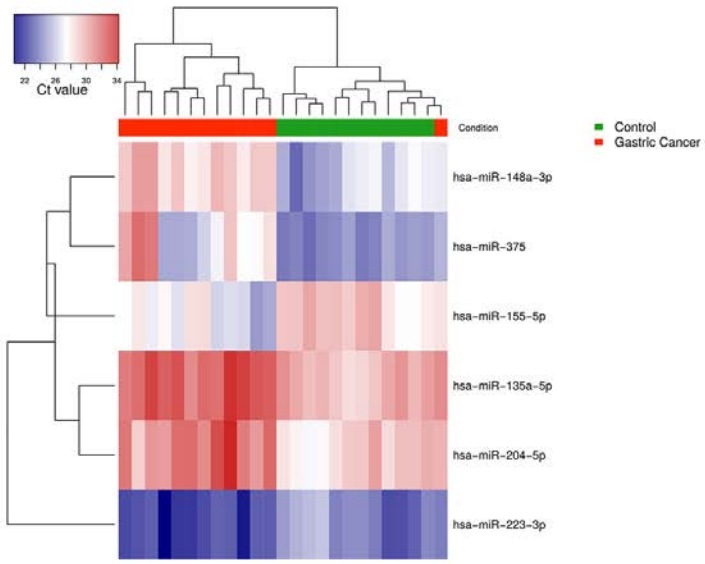

Fig 2. Heat-map diagram of a two-way hierarchical clustering analysis consisting of the 6 most differentially expressed miRNAs in GC tissue and normal gastric mucosa. The red and blue colors indicate expression level of miRNAs in terms of normalized Ct value. Upper color labeling shows GC patient samples in red and controls in green. Distance of hierarchical clustering was measured using the Euclidean method.

doi:10.1371/journal.pone.0132327.g002

(red) and a target mRNA node (pink) connected through one directed edge. Overall, the network included 593 validated targets, 419 of them assigned to miR-375, 88 assigned to miR-204-5p, 83 assigned to miR-148a-3p and 21 assigned to miR-223-3p. The network analysis revealed that miR-148a-3p and miR-375 had six shared targets (MPP5, DNMT3B, PAPD4, RASSF8, PBXIP1 and RAB10), miR-204-5p and miR-375 also had six shared targets (SERP1, CTSC, EFNB2, HSP90AA1, TCF12 and IL1RAP), miR-148a-3p and miR-204-5p had two shared targets (AURKB and CDC25B), miR-223-3p and miR-148a-3p also had two shared targets (HSP90B1 and IRS1), miR-223-3p and miR-375 had one shared target (PARP1) and miR-148a-3p, miR-204-5p and miR-375 also had one shared target (BCL2). Target genes which were regulated simultaneously by two or three miRNAs are represented in orange and green colors, respectively (Fig 5).

Disease-associated gene enrichment analysis

In order to identify whether the disease-associated genes were significantly enriched in our set of miRNA targets, enrichment analysis was performed. First, the gene list was retrieved from DisGeNet database. As query for the database we used the term "gastric adenocarcinoma" (umls: C0149826). After filtering out miRNA, lncRNA and genes which were not in

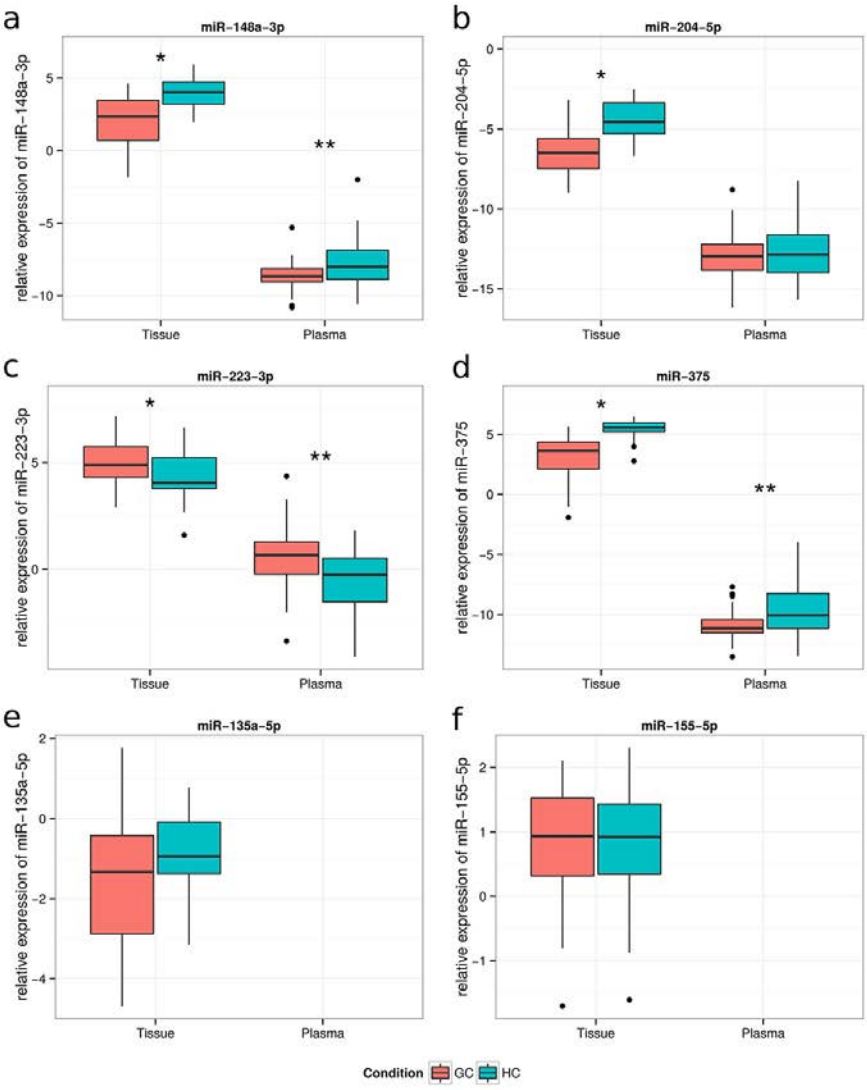
a
miR−148a−3p
relative expression of miR−148a−3p
Tissue
Plasma
b
miR−204−5p
relative expression of miR−204−5p
c
miR−223−3p
relative expression of miR−223−3p
d
miR−375
relative expression of miR−375
e
miR−135a−5p
relative expression of miR−135a−5p
f
miR−155−5p
relative expression of miR−155−5p
Condition GC HC

Fig 3. Expression levels of candidate miRNA in tissue and plasma samples. The boxplots for miR-148a-3p (**a**); miR-204-5p (**b**); miR-223-3p (**c**); miR-375 (**d**); miR-135a-5p (**e**) and miR-155-5p (**f**) represent the results of qRT-PCR comparing GC samples with healthy controls. qRT-PCR data are represented as log2 2-(deltaCt) values. The red and blue colors shows expression levels of candidate miRNAs in tissue and plasma samples, respectively. *—FDR adjusted p < 0.05 by Mann-Whitney U test in tissue. **- FDR adjusted p < 0.05 by Mann-Whitney U test in plasma.

doi:10.1371/journal.pone.0132327.g003

miRTarbase and miRecords, 115 genes were identified to be associated with GC. In the network analysis 20 of GC-associated genes were overlapped, 4 (BCL10, YAP1, IGFBP3 and JAG1) of them assigned to miR-375, 6 (IL8, MMP9, IL1B, CDX2, SERPINE1 and SPARC) assigned to miR-204-5p, 4 (CDKN1B, APC, NR1I2 and CCKBR) assigned to miR-148a-3p and 2 (STMN1 and IGF1R) assigned to miR-223-3p. Target genes which were associated with GC are represented in blue (Fig 5). Finally, disease-associated gene enrichment analysis was performed

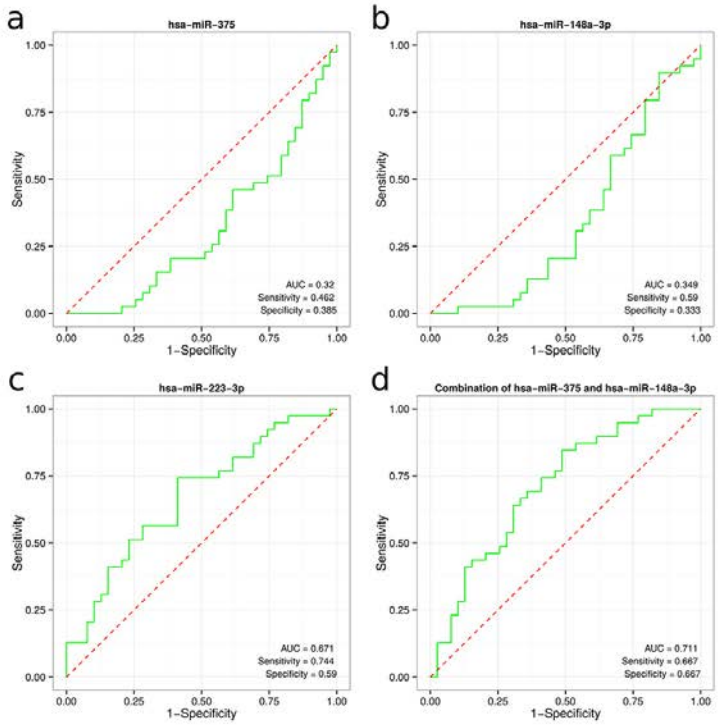

Fig 4. Network of candidate miRNAs and their putative target genes. Network includes the individual miRNAs (red circles) and four types of their predicted mRNA target genes (hexagons), obtained from miRTarBase and miRecords databases. The pink color represents target genes which are regulated by a single miRNA. The orange and green colors indicate target genes regulated simultaneously by two or three distinct miRNAs, respectively. GC-associated target genes retrieved from DisGeNet database are represented by blue hexagons. The databases included in the regulatory interaction networks are identified by the color of the connecting arrows: miRTarBase (blue) and miRecords (red).

doi:10.1371/journal.pone.0132327.g004

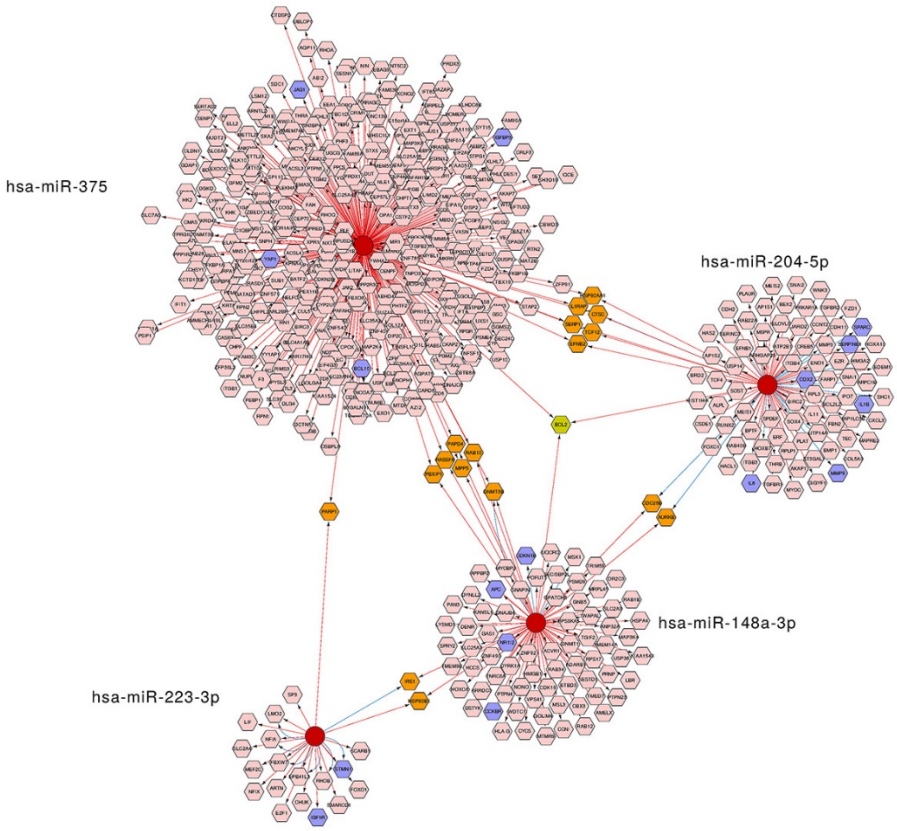

Fig 5. Expression levels of *BCL2* and *DNMT3B* in GC tissue and correlation analysis with their putatively targeting miRNAs. (**a**) Expression levels of *BCL2* and *DNMT3B* was analyzed using qRT-PCR. The data are represented as log2 2-(deltaCt) values; Pearson correlation analysis: (**b**) between relative expression levels of *DNMT3B* and relative expression levels miR-375; (**c**) between relative expression levels of *DNMT3B* and relative expression levels miR-148a-3p; (**d**) between relative expression levels of *BCL2* and relative expression levels miR-148a-3p; (**e**) between relative expression levels of *BCL2* and relative expression levels miR-204-5p; (**f**) between relative expression levels of *BCL2* and relative expression levels miR-375 in gastric tissue samples. P value below 0.05 was considered significant.

doi:10.1371/journal.pone.0132327.g005

using a hypergeometric distribution-based test which led to assess whether the number of GC-associated genes is larger than expected in the set of target genes of selected miRNAs. Enrichment analysis revealed that GC-associated genes were significantly over-represented ($p < 0.05$)

Table 3. Enrichment analysis of GC-associated genes in miR-148a-3p, miR-204-5p, miR-223-3p and miR-375 target genes by using hypergeometrical distribution.

miRNA	Validated targets of miRNA	GC-related target genes of miRNA	GC-unrelated targets of miRNA	Number of GC related genes	Number of GC unrelated genes	p-value
miR-148a-3p	83	6	77	115	12210	1.16E-04
miR-204-5p	88	4	84	115	12210	0.0103796
miR-223-3p	19	2	17	115	12210	0.0089086
miR-375	419	4	415	115	12210	0.5452276

GC–gastric cancer.

doi:10.1371/journal.pone.0132327.t003

in miR-148a-3p, miR-204-5p and miR-223-3p, while no significant enrichment was observed in miR-375 target genes ($p > 0.05$) (Table 3).

BCL2 and *DNMT3B* over-expression and inverse correlation with targeting miRNAs in gastric cancer tissues

The bioinformatical screening of potential miRNA targets identified *BCL2* as a putative target for miR-148a-3p, miR-204-5p and miR-375 and *DNMT3B* for miR-148a-3p and miR-375. All of these miRNAs in our study were found to be down-regulated in gastric cancer tissue. To further support these results, first the expression analysis of *BCL2* and *DNMT3B* genes was performed. The data of qRT-PCR showed significant increase in expression levels of both *BCL2* and *DNMT3B* genes in gastric cancer tissue. *BCL2* showed a 2.7-fold increase, while *DNMT3B* had a 16.7-fold increase in mRNA levels (Fig 6). Pearson's correlation analysis was performed in order to unveil the relationship among the *BCL2* and *DNMT3B* mRNAs and their targeting miRNA levels in normal and cancerous gastric tissues (Fig 6). An inverse correlation was observed for both of the predicted *DNMT3B*-targeting miRNAs: miR-375 ($r = -0.49$; $p = 0.00001$) and miR-148a-3p ($r = -0.26$; $p = 0.0328$). A negative correlation was observed between *BCL2* and miR-375 ($r = -0.32$; $p = 0.0116$), whereas no relationship was found between *BCL2* and miR-148a-3p ($r = -0.06$; $p = 0.6631$) or *BCL2* and miR-204-5p ($r = -0.02$; $p = 0.8546$).

Discussion

Altered miRNA expression profiles have been reported in GC tissue and blood samples [13] [20][14][38]. Some of these deregulated miRNAs have been linked with survival, metastatic behavior of tumor and other clinicopathological features of GC [39][40]. Furthermore, miRNAs have been shown to regulate tumor growth by affecting proliferation, adhesion, invasion and migration in GC cell lines [41]. More importantly, target genes of deregulated miRNAs in GC are involved in apoptosis, cell cycle and other crucial carcinogenesis pathways [12]. Therefore, investigation of miRNAs and their potential target genes in GC may serve for further development of important diagnostic and treatment alternatives. It is evident that miRNAs are major players in gastric carcinogenesis, but we still lack precise data about the mechanisms and potential clinical applications of these molecules in GC.

Our present study aimed to determine the profile of deregulated miRNAs in GC tissues, assess them in plasma samples and to evaluate potential target genes of deregulated miRNAs. Using TaqMan miRNA TLDA cards, encompassing 377 miRNAs primers, we found 15

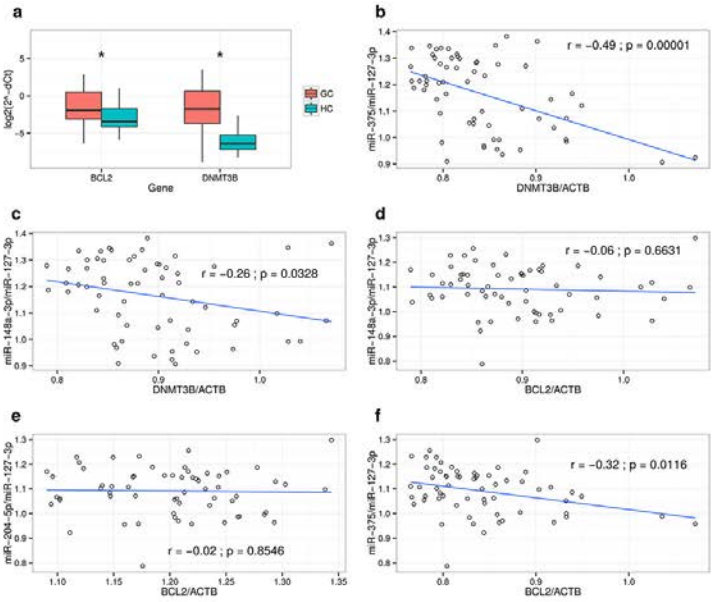

Fig 6. Receiver operating characteristic (ROC) curves of differentially expressed miRNAs in plasma between GC patients and healthy controls. ROC curves of miR-375 (**a**); miR-148a-3p (**b**); and miR-223 (**c**); the combination of miR-375 and miR-148a-3p (**d**).

doi:10.1371/journal.pone.0132327.g006

differentially expressed miRNAs between cancerous GC and healthy gastric tissues. Eight miRNAs were down-regulated (let-7a-5p, let-7g-5p, miR-26b-5p, miR-30b-5p, miR-135a-5p, miR-148a-3p, miR-204-5p, miR-375), while seven miRNAs were up-regulated (miR-146b-5p, miR-155-5p, miR-214-3p, miR-223-3p, miR-224-5p, miR-331-3p and miR-484). Our profiling results did not reveal some of the commonly deregulated miRNAs, which have been previously reported to be deregulated in other studies, including miR-21-5p, miR-25-3p, miR-92a-3p, miR-29c-3p, and some others [15][19][14]. One of the possible explanations for these missing miRNAs might be linked with very strict statistical criteria which were applied for TLDA analysis of our data (FDR adjusted p-value <0.01 and fold change >2). Furthermore, some level of discrepancy in miRNA profiling results among the studies might result from differences in anatomical location of the stomach, histological subtype, statistical analysis and especially profiling methods [18]. A study by Mestdagh et al. shows that different platforms of miRNA profiling have different detection rates, specificity and reproducibility, and that the most suitable platform should be chosen on the basis of the experimental setting and the specific research questions (Mestdagh et al., 2014).

In the second stage of our study we used qRT-PCR in replication cohort including six most deregulated miRNAs from the profiling phase (miR-135a-5p, miR-148a-3p, miR-155-5p, miR-204-5p, miR-223-3p and miR-375). We showed that miR-148a-3p, miR-204-5p, miR-223-3p

and miR-375 were consistently deregulated between normal and cancerous GC tissues. The results of our replication cohort are supported by previous reports. Several studies have shown that miR-148a-3p was significantly down-regulated in GC cell lines and tissue samples compared to the adjacent normal tissues [42][43]. Low expression levels of miR-375 were also reported by studies, hypothesizing that miR-375 was associated with gastric carcinogenesis [44][16]. We identified that miR-223-3p was significantly up-regulated in GC tissue compared to normal tissue, as suggested by several previous reports [15][19]. MiR-204-5p has been less frequently reported in GC miRNA profiling studies; nevertheless, our results are in line with a previous study which showed significant down-regulation of this miRNA in GC tissue and GC cell lines [45]. MiR-135a-5p expression was lower in GC tissues than in control subjects; however, the observed down-regulation trend was similar to previous reports [15] and might have reached required significance with a higher number of individuals within the groups. It is worth pointing out that we did not find differences for miR-155-5p in our replication cohort comparing control subjects and GC patients. Most likely this finding is linked with the fact that, in the initial profiling cohort, control subjects were *H. pylori* free, while in the replication set, some of the control subjects had positive serology for this bacterium. It is well known that miR-155-5p is involved in inflammatory pathways including *H. pylori* gastritis [46]; therefore, this might give a certain bias to our results. Our results are in line with previous study by Link et al. (2015), where the difference in the expression of miR-155 in biopsies from GC compared to controls did not reach statistical significance [18]. In the replication stage of our miRNA profiling study we selected only six miRNAs, which showed the highest biological relevance and statistical significance. We agree that it would be worthwhile to look at all significantly deregulated miRNAs and that could be a potential task in further studies.

To investigate the potential role of differentially expressed miRNAs as biomarkers in GC, we analyzed deregulated miRNAs in plasma samples. Circulating miRNAs, particularly the combination of multiple miRNAs, may present as promising biomarkers for the diagnosis of gastrointestinal cancers [21]. A study by Zhu et al. demonstrated that a panel of five miRNAs may serve as a potential biomarker in detecting early stage GC [47]. To date, reported miRNA profiles in GC serum or plasma differ considerably among separate studies [21]. We found that expression levels of miR-148a-3p and miR-375 were down-regulated in GC plasma; while miR-223-3p was significantly up-regulated. Individual analysis of ROC curves for miR-148a-3p, miR-375 and miR-223-3p expression in plasma samples suggests poor diagnostic performance for GC and these results are in line with certain previous studies [21]. However, when combining two down-regulated miRNAs (miR-148a-3p and miR-375), we obtained an AUC value of 0.71, which corresponds to moderate separation between the GC and control samples. Based on our findings, these circulating miRNAs have relatively weak diagnostic value and may not be applicable as sole biomarkers for GC. We could postulate that with increasing number of miRNAs analyzed in the plasma or in adjunction with other cancer related biomarkers, higher diagnostic accuracy might be achieved, but these speculations need further research.

In the third stage of our study, to gain further insight into the pathogenic role in gastric cancerogenesis of miR-148a-3p, miR-204-5p, miR-223-3p and miR-375, we obtained miRNA validated target genes and performed disease-associated gene enrichment analysis. MiR-148a-3p, miR-204-5p and miR-223-3p, which were significantly enriched in GC-associated target genes, are clearly implicated in GC-related pathways. MiR-148a-3p, down-regulated in GC, was shown to influence tumor cell growth, migration, adhesion, invasion and angiogenesis in GC by targeting CDKN1B (p27), NR1I2 (PAR2) and CCKBR genes [42][48]. MiR-204-5p may act as a tumor-suppressor by targeting IL-8, SOX4, USP47 and RAB22A genes and regulating the apoptosis, proliferation, invasion and tumor progression in GC [49][45]. Important studies have shown that miR-223-3p, up-regulated in GC, could directly target STMN1and IGF1R

genes, resulting in significantly suppressed proliferation, growth rate and colony formation *in vitro* and *in vivo* [50][51]. In our disease-associated target gene enrichment analysis, miR-375 was not significantly enriched in GC-associated target genes. However, a study by Shen et al. showed that miR-375 overexpression suppressed the proliferation of human gastric cancer cells *in vitro* [52]. Another study by Xu et al. showed that miR-375 may be negatively regulated by Snail and involved in gastric cancer cell migration and invasion, potentially by targeting JAK2 [53].

Our network analysis revealed that potential targets of these miRNAs include important cancer-related genes which are regulated simultaneously by two or three distinct miRNAs. For example, miR-148a-3p, miR-204-5p and miR-375, found to be down-regulated in GC, simultaneously target *BCL2* oncogene, which acts as an anti-apoptotic factor and regulates apoptosis (Zhang et al., 2011). A study by Sacconi *et al.* showed that *BCL2* gene is an important component of the complex molecular network underlying poor response of gastric tumors to anticancer treatment and can be used as independent prognostic factor for GC patients [54]. *DNMT3B* is an oncogene which was found to be targeted by miR-375 and miR-148a-3p. It was revealed that *DNMT3B* gene is responsible for de novo methylation during embryogenesis and promotes tumor genesis of gastric cancer [55][38]. To support the observations revealed by our bioninformatical analysis, we evaluated the expression of *BCL2* and *DNMT3B* in GC tissues and found that both of them are up-regulated. Furthermore, we showed a link between expression of target genes and targeting miRNAs—significant correlation was determined for both of the predicted *DNMT3B*-targeting miRNAs (miR-375 and miR-148a-3p) and for one of *BCL2* targeting miRNAs (miR-375). It is well known that in certain cases, predicted target genes of miRNAs do not always correlate with real time alterations in the tissue [56]. Nevertheless, it is very likely that the relationships between miRNAs and their targets are not one-to-one but multiple-to-multiple in GCs, and that these complex relationships may be related to gastric carcinogenesis [57]. Investigation of other potential target genes identified by our bioinformatical tools and their validation by additional functional analysis in cell lines would be a valuable target in further research studies.

Our study was underpowered to determine pattern of miRNA expression among different anatomical and histological subtypes of GC due to a relatively small number of individuals within the groups. Therefore, larger scale studies with high numbers of individuals stratified by different clinical and pathological features of GC are further needed to outline potential differences among the subgroups of GC patients. There are some limitations to our disease-associated target gene enrichment analysis, possibly related to the incompleteness of the DisGeNET database due to inaccuracies derived from text-mining. As a result of this, not all of the GC-associated genes could have been included in our set of genes. Moreover, in our analysis we used all miRNA-target interactions retrieved from miRecords and miRTarbase databases, including weak evidence interactions based on microarray method, which could have caused some false-positive or indirect miRNA-target interactions.

Conclusions

Our study revealed miRNA profile in GC tissues and showed that miR-148a-3p, miR-223-3p and miR-375 are deregulated in GC plasma samples, but these circulating miRNAs showed relatively weak diagnostic performance as sole biomarkers. Target gene analysis demonstrated that *BCL2* and *DNMT3B* expression in GC tissue correlated with their targeting miRNA expression.

Supporting Information

S1 Table. Raw data of miRNA profiling with Taqman low density array in gastric cancer patients and healthy controls.
(XLS)

S2 Table. Raw data of miR-135a-5p, miR-148a-3p, miR-155-5p, miR-204-5p, miR-223-3p and miR-375 expression in tissues of gastric cancer patients and healthy controls.
(XLSX)

S3 Table. Raw data of miR-148a-3p, miR-204-5p, miR-223-3p and miR-375 expression in plasma samples of gastric cancer patients and healthy controls.
(XLSX)

S4 Table. Raw data of BCL2 and DNMT3B expression in tissues of gastric cancer patients and healthy controls.
(XLSX)

Author Contributions

Conceived and designed the experiments: JK LK PM JS. Performed the experiments: VS S. Juzėnas S. Jarmalaite. Analyzed the data: S. Juzėnas VS JK. Contributed reagents/materials/analysis tools: LJ GK S. Jarmalaite. Wrote the paper: JK S. Juzėnas AL VS.

References

1. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993; 75:843–54. PMID: 8252621
2. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 1993; 75:855–62. PMID: 8252622
3. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136:215–33. doi: 10.1016/j.cell.2009.01.002 PMID: 19167326
4. Link A, Goel A. MicroRNA in gastrointestinal cancer: a step closer to reality. Adv Clin Chem. 2013; 62:221–68. PMID: 24772669
5. Lujambio A, Lowe SW. The microcosmos of cancer. Nature. 2012; 482:347–55. doi: 10.1038/nature10888 PMID: 22337054
6. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA. 2002; 99:15524–9. PMID: 12434020
7. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006; 9:189–98. PMID: 16530703
8. Yamakuchi M, Lotterman CD, Bao C, Hruban RH, Karim B, Mendell JT, et al. P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis. Proc Natl Acad Sci USA. 2010; 107:6334–9. doi: 10.1073/pnas.0911082107 PMID: 20308559
9. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA. Cancer J Clin. 2014; 61:69–90.
10. Bornschein J, Rokkas T, Selgrad M, Malfertheiner P. Gastric cancer: clinical aspects, epidemiology and molecular background. Helicobacter. 2011; 16 Suppl 1:45–52. doi: 10.1111/j.1523-5378.2011.00880.x PMID: 21896085
11. Bornschein J, Leja M, Kupcinskas J, Link A, Weaver J, Rugge M, et al. Molecular diagnostics in gastric cancer. Front. Biosci. Landmark Ed. 2014; 19:312–38. PMID: 24389187
12. Link A, Kupcinskas J, Wex T, Malfertheiner P. Macro-role of MicroRNA in gastric cancer. Dig Dis. 2012; 30:255–67. doi: 10.1159/000336919 PMID: 22722550
13. Volinia S, Calin GA, Liu C-G, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA. 2006; 103:2257–61. PMID: 16461460

14. Shrestha S, Hsu S-D, Huang W-Y, Huang H-Y, Chen W, Weng S-L, et al. A systematic review of microRNA expression profiling studies in human gastric cancer. Cancer Med. 2014; 3:878–88. doi: 10.1002/cam4.246 PMID: 24902858

15. Kim CH, Kim HK, Rettig RL, Kim J, Lee ET, Aprelikova O, et al. miRNA signature associated with outcome of gastric cancer patients following chemotherapy. BMC Med. Genomics. 2011; 4:79. doi: 10.1186/1755-8794-4-79 PMID: 22112324

16. Ueda T, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, et al. Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. Lancet Oncol. 2010; 11:136–46. doi: 10.1016/S1470-2045(09)70343-2 PMID: 20022810

17. Xiao B, Liu Z, Li B-S, Tang B, Li W, Guo G, et al. Induction of microRNA-155 during Helicobacter pylori infection and its negative regulatory role in the inflammatory response. J Infect Dis. 2009; 200:916–25. doi: 10.1086/605443 PMID: 19650740

18. Link A, Schirrmeister W, Langner C, Varbanova M, Bornschein J, Wex T, et al. Differential expression of microRNAs in preneoplastic gastric mucosa. Sci Rep. 2015; 5:8270. doi: 10.1038/srep08270 PMID: 25652892

19. Li X, Luo F, Li Q, Xu M, Feng D, Zhang G, et al. Identification of new aberrantly expressed miRNAs in intestinal-type gastric cancer and its clinical significance. Oncol Rep. 2011; 26:1431–9. doi: 10.3892/or.2011.1437 PMID: 21874264

20. Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, et al. E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell. 2008; 13:272–86. doi: 10.1016/j.ccr.2008.02.013 PMID: 18328430

21. Wang R, Wen H, Xu Y, Chen Q, Luo Y, Lin Y, et al. Circulating microRNAs as a novel class of diagnostic biomarkers in gastrointestinal tumors detection: a meta-analysis based on 42 articles. PLoS One. 2014; 9:e113401. doi: 10.1371/journal.pone.0113401 PMID: 25406082

22. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at histo-clinical classification. Acta Pathol Microbiol Scand. 1965; 64:31–49. PMID: 14320675

23. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 2006; 34:D140–4. PMID: 16381832

24. Dvinge H, Bertone P. HTqPCR: high-throughput analysis and visualization of quantitative real-time PCR data in R. Bioinformatics. 2009; 25:3325–6. doi: 10.1093/bioinformatics/btp578 PMID: 19808880

25. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004; 64:5245–50. PMID: 15289330

26. Genovesi LA, Anderson D, Carter KW, Giles KM, Dallas PB. Identification of suitable endogenous control genes for microRNA expression profiling of childhood medulloblastoma and human neural stem cells. BMC Res Notes. 2012; 5:507. doi: 10.1186/1756-0500-5-507 PMID: 22980291

27. Lamba V, Ghodke-Puranik Y, Guan W, Lamba JK. Identification of suitable reference genes for hepatic microRNA quantitation. BMC Res Notes. 2014; 7:129. doi: 10.1186/1756-0500-7-129 PMID: 24606728

28. Benz F, Roderburg C, Vargas Cardenas D, Vucur M, Gautheron J, Koch A, et al. U6 is unsuitable for normalization of serum miRNA levels in patients with sepsis or liver fibrosis. Exp Mol Med. 2013; 45:e42. doi: 10.1038/emm.2013.81 PMID: 24052167

29. Zheng G, Wang H, Zhang X, Yang Y, Wang L, Du L, et al. Identification and validation of reference genes for qPCR detection of serum microRNAs in colorectal adenocarcinoma patients. PLoS One. 2013; 8:e83025. doi: 10.1371/journal.pone.0083025 PMID: 24349425

30. Bae I-S, Chung KY, Yi J, Kim T Il, Choi H-S, Cho Y-M, et al. Identification of reference genes for relative quantification of circulating microRNAs in bovine serum. PLoS One. 2015; 10:e0122554. doi: 10.1371/journal.pone.0122554 PMID: 25826387

31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method Methods. 2001; 25:402–8. PMID: 11846609

32. Kutmon M, Kelder T, Mandaviya P, Evelo CTA, Coort SL. CyTargetLinker: a cytoscape app to integrate regulatory interactions in network analysis. PLoS One. 2013; 8:e82160. doi: 10.1371/journal.pone.0082160 PMID: 24340000

33. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, et al. A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol. 2009; 10:R64. doi: 10.1186/gb-2009-10-6-r64 PMID: 19531210

34. Perkins JR, Dawes JM, McMahon SB, Bennett DLH, Orengo C, Kohl M. ReadqPCR and NormqPCR: R packages for the reading, quality checking and normalisation of RT-qPCR quantification cycle (Cq) data. BMC Genomics. 2012; 13:296. doi: 10.1186/1471-2164-13-296 PMID: 22748112

35. Lauss M, Frigyesi A, Ryden T, Höglund M. Robust assignment of cancer subtypes from expression data using a uni-variate gene expression average as classifier. BMC Cancer. 2010; 10:532. doi: 10.1186/1471-2407-10-532 PMID: 20925936

36. Sing T, Sander O, Beerenwinkel N, Lengauer T. ROCR: visualizing classifier performance in R. Bioinformatics. 2005; 21:3940–1. PMID: 16096348

37. Xiang M, Zeng Y, Yang R, Xu H, Chen Z, Zhong J, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem Biophys Res Commun. 2014; 454:210–4. doi: 10.1016/j.bbrc.2014.10.064 PMID: 25450382

38. Bass AJ, Thorsson V, Shmulevich I, Reynolds SM, Miller M, Bernard B, et al. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014; 513:202–9. doi: 10.1038/nature13480 PMID: 25079317

39. Li X, Zhang Y, Zhang Y, Ding J, Wu K, Fan D. Survival prediction of gastric cancer by a seven-microRNA signature. Gut. 2010; 59:579–85. doi: 10.1136/gut.2008.175497 PMID: 19951901

40. Minn Y-K, Lee DH, Hyung WJ, Kim JE, Choi J, Yang S-H, et al. MicroRNA-200 family members and ZEB2 are associated with brain metastasis in gastric adenocarcinoma. Int J Oncol. 2014; 45:2403–10. doi: 10.3892/ijo.2014.2680 PMID: 25270520

41. Han T-S, Hur K, Xu G, Choi B, Okugawa Y, Toiyama Y, et al. MicroRNA-29c mediates initiation of gastric carcinogenesis by directly targeting ITGB1. Gut. 2014; 64(2):203–14.

42. Guo S-L, Peng Z, Yang X, Fan K-J, Ye H, Li Z-H, et al. miR-148a promoted cell proliferation by targeting p27 in gastric cancer cells. Int J Biol Sci. 2011; 7:567–74. PMID: 21552422

43. Kim SY, Jeon TY, Choi CI, Kim DH, Kim DH, Kim GH, et al. Validation of circulating miRNA biomarkers for predicting lymph node metastasis in gastric cancer. J Mol Diagn. 2013; 15:661–9. doi: 10.1016/j.jmoldx.2013.04.004 PMID: 23806809

44. Ding L, Xu Y, Zhang W, Deng Y, Si M, Du Y, et al. MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. Cell Res. 2010; 20:784–93. doi: 10.1038/cr.2010.79 PMID: 20548334

45. Zhou X, Li L, Su J, Zhang G. Decreased miR-204 in H. pylori-associated gastric cancer promotes cancer cell proliferation and invasion by targeting SOX4. PLoS One. 2014; 9:e101457. doi: 10.1371/journal.pone.0101457 PMID: 24984017

46. Tang B, Xiao B, Liu Z, Li N, Zhu E-D, Li B-S, et al. Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation. FEBS Lett. 2010; 584:1481–6. doi: 10.1016/j.febslet.2010.02.063 PMID: 20219467

47. Zhu C, Ren C, Han J, Ding Y, Du J, Dai N, et al. A five-microRNA panel in plasma was identified as potential biomarker for early detection of gastric cancer. Br J Cancer. 2014; 110:2291–9. doi: 10.1038/bjc.2014.119 PMID: 24595006

48. Fujimoto D, Hirono Y, Goi T, Katayama K, Matsukawa S, Yamaguchi A. The activation of proteinase-activated receptor-1 (PAR1) promotes gastric cancer cell alteration of cellular morphology related to cell motility and invasion. Int J Oncol. 2013; 42:565–73. doi: 10.3892/ijo.2012.1738 PMID: 23242308

49. Li G, Luna C, Qiu J, Epstein DL, Gonzalez P. Role of miR-204 in the regulation of apoptosis, endoplasmic reticulum stress response, and inflammation in human trabecular meshwork cells. Invest. Ophthalmol Vis Sci. 2011; 52:2999–3007. doi: 10.1167/iovs.10-6708 PMID: 21282569

50. Kang W, Tong JHM, Chan AWH, Lung RWM, Chau SL, Wong QWL, et al. Stathmin1 plays oncogenic role and is a target of microRNA-223 in gastric cancer. PLoS One. 2012; 7:e33919. doi: 10.1371/journal.pone.0033919 PMID: 22470493

51. Jia CY, Li HH, Zhu XC, Dong YW, Fu D, Zhao QL, et al. MiR-223 suppresses cell proliferation by targeting IGF-1R. PLoS One. 2011; 6:e27008. doi: 10.1371/journal.pone.0027008 PMID: 22073238

52. Shen Z-Y, Zhang Z-Z, Liu H, Zhao E-H, Cao H. miR-375 inhibits the proliferation of gastric cancer cells by repressing ERBB2 expression. Exp Ther Med. 2014; 7:1757–61. PMID: 24926380

53. Xu Y, Jin J, Liu Y, Huang Z, Deng Y, You T, et al. Snail-regulated MiR-375 inhibits migration and invasion of gastric cancer cells by targeting JAK2. PLoS One. 2014; 9:e99516. doi: 10.1371/journal.pone.0099516 PMID: 25055044

54. Sacconi A, Biagioni F, Canu V, Mori F, Di Benedetto A, Lorenzon L, et al. miR-204 targets Bcl-2 expression and enhances responsiveness of gastric cancer. Cell Death Dis. 2012; 3:e423. doi: 10.1038/cddis.2012.160 PMID: 23152059

55. Linhart HG, Lin H, Yamada Y, Moran E, Steine EJ, Gokhale S, et al. Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. Genes Dev. 2007; 21:3110–22. PMID: 18056424

56. Steponaitiene R, Kupcinskas J, Langner C, Balaguer F, Venclauskas L, Pauzas H, et al. Epigenetic silencing of miR-137 is a frequent event in gastric carcinogenesis. Mol Carcinog. 2015; doi: 10.1002/mc.22287

57. Hashimoto Y, Akiyama Y, Yuasa Y. Multiple-to-multiple relationships between microRNAs and target genes in gastric cancer. PLoS One. 2013; 8:e62589. doi: 10.1371/journal.pone.0062589 PMID: 23667495

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2017 May 21; 23(19): 3480-3487

DOI: 10.3748/wjg.v23.i19.3480

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Case Control Study

Polymorphisms of microRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* in gastric cancer

Vytenis Petkevicius, Violeta Salteniene, Simonas Juzenas, Thomas Wex, Alexander Link, Marcis Leja, Ruta Steponaitiene, Jurgita Skieceviciene, Limas Kupcinskas, Laimas Jonaitis, Gediminas Kiudelis, Peter Malfertheiner, Juozas Kupcinskas

Vytenis Petkevicius, Violeta Salteniene, Limas Kupcinskas, Laimas Jonaitis, Gediminas Kiudelis, Juozas Kupcinskas, Department of Gastroenterology, Lithuanian University of Health Sciences, 50009 Kaunas, Lithuania

Simonas Juzenas, Ruta Steponaitiene, Jurgita Skieceviciene, Limas Kupcinskas, Laimas Jonaitis, Gediminas Kiudelis, Juozas Kupcinskas, Institute for Digestive Research, Lithuanian University of Health Sciences, 50009 Kaunas, Lithuania

Thomas Wex, Alexander Link, Peter Malfertheiner, Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, 39106 Magdeburg, Germany

Thomas Wex, Medical Laboratory for Clinical Chemistry, Microbiology and Infectious Diseases, Department of Molecular Genetics, Otto-von-Guericke University, 39106 Magdeburg, Germany

Marcis Leja, Institute for Clinical and Preventive Medicine, University of Latvia, 1586 Riga, Latvia

Marcis Leja, Faculty of Medicine, University of Latvia, 1586 Riga, Latvia

Marcis Leja, Riga East University Hospital, 1079 Riga, Latvia

Author contributions: Petkevicius V and Salteniene V contributed equally to this work; Wex T, Kupcinskas L, Malfertheiner P and Kupcinskas J designed the research; Juzenas S, Steponaitiene R, Skieceviciene J, Jonaitis L and Kiudelis G performed analysis and interpretation of data; Petkevicius V, Salteniene V and Kupcinskas J drafted the manuscript; Wex T, Link A, Leja M and Kupcinskas L provided critical revision of the manuscript for important intellectual content.

Supported by Lithuanian Research Council Grant, No. MIP-14418.

Institutional review board statement: The study was approved by the Ethics Committees of the Otto-von-Guericke University Magdeburg (Protocols No. 63/08 and No. 34/08), Lithuanian University of Health Sciences (Protocol No. BE-10-2) and Central Medical Ethics Committee of Latvia (Protocol No. 91-29.1/20 dated 22.09.2011).

Informed consent statement: All patients have signed an informed consent form to participate in the study.

Conflict-of-interest statement: The authors declare no conflicts.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Juozas Kupcinskas, MD, PhD, Gastroenterologist, Chief Researcher, Department of Gastroenterology, Lithuanian University of Health Sciences, Eiveniu str. 2, Kaunas 50009, Lithuania. juozas.kupcinskas@lsmuni.lt
Telephone: +370-37-326508

Received: December 23, 2016
Peer-review started: December 25, 2016
First decision: January 19, 2017
Revised: February 23, 2017
Accepted: March 21, 2017
Article in press: March 21, 2017
Published online: May 21, 2017

Abstract

AIM

To evaluate associations between miRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* polymorphisms and gastric cancer (GC) in European population.

METHODS

Gene polymorphisms were analyzed in 508 controls and 474 GC patients from 3 tertiary centers in Germany, Lithuania and Latvia. Controls were patients from the out-patient departments, who were referred for upper endoscopy because of dyspeptic symptoms and had no history of previous malignancy. Gastric cancer (GC) patients had histopathological verification of gastric adenocarcinoma. Genomic DNA was extracted using salting out method from peripheral blood mononuclear cells. *IL12B* T>G (rs1368439), *INSR* T>C (rs1051690), *CCND1* A>C (rs7177) and *IL10* T>C (rs3024498) SNPs were genotyped by the real-time polymerase chain reaction. Associations between gene polymorphism and GC were evaluated using multiple logistic regression analysis with adjustment for sex, age and country of birth.

RESULTS

We observed similar distribution of genotypes and allelic frequencies of all polymorphisms between GC patients and controls except of *INSR* rs1051690. The frequency of the T allele of *INSR* gene was significantly higher in GC patients than in controls (23.26% and 19.19% respectively, $P = 0.028$). CT genotype was also more prevalent in patients compared to control group (38.48% and 30.12% respectively, $P < 0.021$). Logistic regression analysis revealed that only one polymorphism (rs1051690 in *INSR* gene) was associated with increased risk of GC. Carriers of CT genotype had higher odds of GC when compared to CC genotype (OR = 1.45, 95%PI: 1.08-1.95, $P = 0.01$). Similar association was observed in a dominant model for *INSR* gene, where comparison of TT+CT *vs* CC genotypes showed an increased risk of GC (OR = 1.44, 95%PI: 1.08-1.90, $P = 0.01$). Other analyzed SNPs were not associated with the presence of GC.

CONCLUSION

INSR rs1051690 SNP is associated with increased risk of GC, while polymorphisms in *IL12B*, *CCND1* and *IL10* genes are not linked with the presence of GC.

Key words: Gastric cancer; miRNA; Target genes; Single-nucleotide polymorphisms

Core tip: Several studies have evaluated an association between single-nucleotide polymorphisms (SNPs) and gastric cancer (GC) risk. Here we used novel approach. Using bioinformatical analysis tools, several SNPs were identified as potential target sites of microRNAs that previously have been linked with gastric carcinogenesis. This study evaluated an association between SNPs in the *INSR* (rs1051690), *IL12B* (rs1368439), *CCND1* (rs7177), and *IL10* (rs3024498) genes and risk of GC in subjects of European descent. The study found that *INSR* rs1051690 SNP was associated with increased risk of GC, while polymorphisms in *IL12B*, *CCND1* and *IL10* genes showed no association with GC.

Petkevicius V, Salteniene V, Juzenas S, Wex T, Link A, Leja M, Steponaitiene R, Skieceviciene J, Kupcinskas L, Jonaitis L, Kiudelis G, Malfertheiner P, Kupcinskas J. Polymorphisms of microRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* in gastric cancer. *World J Gastroenterol* 2017; 23(19): 3480-3487 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i19/3480.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i19.3480

INTRODUCTION

Gastric cancer (GC) is one of the most prevalent cancers across the globe. Despite decline in the incidence over the last century, GC remains the third leading cause of cancer-related mortality worldwide[1,2]. Furthermore, an upward trend of GC incidence was observed in young patients in recent years[3]. The incidence and mortality of GC vary widely across different countries. Based on the GLOBOCAN 2012 estimates, the highest incidence is in East Asia. High rates are also observed in Central and Eastern Europe, where age-standardized GC mortality rates per 100000 are 16.8 in men and 7.1 in women[1] and prevalence of *H. pylori* infection remains burdensome[4].

Both environmental and genetic factors play a role in etiology of GC; however, as in most cancers, pathogenetic mechanisms in GC are still not fully understood. Demographic and environmental risk factors for GC include older age, male sex, family history, tobacco smoking, *H. pylori* infection and obesity[5]. In recent years, different studies, including genome-wide association studies, examined genetic risk factors for GC. A number of gene polymorphisms have been shown to be related to gastric carcinogenesis, but this field mandates further research[6,7].

The discovery of microRNAs (miRNAs) has opened new opportunities for understanding of pathophysiology and molecular biology of GC[8]. Small non-coding miRNAs molecules (approximately 18-25 nucleotides) regulate gene expression through sequence-specific pairing with the target mRNA and inhibition of its translation[9]. Previous studies have revealed that certain single-nucleotide polymorphisms (SNPs) of miRNA encoding genes may alter miRNA expression and influence cancer development[10,11]. Moreover, genetic variations within miRNA binding sites affect the miRNA-mRNA interaction. SNPs within a miRNA target can

reinforce, weaken or disrupt the binding with miRNAs and change the expression of mRNA targets[12-14].

Target gene identification may help to reveal specific functions of individual miRNAs. This process is challenging because miRNAs may bind to multiple target mRNAs. In order to identify potential miRNA targets computational modeling and experimental approaches are applied[15]. In this study, selection of SNPs was carried out using freely available online database for miRNA target gene prediction[16]. Using this bioinformatical approach we selected four SNPs: *IL12B* (rs1368439), *INSR* (rs1051690), *CCND1* (rs7177) and *IL10* (rs3024498) as putative miRNA-binding sites. Selected SNPs within the above mentioned genes are potential target sites of miR-27, miR-146a, miR-223 and miR-107, that have been linked with gastric carcinogenesis in different studies[8,17].

The aim of this study was to evaluate potential associations between gene polymorphisms of predicted miRNA target genes *IL12B* (rs1368439), *INSR* (rs1051690), *CCND1* (rs7177) and *IL10* (rs3024498) and the presence of GC in European population. To date, these genetic variations have not been evaluated in case-control studies of GC.

MATERIALS AND METHODS

Study subjects

Patients and controls were recruited during the years 2005-2013 at three gastroenterology centers in Germany (Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg), Lithuania (Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas) and Latvia (Riga East University Hospital and Digestive Diseases Centre GASTRO, Riga). Controls were patients from the out-patient departments, who were referred for upper endoscopy because of dyspeptic symptoms and had no history of previous malignancy. GC patients had histopathological verification of gastric adenocarcinoma and were recruited from out-patient and stationary departments. The data from the most of the patients were in focus of our previous studies to genetic predisposition of GC[18-20].

In total, 982 individuals were included in this study (508 controls and 474 GC). There were 206 subjects from Germany (104 controls and 102 GC), 285 subjects from Latvia (146 controls and 139 GC) and 491 subjects from Lithuania (258 controls and 233 GC). All patients were of European descent.

DNA extraction and genotyping

Genomic DNA from samples was extracted using salting out method from peripheral blood mononuclear cells and stored at -20 ℃ until analysis. *IL12B* T>G (rs1368439), *INSR* T>C (rs1051690), *CCND1* A>C (rs7177) and *IL10* T>C (rs3024498) SNPs were genotyped by real time PCR (RT-PCR), using TaqMan® assays with a 7500 TM real-time cycler, in accordance with the manufacturer's instructions (Life Technologies, CA, United States). Dubious samples had repetitive genotyping analysis. Duplicate genotyping was performed in 5% of all samples with one hundred percent concordance rates.

Selection of putative miRNA target gene SNPs

In order to select the candidate SNPs falling within 3'-UTR of genes which are putative targets of frequently deregulated miRNAs in GC, the mirsnpscore database was used (http://www.bigr.medisin.ntnu.no/mirsnpscore). The database contains *in silico* predictions of SNP effects on miRNA-target gene regulation, which are measured by ΔS score. The higher the ΔS score, the higher the possibility that the miRNA-mRNA interaction is disrupted[16]. The candidate SNPs had to meet the following criteria: a minor allele frequency (MAF) > 0.2, the ΔS value > 0.25 and the target gene had to be previously reported as associated with GC. The MAFs and positions of SNPs for Central European population (CEU) were retrieved from 1000 Genomes Browser [phase 3, dbSNP build 149 (Homo sapiens Annotation Release 105)][21]. The list of selected miRNA target gene polymorphisms is presented in Table 1.

Statistical analysis

Age is shown as means ± SD. Mean values of age was compared using Student's *t*-test. Categorical data are presented as frequencies and comparisons were performed using the χ^2 test. Each polymorphism was tested to ensure the fitting with Hardy-Weinberg equilibrium with alpha threshold of 0.05. Associations between GC and gene polymorphisms were calculated using multiple logistic regression analysis and expressed as OR with 95%CI. The ORs were adjusted for sex, age and country of birth. The ORs and 95%CI were calculated for each genotype compared with the wild-type allele homozygous group. Recessive (variant homozygous genotypes *vs* heterozygotes for the variant and homozygotes for the wild-type allele) and dominant (homozygotes variant + heterozygotes versus homozygotes for the wild-type allele) models were also evaluated. The Bonferroni-corrected alpha level was set at 0.013 (0.05/4 SNPs).

The analysis was performed using freely available statistical program PLINK v.1.9 available at pngu.mgh.harvard.edu/~purcell/plink.

RESULTS

Characteristics of the study group

The characteristics of control (n = 508) and GC (n = 474) groups are presented in Table 2. Control subjects were significantly younger than GC patients (P < 0.001). Proportion of men was considerably higher in

Table 1 Selected target genes and their corresponding single-nucleotide polymorphisms

Chromosome	Target gene	miRNA	Delta S	SNP ID	Position	MAF
5	*IL12B*	miR-27	0.3249	rs1368439	15874204	0.202
19	*INSR*	miR-146a	0.2591	rs1051690	7116963	0.242
11	*CCDN1*	miR-223	0.4614	rs7177	69466115	0.419
1	*IL10*	miR-107	0.7057	rs3024498	206941529	0.267

SNP ID: Single-nucleotide polymorphisms number; MAF: Minor allele frequency.

Table 2 Characteristics of gastric cancer patients and control subjects *n* (%)

	Controls (*n* = 508)	Gastric cancer patients (*n* = 474)	*P* value
Age (mean ± SD)	58.1 ± 17. 4	62.5 ± 18. 4	< 0.001[1]
Gender			
Male	139 (27.4)	288 (60.8)	< 0.001[2]
Female	366 (72.0)	178 (37.5)	
Unknown	3 (0.6)	8 (1.7)	
Country of birth			
Latvia	146 (28.7)	139 (29.3)	0.866[2]
Lithuania	258 (50.8)	233 (49.2)	
Germany	104 (20.5)	102 (21.5)	

[1]Student *t*-test; [2]χ^2 test.

GC group than in control group, 60.8% and 27.4% respectively ($P < 0.001$). Individuals in both groups did not differ significantly by country of birth. In order to avoid the potential influence of gender, age and country of birth, these variables were included in further logistic regression analysis.

Hardy-Weinberg equilibrium

The distributions of all analyzed genotypes in the control group did not differ from those predicted by a Hardy-Weinberg equilibrium: $P = 0.013$ for *IL12B* (rs1368439), $P = 0.819$ for *INSR* (rs1051690), $P = 0.856$ for *CCND1* (rs7177) and $P = 0.412$ for *IL10* (rs3024498).

Association analysis of rs1368439, rs1051690, rs7177 and rs3024498 SNPs with gastric cancer

Genotype and allele distributions for analyzed gene polymorphisms are shown in Table 3. No significant differences in the frequencies of the *IL12B, CCND1* and *IL10* genotypes or alleles between control and GC groups were found. The rare G allele of *IL12B* gene had the lowest frequency (14.47% in controls and 15.30% in patients). C allele of *CCND1* gene was found in 44.18% of controls and 43.13% of GC patients, while C allele of *IL10* gene - in 28.04% and 25.53% respectively. Distribution of *INSR* genotypes and alleles differed between control and GC patients groups. The frequency of T allele was 19.19% in controls and 23.26% in GC patients ($P = 0.028$). Distribution of TT genotypes was similar in both groups, while CT genotype was more prevalent in patients than in controls (38.48% and 30.12% respectively, $P = 0.021$). Logistic regression analysis revealed that only one polymorphism (rs1051690 in *INSR* gene) was associated with increased risk of GC. Carriers of CT genotype had higher odds of GC when compared to CC genotype (OR = 1.45, 95%PI: 1.08-1.95, $P = 0.01$). A similar association was observed in a dominant model for *INSR* (rs1051690), where comparison of TT + CT *vs* CC genotypes showed an increased risk of GC ($P = 0.01$). A tendency for T allele *vs* C allele to be associated with higher risk of GC was observed; however, the difference did not reach the adjusted significance threshold (OR = 1.32, 95%PI: 1.04-1.67, $P = 0.02$). No associations with GC risk was found for other analyzed SNPs (Table 3).

DISCUSSION

This study evaluated the association between SNPs in the *INSR* (rs1051690), *IL12B* (rs1368439), *CCND1* (rs7177), and *IL10* (rs3024498) genes and risk of GC in subjects of European descent. These SNPs were selected as candidate miRNA-related genetic alterations that may change the expression of miRNAs linked to GC and potentially mediate carcinogenesis. The study found that *INSR* rs1051690 SNP was associated with increased risk of GC, while no link has been found for the polymorphisms in *IL12B, CCND1* and *IL10* genes and GC risks. To our best knowledge this is the first study which evaluated the effect of these SNPs for the development of GC.

The biological actions of insulin are mediated by *INSR* gene. de-Freitas-Junior *et al*[22] demonstrated that changes in the *INSR* gene can affect the insulin signaling pathway by modulating E-cadherin glycosylation and destabilization of cellular membranes that may have detrimental effects in gastric carcinogenesis. A recent study also identified *INSR* as new candidate gene for diffuse gastric cancer susceptibility[23]. Landi *et al*[13] showed that alleles regulate differentially the amount of a reporter gene (luciferase) in an *in vitro* assay and may have a functional role in regulating the expression of INSR proteins. Several studies have described the role of miRNAs in the regulation of *INSR* gene in different cancers[24,25]. In our study we selected rs1051690 of *INSR* gene which is a potential binding site for miR-146a[16]. Previous case-control studies carried out in Czech Republic, Spain and Israel revealed an association between rs1051690 and colorectal cancer[13,26,27]. The findings of our study are partly in line with the latter

Table 3 Genotype and allele frequencies in control and gastric cancer patients and odds ratio of gastric cancer by genotypes *n* (%)

Genotype	Controls (n = 508)	Gastric cancer patients (n = 474)	OR	95%CI	P value
IL12B (rs1368439)					
TT	366 (72.05)	338 (71.31)	1.00		
TG	137 (26.97)	127 (26.79)	0.87	0.63-1.18	0.39
GG	5 (0.98)	9 (1.90)	1.27	0.39-4.14	0.68
GG *vs* TG + TT			1.33	0.41-4.30	0.63
GG + TG *vs* TT			0.88	0.65-1.20	0.42
Allele T	869 (85.53)	803 (84.70)	1.00	0.83-1.45	0.63
Allele G	147 (14.47)	145 (15.30)	1.10		
INSR (1051690)					
CC	334 (65.75)	272 (57.51)	1.00		
CT	153 (30.12)	182 (38.48)	1.45	1.08-1.95	0.01
TT	21 (4.13)	19 (4.02)	1.30	0.66-2.60	0.44
TT *vs* CT + CC			1.15	0.58-2.30	0.70
TT + CT *vs* CC			1.44	1.08-1.90	0.01
Allele C	821 (80.81)	726 (76.74)	1.00		
Allele T	195 (19.19)	220 (23.26)	1.32	1.04-1.67	0.02
CCND1 (rs7177)					
AA	160 (31.56)	159 (33.62)	1.00		
AC	245 (48.32)	220 (46.51)	0.92	0.68-1.26	0.63
CC	102 (20.12)	94 (19.87)	1.07	0.73-1.58	0.70
CC *vs* AC + AA			1.12	0.80-1.60	0.50
CC + AC *vs* AA			0.97	0.72-1.30	0.83
Allele A	565 (55.72)	538 (56.87)	1.00		
Allele C	449 (44.28)	408 (43.13)	1.02	0.84-1.24	0.81
IL10 (rs3024498)					
TT	252 (50.30)	259 (54.87)	1.00		
TC	217 (43.31)	185 (39.19)	0.92	0.69-1.22	0.57
CC	32 (6.39)	28 (5.93)	1.05	0.58-1.87	0.88
CC *vs* TC + TT			1.08	0.29-1.61	0.78
CC + TC *vs* TT			0.94	0.71-1.23	0.64
Allele T	721 (71.96)	703 (74.47)	1.00		
Allele C	281 (28.04)	241 (25.53)	1.03	0.83-1.30	0.78

studies, suggesting that this SNP might mediate not only colorectal but also GC risks, pointing to a potential joint mechanism of gastrointestinal cancers. A study by Xiao *et al*[28] showed that miR-146a was upregulated in 20 gastric cancer tissues compared with matched non-tumor adjacent tissues. Due to the design of the study we were not able to evaluate whether rs1051690 could mediate the expression of miR-146a and this remains to be evaluated in further studies.

Chronic inflammation plays a crucial role in GC development, thus multiple genes in inflammatory pathways may be associated with GC risk[29]. To date, different gene polymorphism related to inflammatory pathways have been evaluated, with *IL-1B* and *IL-1RN* being the most widely studied ones[18,30-34]. Computational analysis tools that we used in our study suggested two genes polymorphisms - *IL12B* (rs1368439) and *IL10* (rs3024498) - situated in inflammatory pathways, that might be the involved in miRNA-target gene interaction[16]. The other studies evaluated some gene polymorphisms located in IL12 and IL10; however, they were different from the ones selected for our study. *IL12B* encodes a subunit p40 of interleukin (IL) 12. Proinflammatory cytokine IL12 is expressed by activated macrophages and favors the differentiation of T helper 1 (Th1) cells[35]. Th1 lymphocytes prevail over Th2 in *H. pylori* associated chronic gastritis[36]. *IL10* down-regulates the expression of Th1 cytokines and enhances B cell survival, proliferation, and antibody production[37]. Our study did not find significant association between polymorphisms in *IL12B* or *IL10* genes with GC risk. Our results support the previous data to other populations, which analyzed associations between SNPs in genes regulating the inflammatory response and GC[30-32,34].

CCND1 is an important regulator of the cell cycle. It plays essential role in the activation of G1/S transition, which increases cell proliferation and growth. Mutations, amplification and overexpression of this gene are observed frequently in a variety of tumors and may contribute to tumorigenesis[38]. The study by Ma *el al*[39] confirmed that high *CCND1* expression was related with poor prognosis in patients with resected gastric adenocarcinoma. A meta-analysis of associations between the most extensively studied *CCND1* polymorphism rs9344 and GC demonstrated negative results[40]. In our study we did not find an association between *CCND1* (rs7177) SNP and the risk of GC. One study found no association between rs7177 and risk of head and neck cancer in a case control study[41], but no data is available until now for GC.

Target site polymorphisms in gene may strengthen or weaken the miRNA-mRNA interaction and change expression of gene[42]. This field still remains poorly

explored in different cancers including GC. The importance of miRNA related SNPs in gene regulation and the mechanism by which these SNPs can induce alteration in molecular pathways is largely unknown. Wang *et al*[43] suggested that rs4901706 SNP of *C14orf101* gene in the microRNA binding site might be used as a valuable biomarker when predicting GC risk. One other study showed that that polymorphisms of the microRNA-binding sites in the 3′ UTR region of integrin are associated with GC susceptibility (rs2675), tumor stage (rs2675, rs17664, and rs3809865), and lymphatic metastasis (rs17664) in Chinese Han population[44]. In our previous studies we could not determine the link between *miR-27a*, *miR-146a, miR-196a-2, miR-492* and *miR-608* gene polymorphisms and the risk of gastric[45] or colorectal cancers[46].

Our study carriers certain limitations that have to be taken into account. First of all, the selection of putative miRNA target genes and corresponding gene polymorphism is based on bioinformatical databases that may over- or underestimate real interaction effects. Future studies are needed to validate our findings in other cohorts and to investigate whether the gene variant affecting the insulin receptor (*INSR* gene) leads to changes in the expression level of the receptor. Since this is the first study on these SNPs in GC, direct comparison with the results of other studies is not possible yet. Nevertheless, overall our data provide important novel aspects on genetic susceptibility for GC.

The study showed that *INSR* rs1051690 SNP is associated with increased risk of GC. We did not find the association between polymorphisms in *IL12B*, *CCND1* and *IL10* genes and GC risks.

COMMENTS

Background

The discovery of microRNAs (miRNAs) has opened new opportunities for understanding of pathophysiology and molecular biology of gastric cancer (GC). MiRNAs regulate gene expression through sequence-specific pairing with the target messenger RNA (mRNA) and inhibition of its translation. Genetic variations within miRNA binding sites can affect the miRNA-mRNA interaction and change expression of gene. This study evaluated an association between single-nucleotide polymorphisms (SNPs) in the *INSR* (rs1051690), *IL12B* (rs1368439), *CCND1* (rs7177), and *IL10* (rs3024498) genes and risk of GC in subjects of European descent.

Research frontiers

Target site polymorphisms in gene may strengthen or weaken the miRNA-mRNA interaction. This field still remains poorly explored in different cancers including GC. The importance of miRNA related SNPs in gene regulation and the mechanism by which these SNPs can induce alteration in molecular pathways is largely unknown. Studied SNPs were selected as candidate miRNA-related genetic alterations that may change the expression of miRNAs linked to GC and potentially mediate carcinogenesis.

Innovations and breakthroughs

In this study, novel approach was applied. Using bioinformatical analysis tools, several SNPs were identified as potential target sites of microRNAs that previously have been linked with gastric carcinogenesis. The study found that *INSR* rs1051690 SNP was associated with increased risk of GC, while polymorphisms in *IL12B, CCND1* and *IL10* genes showed no association to GC. Our data provide important novel aspects on SNPs of miRNA and their target gene interaction sites in GC.

Applications

Polymorphisms in microRNA binding site might be used as a valuable biomarker when predicting GC risk.

Peer-review

The authors investigated the association of selected polymorphisms with the risk of developing gastric cancer in European population. Their analyses were performed on relatively large population of patients. Overall, the manuscript presents the hypothesis and results well.

REFERENCES

1 GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012. Available from: URL: http://globocan.iarc.fr/Default.aspx

2 **Rahman R**, Asombang AW, Ibdah JA. Characteristics of gastric cancer in Asia. *World J Gastroenterol* 2014; **20**: 4483-4490 [PMID: 24782601 DOI: 10.3748/wjg.v20.i16.4483]

3 **Sonnenberg A**. Time trends of mortality from gastric cancer in Europe. *Dig Dis Sci* 2011; **56**: 1112-1118 [PMID: 21234687 DOI: 10.1007/s10620-010-1553-2]

4 **Kupcinskas J**, Leja M. Management of Helicobacter pylori-related diseases in the Baltic States. *Dig Dis* 2014; **32**: 295-301 [PMID: 24732196 DOI: 10.1159/000357862]

5 **Karimi P**, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. *Cancer Epidemiol Biomarkers Prev* 2014; **23**: 700-713 [PMID: 24618998 DOI: 10.1158/1055-9965.EPI-13-1057]

6 **Xiang B**, Mi YY, Li TF, Liu PF. Updated meta-analysis of the TP53 Arg72Pro polymorphism and gastric cancer risk. *Asian Pac J Cancer Prev* 2012; **13**: 1787-1791 [PMID: 22901123]

7 **Saeki N**, Ono H, Sakamoto H, Yoshida T. Genetic factors related to gastric cancer susceptibility identified using a genome-wide association study. *Cancer Sci* 2013; **104**: 1-8 [PMID: 23057512 DOI: 10.1111/cas.12042]

8 **Link A**, Kupcinskas J, Wex T, Malfertheiner P. Macro-role of microRNA in gastric cancer. *Dig Dis* 2012; **30**: 255-267 [PMID: 22722550 DOI: 10.1159/000336919]

9 **Filipowicz W**, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008; **9**: 102-114 [PMID: 18197166 DOI: 10.1038/nrg2290]

10 **Yang Q**, Jie Z, Ye S, Li Z, Han Z, Wu J, Yang C, Jiang Y. Genetic variations in miR-27a gene decrease mature miR-27a level and reduce gastric cancer susceptibility. *Oncogene* 2014; **33**: 193-202 [PMID: 23246964 DOI: 10.1038/onc.2012.569]

11 **Xu Q**, Liu JW, Yuan Y. Comprehensive assessment of the association between miRNA polymorphisms and gastric cancer risk. *Mutat Res Rev Mutat Res* 2015; **763**: 148-160 [PMID: 25795117 DOI: 10.1016/j.mrrev.2014.09.004]

12 **Chen K**, Song F, Calin GA, Wei Q, Hao X, Zhang W. Polymorphisms in microRNA targets: a gold mine for molecular epidemiology. *Carcinogenesis* 2008; **29**: 1306-1311 [PMID: 18477647 DOI: 10.1093/carcin/bgn116]

13 **Landi D**, Moreno V, Guino E, Vodicka P, Pardini B, Naccarati A, Canzian F, Barale R, Gemignani F, Landi S. Polymorphisms affecting micro-RNA regulation and associated with the risk of dietary-related cancers: a review from the literature and new evidence for a functional role of rs17281995 (CD86) and rs1051690 (INSR), previously associated with colorectal cancer. *Mutat Res* 2011; **717**: 109-115 [PMID: 20971123 DOI: 10.1016/j.mrfmmm.2010.10.002]

14 **Liu C**, Rennie WA, Carmack CS, Kanoria S, Cheng J, Lu J, Ding Y. Effects of genetic variations on microRNA: target interactions. *Nucleic Acids Res* 2014; **42**: 9543-9552 [PMID: 25081214 DOI:

10.1093/nar/gku675]
15 **Thomas M**, Lieberman J, Lal A. Desperately seeking microRNA targets. *Nat Struct Mol Biol* 2010; **17**: 1169-1174 [PMID: 20924405]
16 **Thomas LF**, Saito T, Sætrom P. Inferring causative variants in microRNA target sites. *Nucleic Acids Res* 2011; **39**: e109 [PMID: 21693556 DOI: 10.1038/nsmb.1921]
17 **Juzėnas S**, Saltenienė V, Kupcinskas J, Link A, Kiudelis G, Jonaitis L, Jarmalaite S, Kupcinskas L, Malfertheiner P, Skieceviciene J. Analysis of Deregulated microRNAs and Their Target Genes in Gastric Cancer. *PLoS One* 2015; **10**: e0132327 [PMID: 26172537 DOI: 10.1371/journal.pone.0132327]
18 **Kupcinskas L**, Wex T, Kupcinskas J, Leja M, Ivanauskas A, Jonaitis LV, Janciauskas D, Kiudelis G, Funka K, Sudraba A, Chiu HM, Lin JT, Malfertheiner P. Interleukin-1B and interleukin-1 receptor antagonist gene polymorphisms are not associated with premalignant gastric conditions: a combined haplotype analysis. *Eur J Gastroenterol Hepatol* 2010; **22**: 1189-1195 [PMID: 20631624 DOI: 10.1097/MEG.0b013e32833cf3d5]
19 **Kupcinskas J**, Wex T, Bornschein J, Selgrad M, Leja M, Juozaityte E, Kiudelis G, Jonaitis L, Malfertheiner P. Lack of association between gene polymorphisms of Angiotensin converting enzyme, Nod-like receptor 1, Toll-like receptor 4, FAS/FASL and the presence of Helicobacter pylori-induced premalignant gastric lesions and gastric cancer in Caucasians. *BMC Med Genet* 2011; **12**: 112 [PMID: 21864388 DOI: 10.1186/1471-2350-12-112]
20 **Kupcinskas J**, Wex T, Link A, Bartuseviciute R, Dedelaite M, Kevalaite G, Leja M, Skieceviciene J, Kiudelis G, Jonaitis L, Kupcinskas L, Malfertheiner P. PSCA and MUC1 gene polymorphisms are associated with gastric cancer and pre-malignant gastric conditions [corrected]. *Anticancer Res* 2014; **34**: 7167-7175 [PMID: 25503145]
21 **Auton A**, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR. A global reference for human genetic variation. *Nature* 2015; **526**: 68-74 [PMID: 26432245 DOI: 10.1038/nature15393]
22 **de-Freitas-Junior JC**, Carvalho S, Dias AM, Oliveira P, Cabral J, Seruca R, Oliveira C, Morgado-Diaz JA, Reis CA, Pinho SS. Insulin/IGF-I signaling pathways enhances tumor cell invasion through bisecting GlcNAc N-glycans modulation. an interplay with E-cadherin. *PLoS One* 2013; **8**: e81579 [PMID: 24282611 DOI: 10.1371/journal.pone.0081579]
23 **Donner I**, Kiviluoto T, Ristimäki A, Aaltonen LA, Vahteristo P. Exome sequencing reveals three novel candidate predisposition genes for diffuse gastric cancer. *Fam Cancer* 2015; **14**: 241-246 [PMID: 25576241 DOI: 10.1007/s10689-015-9778-z]
24 **Shrestha S**, Hsu SD, Huang WY, Huang HY, Chen W, Weng SL, Huang HD. A systematic review of microRNA expression profiling studies in human gastric cancer. *Cancer Med* 2014; **3**: 878-888 [PMID: 24902858 DOI: 10.1002/cam4.246]
25 **Gurung B**, Muhammad AB, Hua X. Menin is required for optimal processing of the microRNA let-7a. *J Biol Chem* 2014; **289**: 9902-9908 [PMID: 24563463 DOI: 10.1074/jbc.M113.520692]
26 **Schmit SL**, Gollub J, Shapero MH, Huang SC, Rennert HS, Finn A, Rennert G, Gruber SB. MicroRNA polymorphisms and risk of colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 2015; **24**: 65-72 [PMID: 25342389 DOI: 10.1158/1055-9965.EPI-14-0219]
27 **Mahmoudi T**, Majidzadeh-A K, Karimi K, Karimi N, Farahani H, Dabiri R, Nobakht H, Dolatmoradi H, Arkani M, Zali MR. An exon variant in insulin receptor gene is associated with susceptibility to colorectal cancer in women. *Tumour Biol* 2015; **36**: 3709-3715 [PMID: 25557790 DOI: 10.1007/s13277-014-3010-x]
28 **Xiao B**, Zhu ED, Li N, Lu DS, Li W, Li BS, Zhao YL, Mao XH, Guo G, Yu PW, Zou QM. Increased miR-146a in gastric cancer directly targets SMAD4 and is involved in modulating cell proliferation and apoptosis. *Oncol Rep* 2012; **27**: 559-566 [PMID: 22020746 DOI: 10.3892/or.2011.1514]
29 **Bornschein J**, Leja M, Kupcinskas J, Link A, Weaver J, Rugge M, Malfertheiner P. Molecular diagnostics in gastric cancer. *Front Biosci* (Landmark Ed) 2014; **19**: 312-338 [PMID: 24389187]
30 **Navaglia F**, Basso D, Zambon CF, Ponzano E, Caenazzo L, Gallo N, Falda A, Belluco C, Fogar P, Greco E, Di Mario F, Rugge M, Plebani M. Interleukin 12 gene polymorphisms enhance gastric cancer risk in H pylori infected individuals. *J Med Genet* 2005; **42**: 503-510 [PMID: 15937086 DOI: 10.1136/jmg.2004.022723]
31 **Kang JM**, Kim N, Lee DH, Park JH, Lee MK, Kim JS, Jung HC, Song IS. The effects of genetic polymorphisms of IL-6, IL-8, and IL-10 on Helicobacter pylori-induced gastroduodenal diseases in Korea. *J Clin Gastroenterol* 2009; **43**: 420-428 [PMID: 19077731 DOI: 10.1097/MCG.0b013e318178d1d3]
32 **Persson C**, Canedo P, Machado JC, El-Omar EM, Forman D. Polymorphisms in inflammatory response genes and their association with gastric cancer: A HuGE systematic review and meta-analyses. *Am J Epidemiol* 2011; **173**: 259-270 [PMID: 21178102 DOI: 10.1093/aje/kwq370]
33 **Sugimoto M**, Yamaoka Y, Furuta T. Influence of interleukin polymorphisms on development of gastric cancer and peptic ulcer. *World J Gastroenterol* 2010; **16**: 1188-1200 [PMID: 20222161 DOI: 10.3748/wjg.v16.i10.1188]
34 **Park MJ**, Hyun MH, Yang JP, Yoon JM, Park S. Effects of the interleukin-1β-511 C/T gene polymorphism on the risk of gastric cancer in the context of the relationship between race and H. pylori infection: a meta-analysis of 20,000 subjects. *Mol Biol Rep* 2015; **42**: 119-134 [PMID: 25258120 DOI: 10.1007/s11033-014-3748-7]
35 **Trinchieri G**. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003; **3**: 133-146 [PMID: 12563297 DOI: 10.1038/nri1001]
36 **Guiney DG**, Hasegawa P, Cole SP. Helicobacter pylori preferentially induces interleukin 12 (IL-12) rather than IL-6 or IL-10 in human dendritic cells. *Infect Immun* 2003; **71**: 4163-4166 [PMID: 12819109]
37 **Moore KW**, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; **19**: 683-765 [PMID: 11244051 DOI: 10.1146/annurev.immunol.19.1.683]
38 **Choi YJ**, Anders L. Signaling through cyclin D-dependent kinases. *Oncogene* 2014; **33**: 1890-1903 [PMID: 23644662 DOI: 10.1038/onc.2013.137]
39 **Ma L**, Wang X, Lan F, Yu Y, Ouyang X, Liu W, Xie F, Huang Q. Prognostic value of differential CCND1 expression in patients with resected gastric adenocarcinoma. *Med Oncol* 2015; **32**: 338 [PMID: 25452171 DOI: 10.1007/s12032-014-0338-4]
40 **Zhang Y**, Zeng X, Lu H, Ji H, Zhao E, Li Y. Association between cyclin D1 (CCND1) G870A polymorphism and gastric cancer risk: a meta-analysis. *Oncotarget* 2016; **7**: 66109-66118 [PMID: 27623072 DOI: 10.18632/oncotarget.11848]
41 **Sabir M**, Baig RM, Mahjabeen I, Kayani MA. Significance of cyclin D1 polymorphisms in patients with head and neck cancer. *Int J Biol Markers* 2013; **28**: 49-55 [PMID: 23125009 DOI: 10.5301/JBM.2012.9768]
42 **Mishra PJ**, Mishra PJ, Banerjee D, Bertino JR. MiRSNPs or MiR-polymorphisms, new players in microRNA mediated regulation of the cell: Introducing microRNA pharmacogenomics. *Cell Cycle* 2008; **7**: 853-858 [PMID: 18414050 DOI: 10.4161/cc.7.7.5666]
43 **Wang C**, Zhao Y, Ming Y, Zhao S, Guo Z. A polymorphism at the microRNA binding site in the 3'-untranslated region of C14orf101 is associated with the risk of gastric cancer development. *Exp Ther Med* 2016; **12**: 1867-1872 [PMID: 27602096 DOI: 10.3892/etm.2016.3521]
44 **Song X**, Zhong H, Zhou J, Hu X, Zhou Y, Ye Y, Lu X, Wang J, Ying B, Wang L. Association between polymorphisms of microRNA-binding sites in integrin genes and gastric cancer in Chinese Han population. *Tumour Biol* 2015; **36**: 2785-2792 [PMID: 25472585 DOI: 10.1007/s13277-014-2903-z]
45 **Kupcinskas J**, Wex T, Link A, Leja M, Bruzaite I, Steponaitiene R, Juzenas S, Gyvyte U, Ivanauskas A, Ancans G, Petrenkiene V, Skieceviciene J, Kupcinskas L, Malfertheiner P. Gene polymorphisms of microrna in Helicobacter pylori-induced

high risk atrophic gastritis and gastric cancer. *PLoS One* 2014; **9**: e87467 [PMID: 24475294 DOI: 10.1371/journal.pone.0087467]

46 **Kupcinskas J**, Bruzaite I, Juzenas S, Gyvyte U, Jonaitis L, Kiudelis G, Skieceviciene J, Leja M, Pauzas H, Tamelis A, Pavalkis D, Kupcinskas L. Lack of association between miR-27a, miR-146a, miR-196a-2, miR-492 and miR-608 gene polymorphisms and colorectal cancer. *Sci Rep* 2014; **4**: 5993 [PMID: 25103961 DOI: 10.1038/srep05993]

P- Reviewer: Hudler P, Wang K **S- Editor**: Qi Y **L- Editor**: A **E- Editor**: Zhang FF

SANTRAUKA

Įvadas

Šiuo metu skrandžio vėžys yra visuotinė sveikatos problema pasaulyje. Ligos eigos prognozė priklauso nuo vėžio stadijos diagnozės metu. Deja, daugeliu atvejų, skrandžio vėžio diagnozės metu yra nustatomos metastazės, nes ankstyvosiose stadijose ši liga yra besimptomė [43]. Šios disertacijos tikslas buvo prisidėti prie atradimų, kurie pagerintų vėžio diagnostiką, naudojant neinvazinę diagnostiką. Neseniai atrastos mažos mikro RNR molekulės yra svarbios vėžinių procesų patogenezėje. Mikro RNR – tai audiniams specifiniai biožymenys, kurie įgalina klasifikuoti skirtingus vėžio tipus, remiantis pakitusių mikro RNR raiškos profiliu, tačiau reikalingi išsamesni tyrimai [23, 24].

Darbo naujumas

Šiame tyrime pateikti nauji įrodymai apie: (i) mikro RNR geno taikinio jungimosi srityje esančius vieno nukleotido polimorfizmus skrandžio vėžio atveju, (ii) mikro RNR profilį skrandžio priešvėžinių ligų ir vėžio atveju, taip pat mikro RNR tinkamumą neinvazinei diagnostikai, (iii) naujus mikro RNR potencialius genus taikinius.

Šie duomenys papildo molekulines žinias apie skrandžio vėžio patogenezę. Be to, šie duomenys gali būti naudingi ne tik moksliniams darbams, tačiau ir diagnostikai, ir naujų terapinių priemonių kūrimui.

Darbo tikslas

Šio darbo tikslas nustatyti mikro RNR raiškos profilį ir jų reguliuojamų genų įtaką skrandžio vėžio išsivystymui bei įvertinti šių molekulinių žymenų tinkamumą neinvazinei ligos diagnostikai.

Uždaviniai:

1. Įvertinti mikro RNR genų taikinių *IL12B (rs1368439), INSR (rs1051690), CCND1 (rs7177)* ir *IL10 (rs3024498)* vieno nukleotido polimorfizmų sąsają su skrandžio vėžiu Europiečių populiacijoje.
2. Nustatyti mikro RNR raiškos profilį skrandžio vėžio ir atrofinio gastrito audinyje.
3. Įvertinti pasirinktųjų mikro RNR raišką skrandžio vėžiu sergančiųjų kraujo plazmoje ir jų tinkamumą neinvazinei diagnostikai.

4. Eksperimentiškai blokuojant hsa-miR-20b ir padidinant hsa-miR-451a bei hsa-miR-1468 kiekį, ištirti jų genų taikinių raiškos pokyčius skrandžio vėžio ląstelių linijose.

Medžiagos ir metodai

STUDIJA I

Pirmos studijos dizainas ir mėginiai

I studijos tikslas buvo ištirti sąsają tarp miRNR genų taikinių (*IL12B, INSR, CCND1 ir IL10*) vieno nukleotido polimorfizmų ir skrandžio vėžio (SV) europiečių populiacijoje. Tyrimui buvo naudojama DNR išgryninta iš kraujo. Iš viso buvo ištirti 982 žmonės (474 žmonės, sergantys skrandžio vėžiu ir 508 nesergantys). Iš jų 206 buvo iš Vokietijos (102 sergantys skrandžio vėžiu ir 104 nesergantys), 285 žmonės iš Latvijos (139 sergantys skrandžio vėžiu ir 146 nesergantys), 491 žmonės iš Lietuvos (233 sergantys skrandžio vėžiu, 258 nesergantys).

DNR gryninimas, miRNR genų taikinių vieno nukleotido polimorfizmų atranka ir genotipavimas

Genominė DNR buvo išgryninta naudojant išdruskinimo metodą iš periferinio kraujo branduolį turinčių ląstelių – leukocitų. Naudojantis literatūros duomenimis, pirmiausiai buvo pasirinktos tos miRNR, kurios yra susijusios su skrandžio vėžiu: hsa-miR-27, hsa-miR-146a, hsa-miR-223 and hsa-miR-107. Tuomet, naudojant bioinformatinę duomenų bazę *„Mirsnpscore“* (http://www.bigr.medisin.ntnu.no/mirsnpscore) buvo parinkti šių miRNR galimi genai taikiniai, kurių 3’ galo netransliuojamoje strityje (3’UTR) (angl. *3’ untranslated region)* yra vieno nukleotido polimorfizmai: *IL12B T>G (rs1368439), INSR T>C (rs1051690), CCND1 A>C (rs7177)* ir *IL10 T>C (rs3024498)*. Vieno nukleotipo polimorfizmai buvo genotipuoti naudojant *TaqMan* pradmenis.

STUDIJA II

Antros studijos dizainas

Tiriamoji medžiaga

I-oje studijos dalyje mikro RNR genų raiškos profiliavimas atliktas skrandžio vėžiniame audinyje (n = 13), palyginti su kontroliniu audiniu (n = 12). Ištirta 377-ių mikro RNR genų raiška. Validavimas atliktas skrandžio vėžiniame audinyje (n = 38), palyginti su kontroliniu skrandžio audiniu (n = 39), bei skrandžio vėžiu sergančių (n = 38) ir sveikų žmonių kraujo plazmoje

(n = 39). Po profiliavimo duomenų analizės pasirinktos 6-ios mikro RNR, kurių genų raiška buvo pakitusi labiausiai. Po mikro RNR genų raiškos audinyje analizės, pasirinkitos 4-ios mikro RNR, kurių genų raiškos analizė atlikta kraujo plazmoje.

II studijos dalyje mikro RNR profiliavimas atliktas skrandžio vėžio (n = 20) ir atrofinio gastrito (n = 18) audinyje palyginti su kontroliniu skrandžio audiniu (n = 26). Ištirta 2180-ies mikro RNR genų raiška. Validavimo kohortoje 21-os mikro RNR raiška nustatyta skrandžio vėžio (n = 39) ir 21-os atrofinio gastrito audinyje (n = 40) palyginti su kontroliniu skrandžio audiniu (n = 40), pasirinkus mikro RNR, kurių raiška buvo pakitusi ir kraujo plazmoje kiekvienos ligos atveju. Tiriamų mėginių ir mikro RNR skaičius, taikant skirtingus tyrimų metodus II-ai studijai pateiktas *1-oje lentelėje.*

1 lentelė. *II studijos dizainas*

II studijos dizainas		**Audinio tipas**	**Mėginių skaičius**	**Tirtų mėginių skaičius**	**Naudotas metodas**
I dalis	Profiliavimo kohorta	Skrandžio gleivinė	GC, n = 13 HC, n = 12	377	TL-kPGR (TLDA plokštelė A)
	Validavimo kohorta	Skrandžio gleivinė	GC, n = 38 HC, n = 39	6	TL-kPGR (TaqMan reagentas)
		Kraujo plazma		4	
II dalis	Profiliavimo kohorta	Skrandžio gleivinė	GC, n = 20 AG, n = 18 HC, n = 26	2 180	Mažųjų RNR sekoskaita
	Validavimo kohorta	Skrandžio gleivinė	GC, n = 39 AG, n = 40 HC, n = 40	21	TL-kPGR (TLDA surinkta plokštelė)

GC – skrandžio vėžys, AG – atrofinis gastritis, HC – sveika kontrolė.

Mėginiai ir tyrimo metodai

Kauno regioninis biomedicininių tyrimų etikos komitetas pritarė biomedicininių tyrimų vykdymui *(Bioetikos leidimo nr: BE-2-10, 2011 m.)*. Žmogaus skrandžio audinių mėginiai buvo gauti operacijos arba gastroskopijos metu talpinami į *RNAlater (Ambion, JAV)* ir laikomi –80 °C. Kraujo plazma surinkta iš skrandžio vėžiu sergančių ir nesergančių asmenų. Visiems tiriamiesiems buvo atliktas serologinis kraujo tyrimas specifiniams *H.pylori Ig G* antikūnams nustatyti ELISA metodu, naudojantis komerciniu rinkiniu *(Virion/Serion GmbH, Vokietija)*. Kontrolinė skrandžio audinių grupė sudaryta gavus gydytojo histologo patvirtinimą jog audinyje nėra pakitimų, o kraujo plazmoje nenustatyti imunoglobulino G *(Ig G)* antikūnai prieš *Helicobacter*

pylori bakteriją. Visuminė RNR iš skrandžio audinių buvo išgryninta naudojantis rinkiniais: *„mirVana miRNA Isolation Kit“* (profiliavimo kohortai (II studijos I dalis) *„miRNeasy Mini Kit“* profiliavimo ir validavimo kohortoms (II studijos I ir II dalys), iš plazmos *„miRnease Serum/Plasma Kit“* (II studijos II dalis), remiantis gamintojo rekomendacijomis. Visuminės RNR kokybė ir kiekybė nustatyta spektrofotometru *„NanoDrop 2000“ (Applied Biosystems, JAV).*

Kiekybinė realaus laiko polimerazinė grandininė reakcija

Mikro RNR profiliavimas (II studija I dalis). Mikro RNR, susijusių su skrandžio vėžiu genų raiška atlikta kiekybinės tikrojo laiko polimerazinės grandininės reakcijos (TL-kPGR) metodu, naudojant validuotus *TaqMan* specifinius mikro RNR pradmenis mažo tankio (384 šulinėlių) plokštelėje A (angl. *TaqMan Low Density Array (TLDA) card A)* realaus laiko termocikleryje *„ViiA 7 Real-time PCR System“ (Applied Biosystems).*

Komplementari DNR susintetinta naudojant 500 ng visuminės RNR ir atvirkštinės transkripcijos pradmenis *„Megaplex“ (Applied biosystem),* remiantis gamintojo rekomendacijomis.

Mikro RNR validavimas (II studija I dalis). Mikro RNR genų raiška buvo tiriama skrandžio vėžio audinyje, palyginti su kontroliniu skrandžio audiniu ir žmonių sergančių skrandžio vėžiu kraujo plazmoje, palyginti su nesergančių. TL-kPGR atlikta naudojant validuotus *TaqMan* specifinius mikro RNR pradmenis. Komplementari DNR paruošta naudojant 20 ng visuminės RNR, išgrynintos iš audinio ir atvirkštinės transkripcijos rinkinį *„TaqMan miRNA Reverse transcription kit“* bei *TaqMan* pradmenis kiekvienai mikro RNR atskirai. Kiekybinės PGR reakcijoje buvo atlikti du pakartojimai. TL-kPGR buvo atlikta naudojant realaus laiko termocikler*į „ABI 7500 Fast“ (Applied biosystem).*

Mikro RNR validavimas (II studija II dalis). Mikro RNR genų raiška buvo tiriama skrandžio vėžio ir atrofinio gastrito audinyje, palyginti su kontroliniu skrandžio audiniu, kiekybinės tikrojo laiko polimerazinės grandininės reakcijos (TL-kPGR) metodu, naudojant validuotus *TaqMan* specifinius mikro RNR pradmenis mažo tankio (384 šulinėlių) pagal užsakymą sukurtoje plokštelėje, realaus laiko termocikleryje *„ViiA 7 Real-time PCR System“ (Applied Biosystems).* Koplementari DNR susintetinta naudojant 600 ng visuminės RNR ir atvirkštinės transkripcijos pradmenų rinkinį remiantis gamintojo rekomendacijomis.

Mažųjų mikro RNR bibliotekos sudarymas ir mikro RNR raiškos profilio nustatymas skrandžio audinyje

Mažųjų RNR biblioteka buvo paruošta naudojant *„TruSeq Small RNA Sample Preparation Kit (Illumina)“*, o mėginiai sekvenuoti naujos kartos sekvenatoriumi *HiSeq2500 (Illumina)*, remiantis gamintojo rekomendacijomis.

Sekoskaitos metu gautų sekų pirminė kokybės analizė atlikta taikant šiuos kokybės kriterijus: sekos kokybė > 30, sekos ilgis > 17 bp. Siekiant sutrumpinti tolimesnės bioinformatinės analizės laiką identiškos sekos buvo pašalintos, tačiau informacija apie sekų skaičių (angl. *read counts*) buvo išsaugota. Toliau naudojant *„BLASTN v2.2.30“* buvo pašalintos sekos, kurios nepriklauso žmogaus genomui, remiantis referentinėmis mikro RNR sekomis iš *„miRBase v20“*. Mikro RNR raiškos analizė atlikta naudojant neigiamą binominį apibendrintą linijinį modelį įdiegtą *„Bioconductor DESeq2“* pakete „R“ programinėje aplinkoje (versija 3.2.3). P-vertės, kurios gautos naudojant Wald testą buvo koreguojamos naudojant daugkartinio palyginimo procedūrą, šiuo atveju *Bonferroni*. Šios studijos galia skrandžio vėžio grupėje buvo 80 proc., kai mikro RNR raiškos pokytis yra > 2 ($p < 0,01$), o atrofinio gastrito grupėje 99 proc. kai mikro RNR raiškos pokytis yra > 2 ($p < 0,01$). Taigi mikro RNR, kurių koreguota $p < 0,01$, o raiškos pokytis > 2, turėjo reikšmingai pakitusią raišką. Mikro RNR raiškos profilio validavimui buvo pasirinktos mikro RNR, kurių sekų vidurkis mėginiuose buvo daugiau kaip 100 sekų, raiškos pokytis > 2, o $p < 0,01$. Studijos statistinė galia apskaičiuota naudojant *„RNASeqPower“* analizės paketą.

STUDIJA III

Studijos dizainas

Su skrandžio vėžiu susijusių mikro RNR (miR-20b-5p, miR-451a-5p ir miR-1468-5p) genai taikiniai buvo pasirinkti atlikus analizę duomenų bazėse *(DIANA-microT-CDS, ElMMo, MicroCosm, miRanda, miRDB, PicTar, PITA ir TargetScan).* Skrandžio vėžio ląstelių linijose AGS ir MKN-28 transfekcija atlikta inhibitoriumi miR-20b-5p ir imitatoriais miR-451a-5p bei miR-1468-5p, naudojant Lipofektaminą 3000. Po transfekcijos praėjus 24 ir 48 val. skrandžio ląstelių linijose (AGS ir MKN-28) atlikta mikro RNR genų taikinių raiškos analizė naudojant specifinius *TaqMan* pradmenis TL-kPGR metodu.

Tiriamoji medžiaga

Skrandžio vėžinės ląstelės AGS ir MKN-28 (iš Amerikos tipo kultūrų kolekcijos (angl. *American Type Culture Collection (ATCC))* buvo auginamos inkubatoriuje mitybinėje terpėje *Ham's F-12K (Gibco by Life technologies, JAV)*, papildytoje 10 proc. veršiuko serumu *(Gibco by Life technologies, JAV)* ir 100 IU/ml penicilinu ir 100 µg/ml streptomicinu *(Gibco by Life technologies, JAV)*, esant 37°C temperatūrai, 5 proc. CO_2 ir 95 proc. santykinei drėgmei.

Mikro RNR genų taikinių paieškos analizė

Mikro RNR genų taikinių analizė buvo atlikta naudojantis „R" paketo programine įranga *„Rstudio Deskto0p v 1.0.143"*. Pirmiausiai buvo atrinkti su skrandžio vėžiu susiję genai, naudojantis duomenų baze *„DisGenet"*. Toliau, naudojantis matematiniais prognozavimo įrankiais DIANA-microT-CDS, ElMMo, MicroCosm, miRanda, miRDB, PicTar, PITA ir TargetScan buvo nustatyti mikro RNR (miR-20b-5p, miR-451a-5p ir miR-1468-5p) galimi genai taikiniai. Sekančiame etape šių mikro RNR genų taikinių sąrašas sumažintas, remiantis duomenų bazių *miRecords, miRTarBase* ir *TarBase* duomenimis, pašalinus tuos genus, kurie buvo validuoti kitose studijose. Paskutiniame analizės etape mikro RNR genai taikiniai buvo atrinkti pagal jų funkciją vėžio patogenezėje (vėžio supresoriai ir onkogenai), bei atlikus literatūros analizę.

Skrandžio vėžinių ląstelių transfekcija

Transfekcija atlikta 24-ių šulinėlių plokštelėse. Skrandžio vėžinės ląstelės AGS (n = 7) ir MKN-28 (n = 7) į šulinėlius susėtos 40 000 ląstelių/šulinėlyje tankiu 500 µl mitybinėje terpėje Ham's F-12K, papildytoje 10 proc. veršiuko serumu be antibiotikų. Transfekcija atlikta inhibitoriumi miR-20b-5p ir imitatoriais miR-451a-5p bei miR-1468-5p *(Ambion by Thermo Fisher Scientific, JAV)*, naudojant *Lipofektaminą 3000 (Thermo Fisher Scientific, JAV)*. Ląstelės su transfekcijos reagentu buvo laikomos 37 °C temperatūroje, 5 proc. CO_2 inkubatoriuje. Visuminė RNR išgryninta iš ląstelių po transfekcijos praėjus 24 val. ir 48 val.

Visuminės RNR gryninimas ir genų raiškos analizė

Mikro RNR genų raiškos analizei visuminė RNR buvo išgryninta iš skrandžio vėžio ląstelių AGS ir MKN-28, naudojant rinkinį *„MiRneasy Micro Kit" (Qiagen, Vokietija),* remiantis gamintojo rekomendacijomis.

Mikro RNR atvirkštinė transkripcija atlikta naudojant atvirkštinės transkripcijos rinkinį *„TaqMan Reverse Transcription Kit“* ir RNR pradmenis miR-16 (000391) – endogeninei kontrolei, miR-20b (001014), miR-451a (001141) ir miR-1468 (121107_mat).

Mikro RNR genų taikinių analizei visuminė RNR išgryninta iš transfekuotų vėžinių ląstelių AGS ir MKN-28, naudojant rinkinkinį *„RNeasy Mini Kit“ (Qiagen, Vokietija),* remiantis gamintojo rekomendacijomis. Mikro RNR atvirkštinė transkripcija atlikta naudojant atvirkštinės transkripcijos rinkinį *„Hight-capasity cDNA Reverse Transcriptiom Kit“ (Thermo Fisher Scientific, USA).* Komplementari DNR buvo susintetinta naudojant 1 µg visuminės RNR 20 µl reakcijoje.

Tikrojo laiko kiekybinė relaus laiko polimerazinė reakcija

Genų raiška mikro RNR lygmenyje *(hsa-miR-20b-5p, hsa-miR-451a-5p ir hsa-miR-1468-5p,* ir *hsa-miR-16* kaip endogeninė kontrolė*)* buvo tiriama skrandžio vėžio ląstelėse AGS (n = 7) ir MKN-28 (n = 7), palyginti su nepažeistu skrandžio audiniu (n = 11). Atlikta šių mikro RNR genų taikinių genų raiškos analizė: *hsa-miR-20b-5p* genų taikinių *(EREG, FAT, IRF1, TXNIP, PTEN),* hsa-miR-451a-5p genų taikinių *(CAV1 ir ADAM28)* ir hsa-miR-1468-5p genų taikinių *(TNFα, DNMT1 ir CITED2)*. TL-kPGR atlikta naudojant validuotus *TaqMan* specifinius mikro RNR pradmenis, o mikro RNR genų taikinių raiškos analizė atlikta naudojant *TaqMan* genų pradmenis, remiantis gamintojo rekomendacijomis. Kiekybinės PGR reakcijoje buvo atlikti du pakartojimai. TL-kPGR buvo atlikta naudojant realaus laiko termociklerį *„ABI 7500 Fast“ (Applied biosystem).*

Statistika

Genų raiška išreikšta delta Ct reikšme ir išvestas trijų pakartojimų vidurkis naudojantis *„GraphPad Prism“ (GraphPadSoftware Inc., JAV)* programine įranga. Mann-Whitney testas, naudotas apskaičiuoti skirtumams tarp grupių. Statistiškai patikima reikšmė vertinta, kai $p < 0,05$.

Rezultatai

I STUDIJA

Vieno nukleotido polimorfizmų *(IL12B (rs1368439), CCND1 (rs7177) ir IL10 (rs3024498))* genotipo ir alelių dažnių skirtumų skrandžio vėžiu sergančių ir sveikų pacientų tarpe nebuvo, išskyrus *INSR* vieno nukleotido polimorfizmą (rs1051690). *INSR* geno retojo alelio T dažnis buvo didenis skrandžio vėžiu sergančiųjų tarpe, palyginti su kontrolinės grupės (23,26

proc. ir 19,19 proc., atitinkamai, p = 0,028). CT genotipas taip pat buvo dažnesnis skrandžio vėžiu sergančiųjų tarpe, palyginti su kontrolinės grupės (38,48 proc. ir 30,12 proc. atitinkamai, p < 0,021). Logistinės regresijos analizės metu nustatyta, kad *INSR* geno vieno nukleotido polimorfizmas (rs1051690) susijęs su skrandžio vėžiu. CT genotipas buvo dažnesnis sergančiųjų tarpe, palyginti su kontroline grupe (ŠS – 1,45, 95 proc. PI 1,08–1,95, p = 0,01). Taip pat nustatyta, kad skrandžio vėžio atveju *INSR* geno dominantinis genotipas TT + CT yra dažnesnis, palyginti su kontroline grupe (ŠS – 1,44, 95 proc. PI 1,08–1,90, p = 0,01).

II STUDIJA

Mikro RNR raiškos profiliavimas skrandžio vėžio audinyje ir plazmoje

Naudojant mažo tankio plokštelę (angl. *TaqMan Low Density Array*) skrandžio vėžio audinyje, palyginti su kontroliniu audiniu, buvo nustatyta pakitusi 15-os mikro RNR raiška, iš kurių 7-ių mikro RNR buvo padidėjusi, o 8-ių sumažėjusi, kai raiškos pokytis > 2, p < 0,01. Šešios mikro RNR buvo pasirinktos replikacijai, iš kurių hsa-135a-5p, hsa-miR-148a-3p, hsa-miR-204-5p ir hsa-miR-375 genų raiška buvo sumažėjusi, o hsa-miR-223-3p ir hsa-miR-155-5p buvo padidėjusi. Validavimo studijoje, naudojant mikro RNR validuotus *TaqMan* pradmenis, nustatyta, kad 4-ių (iš 6-ių) mikro RNR (hsa-miR-148a-3p, hsa-miR-204-5p, hsa-miR-375, hsa-miR-223-3p) genų raiška buvo pakitusi skrandžio vėžio audinyje, palyginti su kontroliniu audiniu ta pačia kryptimi, kaip ir profiliavimo studijoje. Tuomet jų (hsa-miR-148a-3p, hsa-miR-204-5p, hsa-miR-375, hsa-miR-223-3p) raiška buvo tirta skrandžio vėžiu sergančių pacientų plazmoje, palyginti su nesergančių. Buvo nustatyta, kad (hsa-miR-148a-3p, hsa-miR-375, hsa-miR-223-3p) raiška buvo pakitusi ta pačia kryptimi, kaip ir audinyje (p < 0,05).

Naudojant mažųjų RNR sekoskaitos metodą mikro RNR profiliavimas atliktas skrandžio vėžio ir atrofinio gastrito audinyje, palyginti su kontroliniu audiniu.

Skrandžio vėžio audinyje, palyginti su kontroliniu, nustatyta 110-ies mikro RNR pakitusi raiška, iš jų 72-iejų buvo padidėjusi, o 38-ių sumažėjusi. Tuo tarpu, skrandžio vėžio audinyje, palyginti su šalia esančiu audiniu, buvo nustatyta 32-iejų mikro RNR pakitusi raiška, iš kurių, 25-ių mikro RNR raiška buvo padidėjusi, o 7-ių sumažėjusi. Tai rodo šalia vėžio esančio nepažeisto audinio panašumą su kontroliniu skrandžio audiniu. Tuo tarpu, 17-kos mikro RNR raiška buvo pakitusi atrofinio gastrito audinyje palyginti su kontroliniu audiniu, iš jų 11-os buvo sumažėjusi, o 6-ių padidėjusi. Skrandžio vėžio audinyje, palyginti su atrofinio gastrito pažeistu audiniu, buvo pakitusi

76-ių mikro RNR raiška, iš jų 54-ių buvo padidėjusi, o 22-jų sumažėjusi. Sekančiame etape buvo nusekvenuota skrandžio vėžiu (n = 2) ir atrofinio gastritu (n = 2) sergančių pacientų plazma.

Mikro RNR profilio validavimui skrandžio vėžio ir atrofinio gastrito grupėse buvo pasirinkta po 21-ą mikro RNR, kurių raiška buvo pakitusi ir kraujo plazmoje (kurių nuskaitymo skaičius buvo > 5). Validavimo studijoje skrandžio vėžio audinyje (n = 39), palyginti su kontroliniu skrandžio audiniu (n = 40), buvo pakitusi 8-ių mikro RNR raiška iš 21-os. Atrofinio gastrito audinyje (n = 40), palyginti su kontroliniu (n = 40), buvo pakitusi 2-jų, iš 21-os. Skrandžio vėžio audinyje, palyginti su atrofinio gastrito audiniu, buvo pakitusi 5-ių mikro RNR raiška, *lentelė 2.*

2 lentelė. *Mikro RNR raiškos pokyčiai skrandžio vėžio ir atrofinio gastrito audinyje, palyginti su kontroliniu skrandžio audiniu, naudojant mažo tankio TaqMan plokšelę.*

Mikro RNR	Pokyčio kryptis	SV vs kontrolė		AG vs kontrolė		SV vs AG	
		Pokytis kartais	p reikšmė	Pokytis kartais	p reikšmė	Pokytis kartais	p reikšmė
hsa-miR-204-5p	down	8,29	$6,6 \times 10^{-12}$	2,23	$1,5 \times 10^{-6}$	3,71	1×10^{-4}
hsa-miR-148a-5p	down	3,24	$3,1 \times 10^{-7}$			2,92	$7,1 \times 10^{-6}$
hsa-miR-375	down	3,07	$2,9 \times 10^{-8}$				
hsa-miR-142-3p	up	2,94	$4,6 \times 10^{-12}$				
hsa-miR-223-3p	up	3,58	$1,1 \times 10^{-12}$			2,54	2×10^{-9}
hsa-miR-215-5p	up	4,41	$3,3 \times 10^{-7}$	7,19	$2,9 \times 10^{-9}$		
hsa-miR-224-5p	up	5,04	$5,7 \times 10^{-8}$			3,10	$4,4 \times 10^{-4}$
hsa-miR-335-3p	up	5,77	$4,6 \times 10^{-12}$			5,12	$1,8 \times 10^{-10}$

SV – skrandžio vėžys, AG – atrofinis gastritas.

III STUDIJA

Mikro RNR hsa-miR-20b-5p, hsa-miR-451a-5p ir hsa-miR-1468-5p genų raiškos pokyčių skrandžio vėžinėse ląstelėse AGS ir MKN-28 analizė

Mikro RNR hsa-miR-451a-5p ir hsa-miR-1468-5p raiška skrandžio vėžio ląstelėse AGS (n = 7) ir MKN-28 (n = 7) buvo sumažėjusi ($p < 0,01$), o hsa-miR-20b-5p padidėjusi ($p < 0,01$), palyginti su skrandžio audiniu (n = 11).

Mikro RNR hsa-miR-20b-5p, hsa-miR-451a-5p ir hsa-miR-1468-5p genų taikinių atranka ir raiškos analizė

Naudojantis genų taikinių prognozavimo programomis buvo nustatyti mikro RNR genai taikiniai: *EREG, FAT4, IRF1, TXNIP ir PTEN* – galimi hsa-miR-20b-5p taikiniai; *CAV1* ir *ADAM28* – hsa-miR-451a-5p, o *TNF, DNMT1* ir *CITED2* – hsa-miR-1468-5p genai taikiniai.

Atlikus transfekciją inhibitoriumi hsa-miR-20b-5p geno *IRF1* raiška padidėjo 1,38 karto ($p < 0,01$) ir 1,43 karto ($p < 0,01$) skrandžio vėžinėse ląstelėse AGS ir MKN-28, atitinkamai, po transfekcijos praėjus 24 val. Tuo tarpu po 48 val. *IRF1* geno raiška padidėjo 1,40 karto ($p < 0,01$) tik ląstelėse AGS.

Geno *PTEN* raiška padidėjo 1,25 karto ($p = 0,02$) ir 1,87 karto ($p < 0,01$) skrandžio vėžinėse ląstelėse AGS ir MKN-28, atitinkamai, po transfekcijos inhibitoriumi hsa-miR-20b-5p praėjus 24 val. Tuo tarpu po 48 val. geno raiškos pokyčio nebuvo nei vienoje ląstelių linijoje.

Geno *TXNIP* raiška padidėjo 1,65 karto ($p = 0,04$) ir 1,70 karto ($p < 0,01$) skrandžio vėžinėse ląstelėse AGS ir MKN-28, atitinkamai, po transfekcijos inhibitoriumi hsa-miR-20b-5p, praėjus 24 val. Tuo tarpu po 48 val. geno raiška padidėjo 1,63 karto ($p < 0,01$) ir 1,80 karto ($p < 0,01$) skrandžio vėžinėse ląstelėse AGS ir MKN-28, palyginti su ląstelėmis, transfekuotomis su neigiama kontrole. Tuo tarpu, genų *EREG* ir *FAT4* raiškos pokyčių nustatyta nebuvo.

Atlikus transfekciją imitatoriumi hsa-miR-451a-5p geno *CAV1* raiška sumažėjo skrandžio vėžinėse ląstelėse AGS 1,76 karto ($p < 0,01$) ir 1,43 karto ($p = 0,03$), po 24 ir 48 val., atitinkamai. Tuo tarpu skrandžio vėžinėse ląstelėse MKN-28 geno *CAV1* raiška sumažėjo 1,46 karto ($p < 0.01$) po transfekcijos praėjus 24 val., palyginti su ląstelėmis, transfekuotomis su neigiama kontrole.

Atlikus transfekciją imitatoriumi hsa-miR-1468-5p, genų *DNMT1* ir *CITED2* raiškos pokyčio nebuvo nei vienoje ląstelų linijoje nei po 24 val, nei po 48 val., palyginti su ląstelėmis, transfekuotomis su neigiama kontrole. Geno *TNFα* raiška buvo nustatyta žemiau slenkstinės (angl. *threshold*) ribos.

Diskusija

Visame pasaulyje sergamumo ir mirtingumo lygis nuo skrandžio vėžio išlieka aukštas. Pagrindiniai skrandžio vėžio rizikos veiksniai yra atrofinis gastritas su *Helicobacter pylori* infekcija, genetinių ir epigenetinių procesų įtaka, netinkami mitybos įpročiai ir kt. [1]. Mikro RNR yra potranskripciniai reguliatoriai, kurie komplementariai susijungia su informacine RNR (iRNR), ko pasekoje įvyksta transliacijos nuslopinimas ir genų nutildymas. Nustatyta, kad mikro RNR raiškos intensyvumas įvairiuose audiniuose, tame tarpe ir

navikiniuose, skiriasi, todėl jos gali būti panaudotos kaip biožymenys ankstyvoje vėžinių ligų diagnostikoje, chemoterapijos efektyvumui įvertinti, ligos eigos prognozavimui ar nežinomos kilmės navikų klasifikavimui. Mikro RNR yra atsparios ribonukleazių sukeltam irimui, ilgiau nei siRNR išlieka stabilios audiniuose ir aptinkamos kraujo serume, plazmoje bei kituose kūno skysčiuose. Mikro RNR reguliuoja geno raišką, prisijungdama prie iRNR 3‘ galo netransliuojamos dalies, todėl geno taikinio vieno nukleotido polimorfizmas gali turėti įtakos mikro RNR prisijungimui.

Šiame darbe buvo nustatyta, kad europiečių populiacijoje geno *INSR (rs1051690)* vieno nukleotido polimorfizas (VNP) yra susijęs su skrandžio vėžiu. Tuo tarpu sąsajos tarp skrandžio vėžio ir genų *IL12B (rs1368439), CCND1 (rs7177) ir IL10 (rs3024498)* VNP nustatyta nebuvo. Tai pirmasis toks atliktas tyrimas skrandžio vėžio atveju. *INSR* geno raiškos pokyčiai buvo nustatyti prostatos, gaubtinės žarnos vėžiu sergančiųjų tarpe [103–105, 20, 111, 112]. Tai rodo, kad *INSR* geno *(rs1051690)* VNP gali būti susijęs navikiniais susirgimais.

Šiame darbe buvo atliktas mikro RNR profiliavimas skrandžio vėžio ir atrofinio gastrito audinyje, naudojant skirtingus metodus, t. y. kiekybinės tikrojo laiko polimerazinės grandininės reakcijos (TL-kPGR) metodą ir mažo tankio *TaqMan* plokštelę (II studija, I dalis) ir mažųjų RNR sekoskaitos metodą (II studija II dalis). II studijos I dalyje mikro RNR profiliavimo metu nustatyta, kad skrandžio vėžio audinyje, palyginti su kontroliniu audiniu, buvo pakitusi 15-os mikro RNR raiška iš 377-ių. Miko RNR raiškos validavimui skrandžio vėžio audinyje buvo pasirinktos 6-ios mikro RNR, iš kurių 4-ių mikro RNR raiška buvo pakitusi ta pačia kryptimi kaip ir profiliavimo metu. Tuo tarpu skrandžio vėžiu sergančiųjų kraujo plazmoje, palyginti su nesergančiųjų, 3-jų mikro RNR raiška buvo pakitusi ta pačia kryptimi kaip ir audinyje, iš 4-ių tirtų.

II studijos II dalyje RNR profiliavimo metu, naudojant mažųjų RNR sekoskaitos metodą, skrandžio vėžio ir atrofinio gastrito audinyje, palyginti su kontroliniu nustatyta 110-ies ir 17-os, atitinkamai, pakitusi mikro RNR raiška, iš 2180-ies, kai raiškos pokytis >2, $p < 0,01$. Tuo tarpu skrandžio vėžio audinyje, palyginti su atrofinio gastrito audiniu, nustatyta 76-ių mikro RNR pakitusi raiška. Mikro RNR profilio validavimui skrandžio vėžio ir atrofinio gastrito grupėse buvo pasirinktos po 21-ą mikro RNR, kurių raiška buvo pakitusi ir skrandžio vėžiu ar atrofiniu gastritu sergančiųjų kraujo plazmoje (kurių nuskaitymo skaičius sekvenuojant buvo > 5). Validavimo metu skrandžio vėžio audinyje, palyginti su kontroliniu audiniu nustatyta aštuonių mikro RNR pakitusi raiška, tuo tarpu atrofinio gastrito audinyje – dviejų. Skrandžio vėžio audinyje, palyginti su atrofiniu gastritu nustatyta penkių mikro RNR pakitusi raiška. Apibendrinant, šiame darbe skrandžio vėžio

audinyje, palyginti su kontroliniu skrandžio audiniu, buvo nustatyta pakitusi 9-ių genų raiška mikro RNR lygyje (hsa-miR-148a-3p, hsa-miR-148a-5p, hsa-miR-204-5p, hsa-miR-375 raiška buvo sumažėjusi, o hsa-miR-142-3p, hsa-miR-215-5p, hsa-miR-223-3p, hsa-miR-224-5p ir hsa-miR-335-3p – padidėjusi). Tuo tarpu atrofinio gastrito audinyje, palyginti su kontroliniu audiniu, buvo pakitusi dviejų mikro RNR (hsa-miR-204-5p ir hsa-miR-215-5p) raiška. Skrandžio vėžio audinyje, palyginti su atrofiniu gastritu nustatyta šių mikro RNR pakitusi raiška: hsa-miR-148a-5p, hsa-miR-204-5p, hsa-miR-223-3p, hsa-miR-224-5p ir hsa-miR-335-3p. Neinvazyvios diagnostikos vertinimui atlikta genų raiškos analizė mikro RNR lygyje pacientų sergančių skrandžio vėžiu kraujo plazmoje, palyginti su nesergančių ir buvo nustatyta trijų mikro RNR pakitusi raiška hsa-miR-148a-3p, hsa-miR-375 ir hsa-miR-223-3p, tačiau jautrumo ir specifiškumo analizės metu nenustatytas jų tinkamumas diagnostikai. Iki šiol yra publikuota daug mikro RNR profiliavimo studijų, kuriose yra nustatyta pakitusi daugiau nei 300-tų genų raiška mikro RNR lygyje skrandžio vėžio atveju (Shrestha et al., 2014).

Shrestha ir jo kolegų apžvalgoje pateikta, kad genų raiška mikro RNR (hsa-miR-148a-3p, hsa-miR-148a-5p, hsa-miR-204-5p, hsa-miR-375, hsa-miR-142-3p, hsa-miR-215-5p, hsa-miR-223-3p, hsa-miR-224-5p ir hsa-miR-335-3p) lygyje buvo pakitusi ta pačia kryptimi, kaip ir šioje studijoje. Tuo tarpu atrofinio gastrito atveju duomenų apie genų raišką mikro RNR lygyje nėra. Šioje studijoje nustatėme, kad sumažėjusi hsa-miR-148a-5p raiška yra susijusi su atrofiniu gastritu ir skrandžio vėžiu. Shui-Long Guo ir kt. nustatė, kad hsa-miR-148a slopinimas sumažino ląstelių proliferaciją skrandžio vėžio ląstelėse, tuo tarpu imitavimas didino [114]. Hsa-miR-204-5p raiškos slopinimas didino skrandžio vėžinių ląstelių proliferaciją *in vitro* [115]. Hsa-miR-215 raiškos lygis koreliavo su naviko diferenciacijos laipsniu, stadija, metastazėmis ir skrandžio vėžio vystumusi [67, 119]. Nustatyta, kad hsa-miR-223 dalyvauja skirtinguose ląstelių procesuose, nuo ląstelės ciklo reguliavimo ir invazyvumo iki kraujodaros ląstelių diferenciacijos ir imuninių ląstelių funkcijos [117]. Hsa-miR-224 slopinimas sumažino vėžinių ląstelių augimą, migraciją, tuo tarpu imitavimas sukelė priešingą efektą. Be to, hsa-miR-224 slopinimas mažino naviko ląstelių augimą *in vivo* [118]. Nustatyta, kad miR-375 turi įtakos skrandžio ląstelių proliferacijai [119].

Svarbu pažymėti, kad šiame tyrime pirmą kartą buvo nustatyta, kad hsa-miR-204-5p ir hsa-miR-215-5p yra susijusios su atrofiniu gastritu (AG). Ankstesniuose tyrimuose buvo nustatyta, kad šios mikro RNR susijusios su vėžiniais procesais, tačiau nėra atlikta tyrimų apie jų raiškos pokyčius uždegiminiuose procesuose. Be to, tai pirmasis tyrimas, kuriame buvo nustatyta pakitusi hsa-miR-148a-5p, hsa-miR-204-5p, hsa-miR-224-5p ir hsa-miR-335-3p genų raiška atrofinio gastrito audinyje, palyginti su skrandžio vėžio

audiniu, todėl jie gali būti panaudoti kaip ankstyvos diagnostikos žymenys. Nustatyta, kad hsa-miR-223-3p genų raiška buvo pakitusi AG ir skrandžio vėžio atveju [123]. Skrandžio vėžio tyrimuose didelis dėmesys skiriams neinvazinei ligos diagnostikai. Wang atlikęs meta-analizę nustatė, skrandžio vėžio atveju kraujyje cirkuliuojančių mikro RNR jautrumas siekė 0,75, o specifiškumas – 0,81. Be to, plazmoje cikuliuojančių mikro RNR raiškos tyrimai buvo tikslesni, nei serume [76]. Kitų mokslininkų atliktuose kraujyje cirkuliuojančių mikro RNR raiškos tyrimuose, skrandžio vėžio atveju, rezultatai yra prieštaringi [66, 90, 124]. Mes šiame tyrime nustatėme, kad skrandžio vėžiu sergančiųjų plazmoje, palyginti su nesergančių, trijų mikro RNR genų raiška (hsa-miR-148a-3p, hsa-miR-375 ir hsa-miR-223-3p) buvo pakitusi, be to, skrandžio vėžio audinyje, palyginti su atrofiniu gastritu mikro RNR hsa-miR-148a-5p ir hsa-miR-223-3p genų raiška taip pat buvo pakitusi. Atlikus šių mikro RNR jautrumo ir specifiškumo analizę paaiškėjo, kad neivazinei ligos diagnostikai jos nėra tinkamos. Tikslinga atlikti mažųjų RNR sekoskaitos analizę, siekiant nustatyti plazmoje cirkuliuojančių mikro RNR tinkamumą ankstyvai ir neivazinei ligų diagnostikai, nes tai yra tikslesnis tyrimo metodas, nei kiekybinės TL-PGR metodas, naudojant vienos mikro RNR pradmenis kiekvienai mikro RNR atskirai.

III šios disertacijos studijoje atlikta mikro RNR genų taikinių eksperimentinė analizė, naudojant mikro RNR inhibitorius ir imitatorius skrandžio vėžio ląstelėse AGS ir MKN-28. Hsa-miR-20b-5p slopinimas didino galimų genų taikinių *IRF1, PTEN* ir *TXNIP* raišką, o hsa-miR-451a-5p imitavimas sumažino galimo geno taikinio *CAV1* raišką skrandžio vėžio ląstelėse AGS ir MKN-28. Hsa-miR-20b priklauso onkogeninių mikro RNR šeimai hsa-miR-17 [122]. Atlikę tyrimą nustatėme, kad skrandžio vėžio ląstelėse AGS ir MKN-28, palyginti su kontroliniu audiniu, hsa-miR-20b-5p geno raiška buvo padidėjusi, be to, nustatėme, kad jos galimų genų taikinių *IRF1, PTEN* ir *TXNIP* raiška padidėjo po transfekcijos su šios mikro RNR inhibitoriumi praėjus 24 val. Tuo tarpu po transfekcijos praėjus 48 val., hsa-miR-20b-5p galimo geno taikinio *TXNIP* raiška padidėjo abiejose ląstelių linijose, o geno taikinio *IRF1* raiška padidėjo tik ląstelėse AGS. Ankstesniuose tyrimuose nustatyta, kad hsa-miR-20b geno raiškos pokyčiai buvo susiję su kepenų [123] ir stemplės vėžiu [124]. Be to, nustatyti kiti šios mikro RNR genai taikiniai: *MMP-2* [125], *HIF1A* [126], *NFAT5* ir *CAMTA1* [127], bei *PTEN* [128, 132–134].

Nustatyta, kad *TXNIP* geno raiška buvo sumažėjusi storosios žarnos ir skrandžio vėžio audinyje, palyginti su šalia esančiu nepažeistu audiniu [131]. Kitose studijose nustatyta, kad *TXNIP* geno raiška galimai reguliuojama skirtingų mikro RNR (hsa-miR-33a [133], hsa-miR-20a [134], hsa-miR-17 [135] ir kt.).

Genas *IRF1* priklauso interferono transkripcijos reguliatorių šeimai (angl. *interferon regulatory transcription factor family*), nustatyta, skrandžio vėžio ląstelėse reguliuoja apoptozę ir slopina vėžinius procesus [136]. Kitoje studijoje nustatyta, kad sėklidės vėžiniame audinyje genas *IRF1* yra galimas hsa-miR-383 taikinys [137]. Mes pirmieji nustatėme hsa-miR-20b geno raiškos pokyčius skrandžio vėžio ląstelių linijose ir atradome šios mikro RNR galimus genus taikinius *TXNIP* ir *PTEN*.

Nustatėme, kad hsa-miR-451a-5p geno raiška skrandžio vėžio ląstelėse AGS ir MKN-28 buvo sumažėjusi, kaip ir Riquelme ir kolegų atliktame tyrime. Be to, atlikę transfekciją hsa-miR-451a imitatoriumi nustatė, kad šios mikro RNR genas taikinys yra *TSC1* [138].

Mes atlikę transfekciją hsa-miR-451a imitatoriumi nustatėme, kad galimo šios mikro RNR geno taikinio *CAV1* raiška skrandžio vėžinėse ląstelėse AGS ir MKN-28 sumažėjo. Nustatyta, kad Kaveolino-1 baltymų šeima yra susijusi su tumorogeneze, o pakitusi *CAV1* geno raiška nustatyta daugelio navikinių ligų atvejais: padidėjusi prostatos vėžinėse ląstelėse [140] ir kasos vėžio audinyje [141] ir sumažėjusi storosios žarnos [142], plaučių vėžio [143] ir kiaušidžių vėžio atveju [139].

Mes pirmieji nustatėme, kad hsa-miR-20b galimai reguliuoja *CAV1* geno raišką.

Tačiau reikėtų paminėti, kad potencialūs mikro RNR genai taikiniai turėtų būti patvirtinti naudojant *Western bloto* (imunobloto) metodą ir liuciferazės tyrimą.

Išvados

1. Geno *INSR* (rs1051690) vieno nukleotido polimorfizmas buvo susijęs su skrandžio vėžiu. Tuo tarpu *IL12B* (rs1368439), *CCND1* (rs7177) ir *IL10* (rs3024498) vieno nukleotido polimorfizmai su liga nebuvo susieti.

2.1. Mikro RNR profiliavimo metu skrandžio vėžio audinyje, palyginti su kontroline grupe, nustatytos devynios pakitusios raiškos mikro RNR: hsa-miR-148a-3p, hsa-miR-148a-5p, hsa-miR-204-5p ir hsa-miR-375 raiška buvo sumažėjusi, o hsa-miR-142-3p, hsa-miR-215-5p, hsa-miR-223-3p, hsa-miR-224-5p ir hsa-miR-335-3p – padidėjusi.

2.2. Mikro RNR profiliavimo metu atrofinio gastrito audinyje, palyginti su kontroline grupe, nustatyta sumažėjusi hsa-miR-204-5p ir padidėjusi hsa-miR-215-5p raiška.

2.3. Skrandžio vėžio audinyje palyginti su atrofiniu gastritu, nustatyta padidėjusi hsa-miR-223-3p, hsa-miR-224-5p ir hsa-miR-335-3p raiška, o hsa-miR-148a-5p, hsa-miR-204-5p – sumažėjusi.

3. Skrandžio vėžiu sergančiųjų plazmoje, palyginti su kontroline grupe, nustatyta sumažėjusi hsa-miR-148a-3p, hsa-miR-375 raiška ir padidėjusi hsa-miR-223-3p raiška. Mikro RNR raiškos jautrumas ir specifiškumas buvo nepakankamas neinvazinei ligos diagnostikai.

4. Hsa-miR-20b-5p slopinimas didino galimų genų taikinių *IRF1, TXNIP* ir *PTEN* raišką, o hsa-miR-451a-5p imitavimas sumažino galimo geno taikinio *CAV1* raišką skrandžio vėžio ląstelėse AGS ir MKN-28.

SUPPLEMENTS

Table 1. *List of deregulated miRNAs determined by sRNA-seq in gastric cancer (n = 20) compared to normal gastric (n = 26) tissues (FDR adjusted p-value < 0.01 and fold change > 2).*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
1	hsa-miR-642a-3p	8.89	down	5.87	2.69×10^{-13}
2	hsa-miR-1224-5p	7.86	down	4.86	1.47×10^{-8}
3	hsa-miR-876-3p	1.02	down	4.86	0.000598
4	hsa-miR-642a-5p	12.14	down	4.56	2.69×10^{-13}
5	hsa-miR-375	45,503.86	down	3.99	3.65×10^{-7}
6	hsa-miR-873-5p	70.94	down	3.86	1.38×10^{-5}
7	hsa-miR-873-3p	10.58	down	3.85	5.29×10^{-5}
8	hsa-miR-4521	6.53	down	3.82	2.42×10^{-6}
9	hsa-miR-148a-5p	1,728.98	down	3.82	3.49×10^{-11}
10	hsa-miR-3065-3p	21.31	down	3.65	6.82×10^{-11}
11	hsa-miR-129-1-3p	7.36	down	3.46	4.14×10^{-5}
12	hsa-miR-1179	4.41	down	3.46	5.78×10^{-5}
13	hsa-miR-1468-5p	81.91	down	3.44	1.54×10^{-12}
14	hsa-miR-1266-5p	10.34	down	3.42	3.95×10^{-11}
15	hsa-miR-625-3p	127.93	down	3.38	7.68×10^{-14}
16	hsa-miR-148a-3p	153,165.30	down	3.22	2.4×10^{-8}
17	hsa-miR-670-3p	6.21	down	3.15	0.000581
18	hsa-miR-3065-5p	27.98	down	3.13	8.61×10^{-7}
19	hsa-miR-204-5p	662.33	down	3.12	0.000279
20	hsa-miR-378b	8.18	down	2.97	1.3×10^{-7}
21	hsa-miR-1251-5p	2.19	down	2.73	0.008831
22	hsa-miR-1295a	2.13	down	2.56	0.006836
23	hsa-miR-203b-3p	29.80	down	2.55	1.48×10^{-5}
24	hsa-miR-4446-3p	2.57	down	2.44	0.006733
25	hsa-miR-135a-5p	126.04	down	2.39	0.002762
26	hsa-miR-2114-5p	3.26	down	2.37	0.008643
27	hsa-miR-130b-5p	85.86	down	2.36	2.31×10^{-8}
28	hsa-miR-378c	1,423.24	down	2.26	9.62×10^{-9}
29	hsa-miR-429	6,414.37	down	2.24	0.000424
30	hsa-miR-153-5p	4.50	down	2.22	0.005605

Table 1. *Continued*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
31	hsa-miR-561-5p	169.72	down	2.17	2.3×10^{-6}
32	hsa-miR-551b-3p	35.32	down	2.12	0.000297
33	hsa-miR-130b-3p	553.26	down	2.09	4.18×10^{-7}
34	hsa-miR-378d	582.11	down	2.06	1.48×10^{-5}
35	hsa-miR-203a	4,186.77	down	2.06	0.003719
36	hsa-miR-629-3p	4.04	down	2.03	0.008496
37	hsa-miR-200c-3p	7,574.98	down	2.02	0.005336
38	hsa-miR-3934-5p	10.20	down	2.02	0.000291
39	hsa-miR-10b-3p	10.30	up	2.01	0.000117
40	hsa-miR-224-3p	5.75	up	2.05	0.001061
41	hsa-miR-152-5p	12.24	up	2.10	0.000502
42	hsa-miR-708-3p	44.62	up	2.11	0.002613
43	hsa-miR-335-5p	366.65	up	2.12	5.05×10^{-5}
44	hsa-miR-199b-5p	2,064.29	up	2.13	4.69×10^{-8}
45	hsa-miR-1185-1-3p	6.50	up	2.16	0.000841
46	hsa-miR-214-3p	251.87	up	2.19	1.59×10^{-6}
47	hsa-miR-181a-3p	247.43	up	2.20	1.48×10^{-9}
48	hsa-miR-218-5p	278.61	up	2.24	0.000195
49	hsa-miR-143-5p	257.75	up	2.24	0.003789
50	hsa-miR-10a-5p	135,330.60	up	2.24	1.28×10^{-7}
51	hsa-miR-146b-5p	8,861.17	up	2.27	8.28×10^{-5}
52	hsa-miR-584-5p	38.72	up	2.35	1.44×10^{-5}
53	hsa-miR-92a-1-5p	11.26	up	2.37	1.66×10^{-5}
54	hsa-miR-487a-3p	7.24	up	2.45	1.35×10^{-6}
55	hsa-miR-181b-3p	2.93	up	2.48	0.000977
56	hsa-miR-199a-3p	21,650.41	up	2.51	2.05×10^{-11}
57	hsa-miR-199b-3p	10,795.71	up	2.51	2.05×10^{-11}
58	hsa-miR-380-3p	2.26	up	2.53	0.000343
59	hsa-miR-499a-5p	13.83	up	2.63	5.19×10^{-7}
60	hsa-miR-34c-5p	48.55	up	2.66	6.17×10^{-6}
61	hsa-miR-199a-5p	3,848.21	up	2.68	2.85×10^{-13}
62	hsa-miR-618	3.32	up	2.71	0.004369
63	hsa-miR-29b-1-5p	6.84	up	2.75	9.11×10^{-6}
64	hsa-miR-145-3p	2,313.02	up	2.79	3.72×10^{-5}
65	hsa-miR-214-5p	111.97	up	2.80	1.09×10^{-12}

Table 1. *Continued*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
66	hsa-miR-143-3p	567,160.44	up	2.81	8.26×10^{-5}
67	hsa-miR-5683	4.12	up	2.86	0.006006
68	hsa-miR-10a-3p	84.49	up	2.91	2.42×10^{-11}
69	hsa-miR-483-3p	2.17	up	3.16	0.001784
70	hsa-miR-431-5p	9.29	up	3.30	1.26×10^{-6}
71	hsa-miR-187-3p	5.99	up	3.32	0.005494
72	hsa-miR-452-5p	77.91	up	3.36	2.69×10^{-13}
73	hsa-miR-615-3p	8.14	up	3.36	3.38×10^{-6}
74	hsa-miR-4661-5p	1.39	up	3.39	0.000775
75	hsa-miR-135b-5p	84.04	up	3.40	1.58×10^{-5}
76	hsa-miR-135b-3p	4.02	up	3.59	9.13×10^{-5}
77	hsa-miR-4510	9.75	up	3.60	8.33×10^{-8}
78	hsa-miR-223-5p	15.87	up	3.61	1.16×10^{-6}
79	hsa-miR-125b-5p	7,254.51	up	3.66	6.82×10^{-11}
80	hsa-miR-100-5p	3,199.35	up	3.66	5.32×10^{-10}
81	hsa-miR-222-5p	2.44	up	3.69	3.81×10^{-6}
82	hsa-miR-1	4.18	up	4.00	0.001837
83	hsa-miR-223-3p	1,440.64	up	4.01	1.06×10^{-6}
84	hsa-l*10t-7c-5p	2,611.21	up	4.05	5.23×10^{-11}
85	hsa-miR-371b-5p	1.28	up	4.05	0.00294
86	hsa-miR-218-1-3p	0.37	up	4.11	0.006816
87	hsa-miR-4485	4.08	up	4.34	9.12×10^{-5}
88	hsa-miR-184	2.71	up	4.59	0.001779
89	hsa-miR-216a-3p	0.59	up	4.61	0.001888
90	hsa-miR-125b-2-3p	300.98	up	4.70	2.35×10^{-10}
91	hsa-miR-23a-5p	11.98	up	4.70	1.2×10^{-9}
92	hsa-miR-6510-3p	1.07	up	4.75	0.001731
93	hsa-miR-675-5p	0.44	up	4.85	0.003018
94	hsa-miR-99a-3p	40.22	up	4.95	2.52×10^{-11}
95	hsa-miR-27a-5p	61.97	up	4.95	2.85×10^{-13}
96	hsa-miR-335-3p	391.85	up	4.96	5.23×10^{-11}
97	hsa-miR-216a-5p	3.56	up	5.01	0.000352
98	hsa-miR-99a-5p	874.77	up	5.30	1.04×10^{-12}
99	hsa-miR-100-3p	19.24	up	5.60	2.69×10^{-13}

Table 1. *Continued*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
100	hsa-miR-1305	0.60	up	5.91	0.000502
101	hsa-miR-125b-1-3p	83.99	up	6.06	2.85×10^{-13}
102	hsa-l*10t-7c-3p	2.95	up	6.64	1.19×10^{-8}
103	hsa-miR-224-5p	98.74	up	7.35	6.93×10^{-15}
104	hsa-miR-196b-3p	1.28	up	7.48	5.78×10^{-5}
105	hsa-miR-549a	0.88	up	10.09	2.42×10^{-6}
106	hsa-miR-196b-5p	76.47	up	11.98	2.18×10^{-10}
107	hsa-miR-196a-5p	128.15	up	16.16	1.09×10^{-12}
108	hsa-miR-552-5p	9.45	up	18.76	1.05×10^{-8}
109	hsa-miR-196a-3p	3.01	up	21.28	1.48×10^{-9}
110	hsa-miR-552-3p	9.88	up	31.66	4.64×10^{-12}

Table 2. *List of deregulated miRNAs determined by sRNA-seq in adjusted gastric cancer (n = 20) compared to normal gastric (n = 26) tissues (FDR adjusted p-value < 0.01 and fold change >2).*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
1	hsa-miR-378g	130.99	down	5.88	0.00000000
2	hsa-miR-4521	6.53	down	4.38	0.00002254
3	hsa-miR-1224-5p	7.86	down	2.91	0.00141988
4	hsa-miR-378i	36.22	down	2.56	0.00423695
5	hsa-miR-6129	6.91	down	2.48	0.00149283
6	hsa-miR-455-3p	121.31	down	2.22	0.00004504
7	hsa-miR-203b-3p	29.80	down	2.11	0.00324285
8	hsa-miR-27a-5p	61.97	up	2.05	0.00879307
9	hsa-miR-422a	15.34	up	2.18	0.00164862
10	hsa-miR-99a-5p	874.77	up	2.18	0.00679974
11	hsa-miR-145-5p	5,478.47	up	2.34	0.00324285
12	hsa-miR-125b-1-3p	83.99	up	2.50	0.00218292
13	hsa-miR-504-5p	8.97	up	2.69	0.00071668
14	hsa-miR-7704	8.62	up	2.70	0.00218292
15	hsa-miR-143-3p	567,160.44	up	2.75	0.00058731
16	hsa-miR-145-3p	2,313.02	up	2.92	0.00007685
17	hsa-miR-4508	3.27	up	3.01	0.00319098

Table 2. *Continued*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
18	hsa-miR-143-5p	257.75	up	3.05	0.00012332
19	hsa-miR-3196	4.09	up	3.19	0.00929309
20	hsa-miR-4485	4.08	up	3.23	0.00879307
21	hsa-miR-6087	4.00	up	3.24	0.00324285
22	hsa-miR-4492	30.48	up	3.25	0.00105693
23	hsa-let-7c-3p	2.95	up	3.37	0.00352797
24	hsa-miR-187-3p	5.99	up	3.95	0.00351760
25	hsa-miR-7641	158.67	up	4.43	0.00324285
26	hsa-miR-619-5p	6.91	up	6.79	0.00000000
27	hsa-miR-552-3p	9.88	up	7.32	0.00068929
28	hsa-miR-5096	9.03	up	7.39	0.00000000
29	hsa-miR-196a-5p	128.15	up	7.47	0.00000414
30	hsa-miR-3648	3.34	up	8.26	0.00003625
31	hsa-miR-196b-5p	76.47	up	8.58	0.00000051
32	hsa-miR-552-5p	9.45	up	9.22	0.00011093

Table 3. *List of deregulated miRNAs determined by sRNA-seq in atrophic gastritis (n = 18) compared to normal gastric (n = 26) tissues (FDR adjusted p-value < 0.01 and fold change > 2).*

	MiRNA ID	Base Mean	Direction of expression	Fold Change	p value
1	hsa-miR-7641	158.67	down	9.80	1.99×10^{-6}
2	hsa-miR-3196	4.09	down	5.40	6.97×10^{-4}
3	hsa-miR-3687	4.83	down	3.92	2.25×10^{-3}
4	hsa-miR-1251-5p	2.19	down	3.54	3.54×10^{-3}
5	hsa-miR-6087	4.00	down	3.42	8.81×10^{-3}
6	hsa-miR-378i	36.22	down	3.02	8.35×10^{-4}
7	hsa-miR-204-5p	662.33	down	2.90	3.79×10^{-3}
8	hsa-miR-2114-3p	3.30	down	2.70	4.37×10^{-3}
9	hsa-miR-1468-5p	81.91	down	2.64	1.07×10^{-6}
10	hsa-miR-3065-5p	27.98	down	2.33	2.05×10^{-3}
11	hsa-miR-3065-3p	21.31	down	2.13	1.23×10^{-3}
12	hsa-miR-378g	130.99	up	2.16	6.97×10^{-4}
13	hsa-miR-142-3p	1,595.41	up	2.17	1.13×10^{-3}

Table 3. *Continued*

	MiRNA ID	Base Mean	Direction of expression	Fold Change	p value
14	hsa-miR-215-5p	36,009.43	up	4.29	8.35×10^{-4}
15	hsa-miR-194-5p	6,574.73	up	4.64	1.39×10^{-4}
16	hsa-miR-196a-5p	128.15	up	5.11	6.97×10^{-4}
17	hsa-miR-215-3p	92.37	up	5.86	1.23×10^{-3}

Table 4. *List of deregulated miRNAs determined by sRNA-seq in gastric cancer (n = 20) compared to atrophic gastritis (n = 18) tissues (FDR adjusted p-value < 0.01 and fold change > 2.*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
1	hsa-miR-802	25.63	down	5.52	0.001945
2	hsa-miR-4521	6.53	down	4.97	5.87×10^{-8}
3	hsa-miR-378g	130.99	down	4.27	1.22×10^{-12}
4	hsa-miR-642a-3p	8.89	down	3.77	3.14×10^{-7}
5	hsa-miR-5189-3p	0.66	down	3.41	0.007108
6	hsa-miR-642a-5p	12.14	down	3.28	8.97×10^{-8}
7	hsa-miR-138-1-3p	1.61	down	3.12	0.002086
8	hsa-miR-1224-5p	7.86	down	3.11	0.000357
9	hsa-miR-4724-5p	0.81	down	3.07	0.007514
10	hsa-miR-5571-3p	13.15	down	3.00	0.000876
11	hsa-miR-1266-5p	10.34	down	2.94	5.87×10^{-8}
12	hsa-miR-194-5p	6574.73	down	2.91	0.007439
13	hsa-miR-625-3p	127.93	down	2.69	2.53×10^{-8}
14	hsa-miR-551b-3p	35.32	down	2.65	6.51×10^{-6}
15	hsa-miR-670-3p	6.21	down	2.61	0.009596
16	hsa-miR-4446-3p	2.57	down	2.60	0.004939
17	hsa-miR-1179	4.41	down	2.57	0.006589
18	hsa-miR-9-3p	15.94	down	2.42	0.001964
19	hsa-miR-148a-5p	1728.98	down	2.36	0.000267
20	hsa-miR-138-5p	108.94	down	2.28	0.000589
21	hsa-miR-3614-5p	14.10	down	2.09	0.006931
22	hsa-miR-378b	8.18	down	2.02	0.002792
23	hsa-miR-708-3p	44.62	up	2.07	0.008108
24	hsa-miR-199b-5p	2064.29	up	2.08	1.11×10^{-6}
25	hsa-miR-511-5p	9.66	up	2.19	0.000666

Table 4. *Continued*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
26	hsa-miR-34c-5p	48.55	up	2.19	0.001426
27	hsa-miR-10a-3p	84.49	up	2.20	8.21×10^{-6}
28	hsa-miR-4510	9.75	up	2.21	0.002792
29	hsa-miR-335-5p	366.65	up	2.24	6.12×10^{-5}
30	hsa-miR-1185-1-3p	6.50	up	2.26	0.000771
31	hsa-miR-760	2.06	up	2.26	0.006748
32	hsa-miR-199b-3p	10795.71	up	2.32	2.67×10^{-8}
33	hsa-miR-199a-3p	21650.41	up	2.32	2.67×10^{-8}
34	hsa-miR-145-3p	2313.02	up	2.33	0.002792
35	hsa-miR-4697-3p	1.96	up	2.42	0.005499
36	hsa-miR-378i	36.22	up	2.51	0.004402
37	hsa-miR-452-5p	77.91	up	2.56	1.34×10^{-7}
38	hsa-miR-214-3p	251.87	up	2.57	6.2×10^{-8}
39	hsa-miR-199a-5p	3848.21	up	2.60	1.42×10^{-10}
40	hsa-let-7c-5p	2611.21	up	2.63	7.05×10^{-5}
41	hsa-miR-214-5p	111.97	up	2.63	9.59×10^{-10}
42	hsa-miR-487a-3p	7.24	up	2.65	2.17×10^{-7}
43	hsa-miR-584-5p	38.72	up	2.72	1.39×10^{-6}
44	hsa-miR-29b-1-5p	6.84	up	2.82	1.07×10^{-5}
45	hsa-miR-6500-3p	1.41	up	3.11	0.001348
46	hsa-miR-100-5p	3199.35	up	3.16	5.53×10^{-7}
47	hsa-miR-125b-5p	7254.51	up	3.23	8.97×10^{-8}
48	hsa-miR-483-3p	2.17	up	3.32	0.001834
49	hsa-miR-615-3p	8.14	up	3.39	9.21×10^{-6}
50	hsa-miR-365b-5p	1.81	up	3.40	0.001475
51	hsa-miR-100-3p	19.24	up	3.42	9.42×10^{-7}
52	hsa-miR-3687	4.83	up	3.53	0.003001
53	hsa-miR-196b-5p	76.47	up	3.64	0.005396
54	hsa-miR-23a-5p	11.98	up	3.71	1.39×10^{-6}
55	hsa-miR-431-5p	9.29	up	3.78	2.66×10^{-7}
56	hsa-miR-335-3p	391.85	up	4.01	2.58×10^{-7}
57	hsa-miR-125b-2-3p	300.98	up	4.04	2.14×10^{-7}
58	hsa-miR-196b-3p	1.28	up	4.07	0.009293
59	hsa-miR-99a-3p	40.22	up	4.09	7.45×10^{-8}
60	hsa-miR-184	2.71	up	4.27	0.005499

Table 4. *Continued*

	miRNA ID	Base Mean	Direction of expression	Fold Change	p value
61	hsa-miR-224-5p	98.74	up	4.41	7.45×10^{-8}
62	hsa-miR-1	4.18	up	4.47	0.001668
63	hsa-miR-7641	158.67	up	4.60	0.002086
64	hsa-miR-27a-5p	61.97	up	4.66	1.42×10^{-10}
65	hsa-miR-99a-5p	874.77	up	4.85	9.59×10^{-10}
66	hsa-miR-675-3p	1.65	up	5.06	0.001342
67	hsa-miR-6510-3p	1.07	up	5.09	0.001765
68	hsa-miR-125b-1-3p	83.99	up	5.12	1.69×10^{-9}
69	hsa-miR-549a	0.88	up	5.33	0.001131
70	hsa-let-7c-3p	2.95	up	5.54	3.5×10^{-7}
71	hsa-miR-216a-5p	3.56	up	5.76	0.000267
72	hsa-miR-663b	0.73	up	6.02	0.000679
73	hsa-miR-4485	4.08	up	9.74	1.9×10^{-8}
74	hsa-miR-552-5p	9.45	up	9.81	3.02×10^{-5}
75	hsa-miR-552-3p	9.88	up	11.31	4.96×10^{-6}
76	hsa-miR-196a-3p	3.01	up	16.81	7.93×10^{-8}

Table 5. *List of deregulated miRNAs determined by sRNA-seq in plasma if patients with gastric cancer (n = 2) compared to health patients (n = 2) (sum of read count > 5).*

	miRNA ID	Mean Counts
1	hsa-miR-486-5p	41,309.3
2	hsa-miR-92a-3p	9,419.3
3	hsa-miR-16-5p	3,339.3
4	hsa-miR-22-3p	2,240
5	hsa-miR-26a-5p	2,179.8
6	hsa-let-7a-5p	1,652.2
7	hsa-miR-451a	1,363.8
8	hsa-let-7f-5p	1,304.2
9	hsa-miR-181a-5p	1,261
10	hsa-miR-103a-3p	1,104.7
11	hsa-miR-423-5p	1,038.3
12	hsa-miR-30d-5p	762.8
13	hsa-miR-21-5p	651.8
14	hsa-miR-10b-5p	548.2

	miRNA ID	Mean Counts
15	hsa-miR-191-5p	526.5
16	hsa-miR-101-3p	481.7
17	hsa-miR-107	406.3
18	hsa-miR-30e-5p	388.2
19	hsa-miR-142-5p	371
20	hsa-miR-15a-5p	362.3
21	hsa-let-7i-5p	362
22	hsa-miR-126-5p	359.3
23	hsa-miR-25-3p	334
24	hsa-miR-143-3p	323.7
25	hsa-miR-10a-5p	284.2
26	hsa-miR-27b-3p	282.8
27	hsa-miR-423-3p	272.8
28	hsa-miR-199a-3p	261.2

Table 5. *Continued*

	miRNA ID	Mean Counts
29	hsa-miR-27a-3p	240.8
30	hsa-miR-30c-5p	223.3
31	hsa-miR-151a-3p	197.2
32	hsa-miR-192-5p	175.8
33	hsa-miR-148a-3p	162
34	hsa-miR-320a	154.7
35	hsa-miR-28-3p	150.5
36	hsa-miR-93-5p	133
37	hsa-miR-199b-3p	130.3
38	hsa-let-7d-5p	129.8
39	hsa-miR-186-5p	129.5
40	hsa-let-7b-5p	123
41	hsa-miR-26b-5p	115.7
42	hsa-let-7g-5p	110
43	hsa-miR-126-3p	108.5
44	hsa-miR-151a-5p	106.5
45	hsa-miR-744-5p	95.2
46	hsa-miR-140-3p	92.3
47	hsa-miR-425-5p	87.8
48	hsa-miR-146a-5p	85.7
49	hsa-miR-19b-3p	85
50	hsa-miR-150-5p	84.7
51	hsa-miR-222-3p	81.7
52	hsa-miR-30a-5p	78.3
53	hsa-miR-486-3p	73.2
54	hsa-miR-378a-3p	70.7
55	hsa-miR-484	58
56	hsa-miR-221-3p	56.3
57	hsa-miR-223-3p	51.5
58	hsa-miR-584-5p	50.5
59	hsa-miR-151b	50.3
60	hsa-miR-182-5p	50
61	hsa-miR-16-2-3p	49.2
62	hsa-miR-122-5p	48.7
63	hsa-miR-144-3p	48.5

	miRNA ID	Mean Counts
64	hsa-miR-30b-5p	47.7
65	hsa-miR-130a-3p	47.3
66	hsa-miR-320b	47.2
67	hsa-miR-92b-3p	46
68	hsa-miR-106b-3p	45
69	hsa-miR-181b-5p	44.5
70	hsa-miR-125a-5p	43.7
71	hsa-miR-98-5p	41.2
72	hsa-miR-148b-3p	40.3
73	hsa-miR-375	39.7
74	hsa-miR-340-5p	38.5
75	hsa-miR-144-5p	32
76	hsa-let-7d-3p	31.8
77	hsa-miR-128-3p	30.8
78	hsa-miR-1307-5p	30.5
79	hsa-miR-3615	29.7
80	hsa-let-7e-5p	27.3
81	hsa-miR-146b-5p	27.2
82	hsa-miR-941	25.3
83	hsa-miR-29a-3p	24.3
84	hsa-miR-21-3p	23.5
85	hsa-miR-363-3p	23
86	hsa-miR-409-3p	22.8
87	hsa-miR-532-5p	21.3
88	hsa-miR-23a-3p	21
89	hsa-miR-17-5p	20
90	hsa-miR-24-3p	19.3
91	hsa-miR-1307-3p	19.3
92	hsa-miR-142-3p	18.8
93	hsa-miR-106b-5p	18
94	hsa-miR-127-3p	17.5
95	hsa-miR-133a-3p	17.3
96	hsa-miR-181c-5p	15.8
97	hsa-miR-99b-5p	15.5
98	hsa-miR-29c-3p	14.7

Table 5. *Continued*

	miRNA ID	Mean Counts
99	hsa-miR-328-3p	14.3
100	hsa-miR-30e-3p	13.7
101	hsa-miR-660-5p	12.5
102	hsa-miR-769-5p	12.2
103	hsa-miR-199a-5p	12
104	hsa-miR-130b-3p	12
105	hsa-miR-339-5p	12
106	hsa-miR-215-5p	11.8
107	hsa-miR-20a-5p	11.2
108	hsa-miR-181a-2-3p	11.2
109	hsa-miR-301a-3p	11.2
110	hsa-miR-335-5p	11.2
111	hsa-miR-421	10.5
112	hsa-miR-15b-5p	10.3
113	hsa-miR-345-5p	10.3
114	hsa-let-7c-5p	10.2
115	hsa-miR-4732-3p	10.2
116	hsa-miR-19a-3p	10
117	hsa-miR-339-3p	10

	miRNA ID	Mean Counts
118	hsa-miR-877-5p	9.7
119	hsa-miR-28-5p	9.5
120	hsa-miR-342-3p	9.5
121	hsa-miR-381-3p	8.7
122	hsa-miR-654-3p	8.5
123	hsa-miR-6852-5p	8.2
124	hsa-miR-210-3p	7.8
125	hsa-miR-361-3p	7.7
126	hsa-miR-4433b-5p	7.7
127	hsa-miR-155-5p	6.5
128	hsa-miR-1285-3p	6.3
129	hsa-miR-4446-3p	6.3
130	hsa-miR-671-3p	6.2
131	hsa-miR-550a-3p	6
132	hsa-miR-361-5p	5.5
133	hsa-miR-320c	5.5
134	hsa-miR-197-3p	5.3
135	hsa-miR-125b-5p	5.3
137	has-miR-410-3p	5.3

NUORAŠAS

KAUNO REGIONINIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS

KMUK Eivenių 2, Centrinis korpusas 71 kab., 50009 Kaunas, tel. +370 37 326168; faks. +370 37 326901, e-mail: **cmeinfo@kmu.lt**

LEIDIMAS ATLIKTI BIOMEDICININĮ TYRIMĄ

2011-03-08 Nr. BE-2-10

Biomedicininio tyrimo pavadinimas: **„Virškinimo sistemos ligų tiriamosios medžiagos biobankas".**	
Protokolo Nr.:	**1**
Data:	**2010-12-27**
Versija:	**1**
Pagrindinis tyrėjas:	**Prof. habil. dr. Limas Kupčinskas** **Prof. habil. dr. Juozas Pundzius**
Biomedicininio tyrimo vieta: Įstaigos pavadinimas: Adresas:	**LSMU MA Gastroenterologijos klinika** **LSMU MA Chirurgijos klinika** **Eivenių g. 2, LT-50009 Kaunas**

Išvada:
Kauno regioninio biomedicininių tyrimų etikos komiteto posėdžio, įvykusio **2011 m. sausio 4 d.** (protokolo Nr. 8/2011) sprendimu pritarta biomedicininio tyrimo vykdymui.

Mokslinio eksperimento vykdytojai įsipareigoja: (1) nedelsiant informuoti Kauno Regioninį biomedicininių Tyrimų Etikos komitetą apie visus nenumatytus atvejus, susijusius su studijos vykdymu, (2) iki sausio 15 dienos – pateikti metinį studijos vykdymo apibendrinimą bei, (3) per mėnesį po studijos užbaigimo, pateikti galutinį pranešimą apie eksperimentą.

Kauno regioninio biomedicininių tyrimų etikos komiteto nariai			
Nr.	Vardas, Pavardė	Veiklos sritis	Dalyvavo posėdyje
1.	Doc. Irena Marchertienė	anesteziologija	taip
2.	Doc. Romaldas Mačiulaitis	klinikinė farmakologija	taip
3.	Prof. Nijolė Dalia Bakšienė	pediatrija	taip
4.	Prof. Irayda Jakušovaitė	filosofija	ne
5.	Dr.Eimantas Peičius	filosofija	taip
6.	Laima Vasiliauskaitė	psichoterapija	taip
7.	Gintaras Česnauskas	chirurgija	ne
8.	Zelmanas Šapiro	terapija	ne
9.	Jurgita Laurinaitytė	bioteisė	ne

Kauno regioninis biomedicininių tyrimų etikos komitetas dirba vadovaudamasis etikos principais nustatytais biomedicininių tyrimų Etikos įstatyme, Helsinkio deklaracijoje, vaistų tyrinėjimo Geros klinikinės praktikos taisyklėmis.

Pirmininkė

LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS * KAUNO REGIONINIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS

Irena Marchertienė

Translation from Lithuanian

KAUNAS REGIONAL BIOMEDICAL RESEARCH ETHICS COMMITTEE
LUHS Eivenių str. 2, central body 71 cab., 5009 Kaunas, Tel. +370 37 326168; Fax +370 37 326901, e-mail: cmeinfo@kmu.lt

AUTHORIZATION FOR BIOMEDICAL RESEARCH

08/03/2011 No. BE-2-10

Biomedical research name: **"Research Material Biobank of Digestive System Diseases"**	
Protocol No.:	1
Date:	27/12/2010
Version:	1
Principal Investigator:	**prof. habil. dr. Limas Kupčinskas** **prof. habil. dr. Juozas Pundzius**
Biomedical Research Location: Institution Name: Address:	**LUHS MA Gastroenterology Clinic** **LUHS MA Surgery Clinic** **LUHS Eivenių str. 2, LT-50009 Kaunas**

Conclusion:
Under the decision of the meeting of Kaunas Regional Biomedical Research Ethics Committee, held on the **4th January 2011** (Protocol No. 8/2011), biomedical research execution was supported.

Scientific experiment promoters undertake: (1) to inform immediately Kaunas Regional Biomedical Research Ethics Committee of all unforeseen cases relating to the implementation of the study, (2) until the 15th January to submit an annual summary of the implementation of the study, (3) and one month after the completion of the study to submit a final report on the experiment.

Members of Kaunas Regional Biomedical Research Ethics Committee			
No.	Name	Activity Area	Participated in the meeting
1.	Assoc.Prof. Irena Marchertienė	Anesthesiology	yes
2.	Assoc. Prof. Romaldas Mačiulaitis	Clinical Pharmacology	yes
3.	Prof. Nijolė Dalia Bakšienė	Pediatrics	yes
4.	Prof. Irayda Jakušovaitė	Philosophy	no
5.	Dr. Eimantas Peičius	Philosophy	yes
6.	Laima Vasiliauskaitė	Psychotherapy	yes
7.	Gintaras Česnauskas	Surgery	no
8.	Zelmanas Šapiro	Therapy	no
9.	Jurgita Laurinaitytė	Biolaw	no

Kaunas Regional Biomedical Research Ethics Committee works in accordance with the ethical principles laid down in the Law on Ethics of Biomedical Research, in Helsinki Declaration, and drug exploration of Good Clinical Practice (GCP).

Chairwoman */signature/* Irena Marchertienė

Seal: /Lithuanian University of Health Sciences
Kaunas Regional Biomedical Research Ethics Committee/

18 September 2014

I, Rūta Palšauskienė, the Notary of Kaunas city Notary's Office No. 5 hereby confirm that this to a true copy of the original document submitted.

Notary's register No. 5-2-7076

Notary's fee 3,- (08 Eur)

Payment for other services provided on the request of the client_____

Notary's signature, seal

Aš, Renata Plukienė, patvirtinu, kad prisiimu atsakomybę už šio dokumento vertimo iš lietuvių kalbos į anglų kalbą teisingumą.

Renata Plukienė, asmens kodas 47905060416, asmens tapatybės kortelės Nr.11673422-, darbovietės adresas Savanorių pr. 192-811, Kaune.

I, Renata Plukienė, confirm that I take the responsibility for the translation of this document from Lithuanian into English.

Renata Plukienė, personal code 47905060416, Identity Card No. 11673422-, workplace address Savanorių pr. 192-811, Kaunas.

Kaunas, du tūkstančiai keturioliktųjų metų rugsėjo mėnesio 18 diena

Kaunas, the 18 of September, two thousand and fourteenth

Aš, Rūta Palšauskienė, Kauno m. 5-ojo notarų biuro notarė (-as) -,
Renatos Plukienės parašo tikrumą liudiju.

I, Rūta Palšauskienė, the Notary of Kaunas city Notary's Office No. 5 -, hereby witness that the signature of Renata Plukienė is true.

Notarinio registro Nr. 5-2-7077
Notaro atlyginimas: 3,- Lt / 0,87 EUR
Atlyginimas už dokumento parengimą:-
Kompensacijos už patikrą registruose dydis:-
Kompensacijos (-ų) už kitas kliento prašymu notaro atliktas paslaugas dydis:-

Notary's register No. 5-2-7077
Notary's fee: 3,- Lt / 0,87 EUR
Fee for preparing the document: -
Fee for Registry review:-
Payment for other services provided on the request of the client:-

Notarė Rūta Palšauskienė Notary's signature

NOTARĖ RŪTA PALŠAUSKIENĖ KAUNO MIESTO

CURRICULUM VITAE

Name, Surname: Violeta Šaltenienė
Address: Lietuvos sveikatos mokslų universitetas, Virškinimo sistemos tyrimų institutas, Klinikinės ir molekulinės gastroenterologijos laboratorija, Mickevičiaus g. 9, LT-44307 Kaunas
E-mail: violeta.salteniene@lsmuni.lt

Education:

2006–2012	Lithuanian University of Health Sciences, Kaunas, Lithuania. Veterinary medicine
2012–2016	Lithuanian University of Health Sciences, Kaunas, Lithuania Ph.D. in Biomedical Sciences

Work experience:

01/2013–present	Junior Scientist, Institute for Digestive Research, Laboratory of Clinical and Molecular Gastroenterology, Kaunas, Lithuania
03/2014–01/2017	Junior Scientist, Project funded by the Research Council of Lithuania (Grant No: MIP-007/2014) "Functional role of miR-20b, miR-451, miR-29c, miR-125b in pathogenesis of gastric and colorectal cancer"

Languages: Lithuanian (native)
Russian (fluent)
English (intermediate)

ACKNOWLEDGEMENTS

This study would not have been possible without the contribution of many competent and kind persons. First, I would like to thank my scientific supervisor prof. Gediminas Kiudelis for guidance and support.

Thanks to Head of Institute for Digestive Research, Lithuanian University of Health Sciences prof. Limas Kupčinskas for providing me a perspective project, large resources of patient material and constructive remarks.

I also would like to thank prof. Sonata Jarmalaitė from Vilnius University, The Life Sciences Centre for provided opportunity to conduct experiments in her laboratory.

I would like to thank Jurgita Skiecevičienė, head of Laboratory of Clinical and Molecular Gastroenterology of Institute for Digestive Research for support and valuable observations.

A special thanks for all laboratory staff: Juozas Kupčinskas, Simas Juzėnas, Greta Streleckienė, Ugnė Gyvytė, Rūta Steponaitienė, Rima Ramonaitė, Lina Baldauskienė, Raminta Bagdanavičienė, Justina Arštikytė for creating a friendly and enjoyable research environment, full help and support.