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EPIGENETIC AND GENETIC ALTERATIONS OF NON-CODING GENOME STRUCTURES IN GASTRIC CANCER PATHOGENESIS

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NEKODUOJANČIŲ GENOMO STRUKTŪRŲ EPIGENETINIAI IR GENETINIAI POKYČIAI SKRANDŽIO VĖŽIO PATOGENEZĖJE

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CONTENTS

ABBREVIATIONS

3'-UTR	3'-untranslated region
AG	atrophic gastritis
AGO	Argonaute protein
AKT2	serine/threonine kinase-2
Bp	base pair
CagA	cytotoxin-associated gene A
Cdc42	cell division cycle-42
CDH1	cadherin-1
CDK6	cyclin dependent kinase-6
cDNA	complementary DNA
CG	chronic gastritis
CI	confidence interval
COX-2	cyclooxygenase-2
CpG	cytosine-guanine dinucleotide
CRC	colorectal cancer
CTNNA1	catenin alpha-1
CUL4A	cullin-4A
DGCR8	DiGeorge critical region 8 protein
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DOT1L	DOT1 like histone lysine methyltransferase
DSC	digestive system cancer
FBXO24	f-box only protein-24
FFPE	formalin-fixed paraffin-embedded
GC	gastric cancer
GI	gastrointestinal
HRAG	high risk atrophic gastritis
IgG	immunoglobulin G
IM	intestinal metaplasia
INSR	insulin receptor
KLF12	Kruppel like factor-12
LINE-1	long interspersed nuclear element-1
lncRNA	long non-coding RNA
LTR	long terminal repeat
MAP3K6	mitogen-activated protein kinase kinase kinase-6
MIR137HG	miR-137 host gene
miRNA, miR	microRNA
MRE	microRNA response element

mRNA	messenger RNA
MSR1	macrophage scavenger receptor-1
MYO1C	myosin IC
N	normal (control)
N-CRC	normal-colorectal cancer (tumour adjacent tissue)
N-GC	normal-gastric cancer (tumour adjacent tissue)
Onco-miRNA	oncogenic microRNA
OR	odds ratio
ORF	open reading frame
PgI, PgII	pepsinogen I, pepsinogen II
PI3K/AKT	phosphoinositide-3-kinase/ protein kinase B
piRNA	piwi-interacting RNA
Pre-miRNA	precursor microRNA
Pri-miRNA	primary microRNA
PRSS1	serine protease-1
qRT-PCR	quantitative real time-polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNU6b	U6 small-nuclear ribonucleoprotein
SD	standard deviation
SINE	short interspersed nuclear element
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
SNP	single nucleotide polymorphism
T-CRC	tumour-colorectal cancer (tumourous tissue)
T-GC	tumour-gastric cancer (tumourous tissue)
TNM	tumour node metastasis
TRBP	TAR RNA-binding protein
TUTase	terminal uridylyl transferase
UICC	the Union for International Cancer Control
VacA	vacuolating cytotoxin gene A
VNTR	variable number tandem repeat
WHO	World Health Organization

INTRODUCTION

Gastric cancer (GC) is the fifth most prevalent malignancy and the third leading cause of cancer-related death worldwide, with 1,000,000 new cases and 750,000 deaths annually [1]. Prevalence and mortality rates of GC vary widely between different geographical regions, making developing countries the most affected [2]. Lithuania belongs to the group of most affected countries in Europe with relatively high incidence of GC (morbidity rate 29.5 cases/100,000 and mortality rate 24.4 cases /100,000 people of both sexes in 2018) [3]. Gastric carcinogenesis is a multifactorial process which involves multiple interactions between *Helicobacter pylori* infection, environmental factors and host hereditary and genetic factors [4]. The well-known pathophysiological cascade, proposed by Colombian pathologist Pelayo Correa [5] is a pathway from stomach inflammation to intestinal type adenocarcinoma and includes the following stages: gastric mucosa inflammation driven by *H. pylori* infection, chronic gastritis, atrophy of gastric mucosa, intestinal metaplasia, gastric epithelial dysplasia and finally intestinal type stomach adenocarcinoma. On the other hand, diffuse type gastric adenocarcinoma circumvents these preneoplastic lesions and arises in the stomach that lacks atrophy and intestinal metaplasia with the absence of a pathogenic role of inflammation [6]. As the carcinogenesis of GC takes years, it is usually diagnosed at the advanced stages when clinical symptoms occur. However, as opposed to many other malignancies, clinical advances in GC screening and treatment did not entwine revolutionary advances over the past decades [7]. While earlier diagnosis would help to improve the efficacy of treatment and disease prognosis for patients with GC, better understanding of the basic mechanisms of cancer is needed [8].

The breakthrough in molecular biology researches over the recent decades demonstrated that a large proportion of the genetic material is composed of protein non-coding DNA sequences [9]. Substantial portion of DNA sequences are transcribed into protein non-coding RNA molecules (miRNAs, siRNAs, piRNAs, snoRNAs etc.), which harbour different functional role in cellular processes, while the other part is present as repetitive genome DNA elements (LTRs, LINEs, SINEs, (mini/micro) satellites) [10, 11]. New discoveries in molecular sciences uncover the profound roles of these molecules in the occurrence and development of various cancer types, including GC.

MicroRNAs (miRNAs) are class of abundant, highly conserved, small, approximately 22 nucleotides in length non-coding RNAs. MiRNAs post-transcriptionally silence target genes through perfect or imperfect pairing

with target mRNA, which results in mRNA degradation or translation repression [12]. Since the discovery of miRNAs in 1993, they have been intensively explored [13–15]. It has been proven that miRNAs play crucial role in cell proliferation, differentiation and apoptosis – the fundamental biological processes within the cell [16]. Evidence of pivotal miRNA role in carcinogenesis appeared in 2002 [17], followed by impressive number of research papers in different malignancies. Different miRNAs have been shown to function as either tumour suppressors or oncogenes, although the repertoire of their target genes is still being explored [18]. Furthermore it is proposed that single nucleotide polymorphisms (SNPs) in miRNA coding genes, which impact miRNA biogenesis or its target binding sites, also affect the process of carcinogenesis [19]. In addition to structural genetic variations, miRNAs can be affected by epigenetic alterations such as promoter DNA methylation or loss of histone acetylation [20].

Epigenetic alteration is a crucial element that ensures the stability of the genome. The mechanism of DNA methylation is the best-studied epigenetic phenomenon with the real biological significance for the initiation of various diseases [21]. Global hypomethylation is a decreased level of total methylation of the genome, which is related to the general loss of genetic stability (activation of oncogenes and endoparasitic DNA and loss of chromosome stability) leading to the initiation of cancerous processes [22]. The loss of genetic stability of the entire genome is associated with a decreased methylation level in repetitive genome elements, which account for a substantial portion of human genome [10]. LINE-1 is one of the major repetitive genome elements, accounting for ~17% of the total genomic DNA sequences [23]. The methylation level of CpG islands, localized within LINE-1 elements directly correlates with the global genomic DNA methylation status and is frequently used as a surrogate biomarker reflecting the global level of total genome DNA methylation [24]. The phenomenon of LINE-1 hypomethylation was investigated in a variety of malignancies and is thought to serve as a potential biological marker for cancer detection, prediction of survival, and the choice of the most appropriate therapeutic strategy [25].

1. AIM AND OBJECTIVES

The **aim** of this study was to assess epigenetic and genetic alterations of non-coding genome structures and their significance in the pathogenesis of gastric cancer.

Objectives:

1. To evaluate the alteration of miR-137 gene expression and promoter methylation status and its implication for gastric cancer and premalignant gastric lesions.
2. To perform a comprehensive analysis on LINE-1 methylation level in different stages of gastric carcinogenesis and to evaluate its prognostic potential.
3. To assess the association between single nucleotide polymorphisms of miR-27a, miR-146a, miR-196a-2, miR-492, miR-608 genes and gastric cancer or premalignant gastric lesions.

1.1. Relevance and novelty of the study

Current study provides: 1) novel evidence on epigenetic and genetic alterations of miR-137 in gastric carcinogenesis; 2) comprehensive LINE-1 methylation analysis and its prognostic potential in GC; 3) novel insights into the implication of genetic variants of miRNA genes and their association with GC and high risk atrophic gastritis. All of the analyses were performed in a cohort of patients of European descent, which, in the whole context of existing literature on current issues, emphasize differences in GC pathogenesis based on subjects' ethnicity. Moreover, all three studies included a set of patients with premalignant gastric lesions (chronic/atrophic gastritis) which was not frequently investigated in analogous studies. Eventually, our results contribute to the overall molecular basis of gastric cancer pathogenesis and may be employed as a reference data for scientific studies in the future.

2. REVIEW OF THE LITERATURE

2.1. Gastric cancer

2.1.1. Gastric cancer epidemiology

Gastric cancer ranks among one of the most common malignancies worldwide with approximately one million new cases and more than three-quarters of a million deaths annually. According to the updated GLOBOCAN 2018 statistics, gastric cancer is the fifth most prevalent cancer and the third leading cause of cancer-related death globally [1]. The prevalence and mortality rates of gastric cancer vary widely between different geographical regions, making developing countries (Eastern European, Eastern Asian, Latin American and Caribbean countries) the most affected [26, 27]. Although the relative rate of gastric cancer is decreasing since it was first described in 1975 [28], the absolute number of affected persons is rising, because of growing population size, big number of senior persons who are at the highest risk and well established medical history documentation. In Lithuania the burden of gastric cancer meets worldwide tendencies and despite the fact, that the morbidity is slightly decreasing, the incidence of gastric cancer still accounts for 29.5 cases/100,000 people with mortality rate of 24.4 cases/100,000 people of both sexes in 2018 (age-standardised rate). To compare with other European countries, Lithuania falls into the group of most frequently affected countries (*e.g.* in Sweden the rates of morbidity and mortality account respectively for 7.7 and 5.5 cases/100,000 people of both sexes in 2018) [3]. According to the statistical data, males are twofold more frequently affected than females worldwide [29]. The highest incidence of the disease ranks in the age group of 60-75 years.

Pathogenesis of gastric cancer usually takes years. It starts asymptomatically and is generally undetectable in early stages. Showing clear clinical symptoms only in the advanced stages, it delays diagnosis of the disease, therefore 1-year and 5-year survival rates after disease onset are only 42% and less than 30%, respectively [30, 31]. The only exception is Japan and the Republic of Korea where well-established mass screening programs (esophagogastroduodenoscopy, radiography or fluoroscopy) are available due to extremely high rates of gastric cancer – this raises 5-year survival rates up to 70% because of the possibility to diagnose the disease in I or II stage [32, 33].

2.1.2. Histological classification of gastric cancer

Histologically gastric tumours can be divided into 2 large groups, regarding to the tissue they arise from: epithelial and non-epithelial (stromal, lymphatic, soft tissue etc.) tumours. The origin of around ~95% of cases is epithelial tissue and those tumours are called adenocarcinomas [34]. Nevertheless, this group of neoplasms is highly heterogeneous considering cell differentiation, growth patterns, extension grades etc.; therefore several histopathological classification systems are applied in order to precisely describe gastric tumours. Two most frequently used classification schemes – Laurén and The World Health Organisation's (WHO) will be presented in a more detailed way.

2.1.2.1. Laurén classification

Gastric tumours classification according to Laurén (1965) [35] is based on the gastric tissue appearance under the microscopic examination and macroscopic differences. Two main histological types are described: intestinal and diffuse.

Intestinal type gastric adenocarcinoma characterization: well to moderate differentiation of tumour cells; slow growth; recognizable glandular structures formation; more common in males and older people. Intestinal type gastric adenocarcinoma is related to the cascade of the processes of precancerous conditions that in the course of time result in cancer [36, 37].

Diffuse type gastric adenocarcinoma characterization: poor differentiation and poor cohesion of tumour cells; cells infiltration into gastric wall; no glands formation; scattering throughout the stomach; aggressive behaviour and tendency to quick metastasis; more common in younger persons [34].

Adenocarcinomas that encompass the features of both types are called **mixed type** gastric adenocarcinomas [36].

2.1.2.2. WHO classification

WHO classification includes 4 categories. They are based on the most prominent histological pattern of gastric carcinoma [36, 38], which often coexists with less dominant histological patterns [39, 40].

Tubular adenocarcinoma: this type of tumour is composed of branching tubes varying in their diameter. This is the most common type of early gastric carcinoma [38].

Papillary adenocarcinoma: this type of tumour is characterized by elongated finger-like outgrowths.

Mucinous adenocarcinoma: this type of tumour is characterized by mucinous pools present outside of the cancer cells that constitute not less than 50% volume of the tumour size.

Poorly cohesive carcinoma: the most specific feature of this type of tumour is diffuse distribution of cancer cells, which may be arranged into small groups or isolated from each other.

Mixed carcinoma includes other rare histological variants of gastric adenocarcinoma that may not be characterized by aforementioned 4 main types [38–40].

2.1.3. Carcinogenesis and risk factors

Gastric carcinogenesis is a multifactorial process which involves multiple interactions between *Helicobacter pylori* (*H. pylori*) infection, environmental factors and host hereditary and genetic factors [4]. However, *H. pylori* infection, as class I carcinogen [41] is assumed as the main cause of sporadic stomach cancer [42]. Acute *H. pylori* infection may cause gastritis, and, if not eradicated, chronic inflammation and subsequent precancerous lesions eventually lead to gastric cancer. This prolonged cancerous process, which lasts decades is called Correa's cascade [5] and includes the following steps: chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, intestinal type gastric cancer. Correa's cascade will be presented more precisely in the following section.

Nevertheless, not all people, who during their lifetime experience *H. pylori* infection, will end up with gastric cancer. It is known, that the prevalence of *H. pylori* infection varies greatly within populations and may be as low as 18.9% in Switzerland and as high as 87.7% in Nigeria [43]. Many of affected people will never experience any clinical symptoms. However, *H. pylori* is responsible for approximately 89% gastric cancer cases [44]. The clinical course of *H. pylori* infection is dependent on the bacterial virulence factors and the host susceptibility. Several factors that increase gastric cancer risk are needed to be taken into account:

Virulence factors of *H. pylori:* the cytotoxin-associated gene A (CagA) coding toxin is recognized as a highly immunogenic oncoprotein, and patients infected by CagA+ strain expose high inflammatory response and are more predisposed to the development of precancerous gastric conditions and gastric cancer [45]. The vacuolating cytotoxin (VacA) is another major factor that determines the virulence of *H. pylori*. Studies showed that people affected with VacA s1/m1 *H. pylori* genotypes are more tended to

progression of preneoplastic gastric lesions than people affected with VacA s2/m2 strains [46].

Diet and lifestyle: the intake of sufficient amount of fresh vegetables and fruits, food rich in fibre, vitamins A, C, E decrease the risk of gastric cancer, while high intake of salt, smoking, consumption of alcohol are established risk factors for gastric cancer [47, 48].

Age, gender, and ethnicity: as mentioned in "Gastric cancer epidemiology" section, at the highest risk to develop gastric cancer are persons of male gender, at the age of 60+ and living in Eastern Asia, Eastern Europe, and South America.

Hereditary factors: mutations in *CDH1* (*e-cadherin*) gene have been identified as genetic cause of hereditary gastric cancer, that accounts for up to 5-10% of all stomach cancer cases, predominantly diffuse type [25]. The risk of inheritance and estimated life time risk to develop gastric adenocarcinoma for family members is quite high, and special prophylactic surveillance is recommended for individuals from risk-families (endoscopic surveillance, total gastrectomy etc.) [51, 52]. Other genetic variants, that might be implicated in the hereditary gastric cancer carcinogenesis were identified in the genes *CTNNA1, FBXO24, MAP3K6, DOT1L, PRSS1, INSR, MSR1* [50, 53], however, only *CDH1* genotyping is used in the clinical practice for identifying risk-persons up to date.

Cancer predisposition syndromes: individuals, diagnosed with one of the below mentioned syndromes are also at the higher risk to develop gastric cancer. These syndromes include: Lynch syndrome, Li-Fraumeni syndrome, familial adenomatous polyposis, Peutz-Jeghers syndrome and other [54].

2.1.4. Premalignant gastric lesions

The well-known pathophysiological cascade, proposed by P. Correa [5] is a pathway from stomach inflammation to intestinal type adenocarcinoma. It starts with uncontrolled gastric mucosa inflammation driven by *H. pylori* infection that in turn leads to chronic gastritis lasting for decades. *H. pylori* acts as gastric pathogen that contributes in changing the stomach environment: alters gastric acid secretion, increases gastric pH therefore leading for invasive colonization of anaerobic flora in the stomach [55]. Atrophic gastritis, characterized by loss of typical gastric glands, is a common endpoint of chronic mucosal inflammation [56]. Subsequently gastric atrophy is followed by intestinal metaplasia, a state characterized as the gastric mucosa replacement by epithelium, which resembles small intestine mucosa [57]. The penultimate stage in gastric carcinogenesis is called gastric epithelial dysplasia, which is characterized as the presence of histologically neoplastic

epithelium without indications of tissue invasion [58]. This well established series of precancerous changes is regarded as essential predisposing factors in the development of intestinal type stomach adenocarcinoma (see Fig. 2.1.4.1).

On the other hand, diffuse type gastric adenocarcinoma circumvents these preneoplastic lesions and arises in the stomach that lacks atrophy and intestinal metaplasia with the absence of a pathogenic role of inflammation [6]. This type of cancer evolves and progress rapidly, is highly metastatic and has poor prognosis.

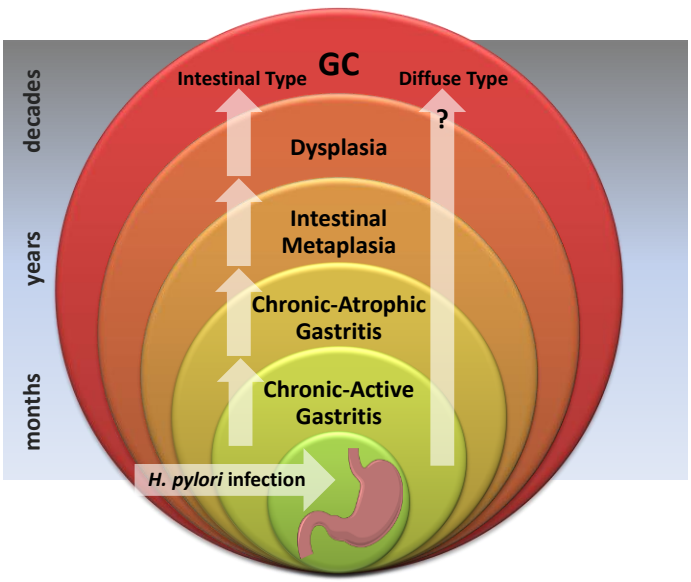

Fig. 2.1.4.1. *The model of Correa's cascade – from H. pylori infection to intestinal type gastric cancer (adapted from [42]).*

2.1.5. Diagnostics and screening

At the early stages gastric cancer rarely causes clinical manifestation. Carcinogenesis takes years and is usually diagnosed at the advanced stages when clinical symptoms occur: dyspepsia, weight loss, abdominal pain, nausea, vomiting, dysphagia, blood in the stools, anaemia, etc. Diagnostics of gastric cancer relies on several approaches that are used in the clinical routine: upper gastrointestinal (GI) tract endoscopy and biopsy sampling, Barium X-ray exam (contrast radiography), endoscopic ultrasonography, computed tomography scan, complete blood count, stool test [36].

Latent course of the disease that in the long run causes advanced and hardly curable stages of gastric cancer requires new, preferably non-invasive biomarkers which could be used for early detection of gastric lesion. Global

gastric cancer screening programs are not feasible in low incidence areas, whereas in several countries, where gastric cancer incidence is extremely high (*e.g.* China, Japan, Korea and Venezuela) mass screening programs are applied in order to reduce the incidence and the mortality of the disease [2]. Upper GI endoscopy is offered as a "gold standard" for persons from high risk areas; however the procedure is invasive and substantially unpleasant with a possibility to experience side effects, such as hemorrhage and perforation [59, 60]. Serologic mass or individual screening method is potentially more acceptable alternative than endoscopic screening. There are several serological biomarkers that are introduced as non-invasive premalignant gastric lesion markers [61]. Serological screening is based on the level of serum pepsinogen I (PgI), pepsinogen II (PgII), anti-*H. pylori* IgG testing and measurement of Gastrin-17 [48]. During the development of gastric atrophy, the level of PgI gradually decreases, while the concentration of PgII remains stable [62]. Low PgI level or decreased PgI/PgII ratio shows the atrophic processes of stomach mucosa. The use of combination measurement of PgI level or PgI/PgII ratio, *H. pylori* antibodies and Gastrin-17 results in better outcomes to diagnose gastric cancer at its early onset or in precancerous lesion state [63]. Gastropanel® is the first non-invasive diagnostic tool, designed by Biohit Oyj (Helsinki, Finland) that enables to diagnose gastric atrophy without invasive procedures with sensitivity and specificity of 71-83% and 95-98%, respectively [63, 64]. This ELISA-based test includes 4 above mentioned markers (PgI, PgII, amidated Gastrin-17 and *H. pylori* IgG antibodies), however they are adequate for patients with atrophic gastritis and the risk for its subsequent intestinal type gastric cancer, but not for diffuse type gastric adenocarcinoma. Several other molecules also showed the potential to become serological markers for gastric atrophy and cancer: ghrelin [65], trefoil factor 3 [66], gastric parietal cell antibody [67], nevertheless their adaptability is still needed to be confirmed by researches.

2.2. Types of non-coding genome sequences

The achievements of molecular biology researches of the last decades revealed, that substantial amount of individuals genetic material is made up of protein non-coding DNA sequences [9]. Human genome sequencing data disclosed that only 2.9% of the genome encodes exon regions of protein coding genes, and the sequences which encodes only amino acids (excluding untranslated regions) constitute 1.2% of genetic material [68]. The rest (~98%) of the genomic material is made up of non-coding DNA, repeated

elements, including tandemly repeated DNA sequences – long terminal repeats (LTRs), long and short interspersed nuclear elements (LINEs and SINEs) as well as shorter tandem repeats (satellites, minisatellites, and microsatellites) [10]. However, substantial portion of the human genome DNA sequences is transcribed into RNA, but non-protein-coding. Protein non-coding RNAs include several types of RNA molecules that differ in length, structure and function: long non-coding RNAs (lncRNAs, >200 nucleotides long), microRNAs (miRNAs, 19 to 25 bases long), short interfering RNAs (siRNAs, 20 to 25 bases long), Piwi-interacting RNAs (piRNAs, 26 to 31 nucleotides long) and small nucleolar RNAs (snoRNAs, 80 to 200 bases long) [11]. These proportions of protein coding and non-coding sequences are supposed to represent the importance of gene regulation function rather than biochemical function in the human genome. Since the present work includes epigenetic and genetic aberrations of miRNAs and LINE-1 under particular clinical conditions, these types of sequences and their function will be presented in a detailed way.

2.2.1. MicroRNA

MicroRNA molecules were discovered in 1993 independently by two research laboratories, led by Victor Ambros [13] and Gary Ruvkun [14], and as a separate class of non-coding regulating RNA molecules was identified in 2001 [69]. Currently, it is one of the fastest growing areas in molecular biology research and now it is believed that miRNAs play crucial role in such biological processes as cell proliferation, differentiation, growth control and apoptosis [70] – the fundamental processes of the cell.

MiRNAs are class of abundant, highly conserved, small, approximately 22 nucleotides in length (19 to 25) non-coding RNAs that are involved in biological machinery called RNA interference (RNAi) [71] as endogenous regulators of gene expression [72]. MiRNAs transcriptional units are localized within different genomic regions, including exons, introns and intergenic locations [73]. MiRNA encoding genes encompasses over 1% of human genome and are localized in all human chromosomes except Y. Currently approximately 2700 mature human miRNA sequences are known according to the 22nd edition of the miRNA database (miRBase v22, March 2018) [74]. Each of them regulates hundreds of mRNA targets and is supposed to manage more than two-thirds of the human genome [75].

2.2.1.1. Biogenesis and mode of action

The classic canonical pathway [76] of miRNAs biogenesis is well understood and it starts with transcription by RNA polymerase II (small group is transcribed by RNA polymerase III) [77, 78]. The initial transcript is known as hairpin-structured pri-miRNA and is subsequently processed into 70- to 100-nucleotides long hairpin-shaped precursor microRNA (pre-miRNA). This process is assisted by RNase III Drosha and RNA-binding protein DGCR8 [79]. Pre-miRNA molecule is further actively transported from nucleus to cytoplasm in the presence of an Exportin-5. In the cytoplasm, the pre-miRNA molecule is cleaved by protein complex consisting of the RNase III family endonuclease Dicer and the TAR RNA binding protein (TRBP) into a 22 nucleotides mature duplex miRNA (miRNA:miRNA*) [16, 80, 81]. Although both arms of formed double-stranded structure could continue to be modified and subsequently loaded into the miRNA-induced silencing complex (RISC), consisting of miRNA and Argonaute family proteins (AGO1-4 in humans), mostly one sequence becomes functionally active [82]. RISC-miRNA complex (miRISC) post-transcriptionally silences target genes through perfect or imperfect pairing with target mRNA which respectively causes either mRNA degradation or translational inhibition [79]. The specificity of miRNA and mRNA interaction is usually determined by the first 2-8 nucleotides in the sequence of the miRNA (seed sequence, starting at the 5′-end and counting toward the 3′-end) [16] and the miRNA response elements (MREs) in the 3′ UTR of target mRNA [83]. However, in humans, the incidence of exact matches on target MRE is rare [84], therefore in most cases miRNAs interaction with the 3′ UTR of target mRNAs results in translation inhibition rather than in target cleavage. Since the exact base match is not required for miRNA:mRNA interaction, one miRNA may interact multiple mRNAs, and one mRNA may be interacted by multiple miRNAs [85].

In addition to the well-known and widely studied canonical biogenesis pathway, several alternative non-canonical miRNA biogenesis mechanisms exist. They encompass Drosha/DGCR8 independent pathways, Dicer independent pathways and Terminal uridylyl transferase (TUTase)-dependent pathways [86, 87].

2.2.1.2. The expression patterns of miRNAs in cancer

The growing number of scientific studies finds relations between specific changes in the expression of miRNAs and various human pathologies, especially among various types of cancer [88, 89]. MiRNA expression

changes may occur at an early stage of carcinogenesis, and these changes may be associated with tumour cell movement, proliferation and survival. The mode of action of specific miRNA in the case of cancer is dependent on the cellular context. If miRNA blocks the oncogene mRNA expression, it acts as a tumour suppressor miRNR, and conversely, if miRNA inhibits the expression of the cancer suppressor gene, it is called oncogenic miRNA (onco-miRNA) [83, 90]. In the process of carcinogenesis particular expression patterns of onco-miRNAs upregulation and tumour suppressor miRNAs downregulation occur (see Fig. 2.2.1.2.1.).

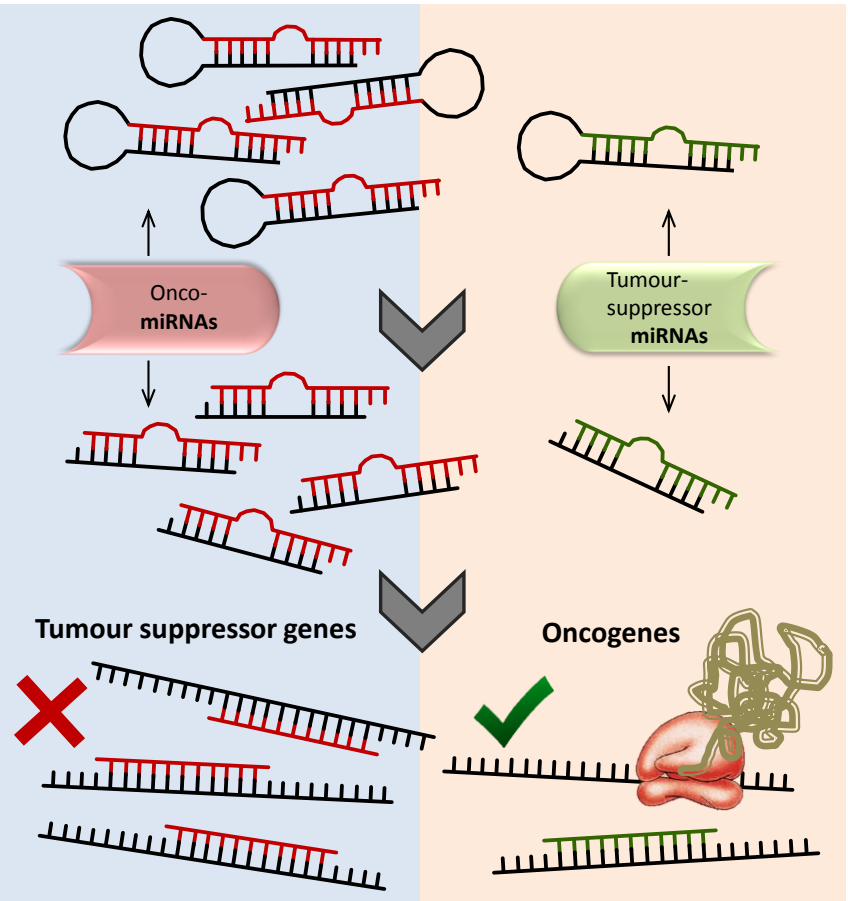

Fig. 2.2.1.2.1. *Expression patterns of onco-miRNAs and tumour suppressor miRNAs and their target genes in cancer.*

Since miRNAs are expressed in a tissue-specific manner, based on the expression profiles of miRNA it is now possible to classify various types and subtypes of cancer and to distinguish the tissues under investigation

according to their origin [91]. Moreover, specific expression pattern of miRNA often correlates with cancer prediction and response to treatment, therefore it is believed that miRNA molecules have a great potential to become biologic markers of various cancerous diseases.

2.2.1.3. Function-related single nucleotide polymorphisms in miRNAs

As it was mentioned before, miRNA exerts its biological function through hybridization with its target mRNA sequence according to Watson-Crick base pairing rules and by formation of a stable double-stranded structure [72]. As a result, even a single nucleotide change in one of the interacting sequences can determine the binding affinity, stability of the contact and its functional outcome. In fact, single nucleotide polymorphisms (SNPs), which can cause alteration of miRNA expression and function, may be present:

I. in the premature miRNA (pri-miR, pre-miR);
II. in the mature miRNA seed region and its target gene binding sites;
III. in the sequences of certain genes implicated in the biogenesis of miRNAs (*e.g.* Drosha, DGCR8) [92].

If the single base change occurs in premature miRNA sequence (pri-miR, pre-miR), there are 3 critical regions that can result functional outcomes:

I. the passenger strand of the mature miRNA
II. the stem-loop structure, which is cut by Dicer during the process of miRNA maturation
III. the promoter region of the pri-miRNA, which can combine with transcription factors [93].

Given the importance of miRNA-related SNPs, there are several online analysis tools and databases (reviewed in [94]), like miRNASNP database, miRdSNP, miRNA SNiPer, MicroSNiPer, PolymiRTS etc. that are used to predict the impact of SNP on potential miRNA target sequence and the secondary structure of RNA. The increasing number of case-control studies revealed the association between miRNA-related polymorphisms and the susceptibility and risk of various malignancies [95], in particular gastric cancer [93], however, most of them have not been functionally validated yet.

2.2.1.4. Site-specific hypermethylation and methylation of miRNAs genes

One of the main processes which steer the onset of cancer is the emergence of common epigenetic modifications that can lead to gene expression abnormality and genome instability. Epigenetic transcriptional regulation is a common feature of both the mRNA and miRNA coding genes. DNA methylation is the most widely studied area of epigenetic alteration in humans [96]. DNA methylation resulting from the covalent addition of a methyl group to the 5 'carbon position of cytosine residues in pyrimidine ring in the context of CpG dinucleotides is essential point in regulation of gene expression and maintenance of genomic structure [97]. CpG dinucleotides are dispersed throughout the whole human genome, but basically they are highly concentrated at specific CpG rich clusters, called CpG islands, located in 5'-flanking promoters of genes [98]. In the context of cancer, site-specific hypermethylation of tumour suppressor genes often occurs, that implies the gain of methyl-group at a position that was originally not methylated. These aberrations may lead to silencing of important tumour suppressor genes or cell cycle control points likewise the hyper activation of oncogenes and growth promoters [99]. Parallel to protein encoding genes, the CpG islands of the miRNR genes are methylated in the presence of enzymes DNA methyltransferases (DNMTs – DNMT1, DNMT3a and DNMT3b), that trigger changes in the three-dimensional DNA configuration which prevent transcription factors from binding, thereby causing gene silencing [100]. In the context of carcinogenesis, methylation of certain miRNA coding gene exerts its effect in double mode:

I. expression of miRNA is repressed;
II. expression of particular miRNA-related target gene is not affected.

It was calculated that over 11% of miRNAs coding genes in humans are embedded in CpG islands and their expression may be altered *via* methylation [101, 102]. The knowledge that has been gained so far suggests that epigenetic miRNA gene modifications are important for the development of various cancers, and epigenetic therapy, using *e.g.* DNA methylation inhibitors has the potential for treating cancerous diseases [99], or early occurrence of methylation of particular DNA sequence in the process of carcinogenesis may serve as the biological biomarker for early diagnosis of the disease [103].

2.2.2. LINE-1

2.2.2.1. Structure and function

Another type of non-coding sequences that cover substantial fraction of human genome is called long interspersed nuclear elements (LINE-1) which are widely distributed throughout the whole genome. LINE-1 sequences are active autonomous repetitive non-LTR DNA retrotransposons that replicate *via* reverse transcription of RNA intermediate and "copy-and-paste" genetic mechanism [25]. Human genome contains more than 500,000 full or truncated LINE-1 copies that constitute ~17% of the genomic sequence [23]. A typical full length and potentially active LINE-1 element is ~6 kb long and consists of 2 non-overlapping long open reading frames (ORF1 and ORF2) that are flanked by 5′- and 3′-untranslated region (UTR) [104]. Both ORFs encode proteins that are essential for retrotransposition - ORF1 encodes a 40 kDa RNA-binding protein (ORF1p), and ORF2 encodes a 146 kDa polypeptide (ORF2p) that provides endonuclease and reverse-transcriptase activities [104–106]. The structure of LINE-1 sequence is presented in Fig. 2.2.2.1.1.

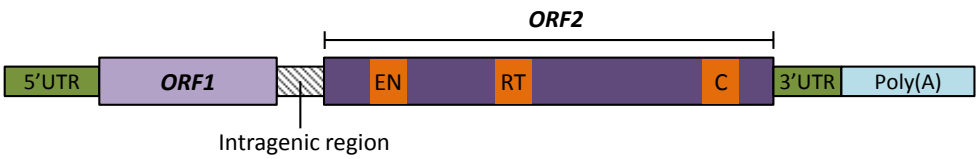

Fig. 2.2.2.1.1. *Structure of LINE-1 sequence (adapted from [107]).*
UTR, untranslated region; EN, endonuclease; RT, reverse transcriptase; C, cysteine-rich domain; ORF, open reading frame.

However, over 99.9% of LINE-1 elements are present in inactive form, caused by 5′ truncations, rearrangements or point mutations in ORFs [108]. For this reason LINE-1 is often considered as an unnecessary "parasitic" DNA. Nevertheless, the crucial roles of LINE-1 elements in various cellular processes are now being intensively investigated. Accumulating evidence suggests that LINE-1 can incessantly modify the genome, thus affecting the state of genes expression in numerous ways. For instance, LINE-1 often insert into protein-coding genes [109] and can cause genetic variability and polymorphism through recombination and rearrangement; the expression of LINE-1 itself may promote transcriptional disruptions or DNA breaks that cause genomic instability [25]. Structural alterations in DNA molecule may drive various human disorders, including cancer.

2.2.2.2. Global hypomethylation and LINE-1 methylation

As described in 2.2.1.4 section, site-specific DNA hypermethylation is one of the crucial processes that stimulate cancer development. On the other hand, the second essential DNA methylation alteration is global hypomethylation or the loss of methyl group at certain genome sites. DNA repeats are rich in CpG content and DNA methylation in repeated transposable elements is the key mechanism that inhibits their mobility and maintains genome stability [110]. In the context of carcinogenesis, the level of methylation in repetitive sequences (LINEs, SINEs, Alu etc.) decreases and results in chromosomal defects and rearrangements that cause genetic instability and loss of genomic integrity [111]. LINE-1 in full-length structure possesses an internal promoter in its 5′ UTR, which ranges from +1 to 909 base pairs and the initial 460-bp region includes 29 CpG sites [112]. Given their genome-wide ubiquity, methylation status of DNA repeats, in particular LINE-1 have been shown to correlate with genome-wide DNA methylation status [113] and the level of LINE-1 methylation is regarded as a surrogate measure of genome-wide DNA methylation. Methylation level of LINE-1 in normal tissue differs regarding to tissue type, however in cancer the methylation status fluctuates during cancer initiation and progression [114]. Significant scientific progress during the past several years introduced the methylation status of LINE-1 as a potential clinical biomarker for disease staging and for prediction of disease-free survival in cancer patients [115].

3. MATERIALS AND METHODS

3.1. Compliance with ethical standards

The present study protocol and the use of biological material were approved by Kaunas Regional Biomedical Research Ethics Committee (Protocol nr. BE-2-10) (see Supplement), Central Medical Ethics Committee of Latvia (Protocol Nr. 01-29.1) and by the Institutional Review Board of Otto-von-Guericke University Magdeburg (Protocol nr. 80/2011). Study was performed according to the guidelines of Declaration of Helsinki and written informed consent was received from all patients.

3.2. Tissue samples collection

Tissue specimens for **Study I** and **Study II** were prospectively collected at two clinical centres – Department of Gastroenterology and Surgery, Hospital of Lithuanian University of Health Sciences (Kaunas, Lithuania) and Department of Gastroenterology, Hepatology and Infectious Diseases Otto-von-Guericke University (Magdeburg, Germany) under the frame of the ERA-Net PathoGenoMics Project. Tissue samples collection consisted of:

- gastric mucosa tissue specimens from 81 primary gastric cancer patients (GC), including tumour tissues (T-GC) and corresponding adjacent normal gastric mucosa (N-GC),
- colonic mucosa tissue specimens from 28 primary colorectal cancer patients (CRC), including tumour tissues (T-CRC) and corresponding adjacent normal colonic mucosa (N-CRC),
- normal gastric mucosa tissues from 31 control subjects (N),
- 44 gastric mucosa tissue specimens from patients with chronic/atrophic gastritis with/without intestinal metaplasia (CG/AG/IM).

Tissue specimens from cancer patients were obtained during surgical interventions, and tissues from controls and gastritis patients were obtained during upper GI endoscopic procedure. Clinicopathological and demographical characteristics of the patients were collected retrospectively according to the data of hospital medical records. Histological characterization of the samples was performed strictly according to currently used medical guidelines, classification systems and criteria's: International Classification of Diseases for Oncology and Laurén criteria was used for histological subtyping of gastric cancer patients; for *H. pylori* status assessment in GC samples we used ELISA IgG test (Virion/Serion GmbH, Germany); all tissue specimens were histologically tested and certified as tumorous or non-tumorous tissue; the updated Sydney classification was

used for histological typing and grading of gastritis patients; samples from N and CG also underwent microbiological, serological and histological evaluation for *H. pylori* status assessment. The detailed clinicopathological and demographical descriptions of patients enrolled in Study I and Study II are provided in publications "*Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* [116] and *"LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis"* [117]. Tissue samples after the process of collection were immediately snap-frozen in liquid nitrogen and stocked in –80°C Biobank until molecular analyses. The survival data for GC patients came from Lithuanian Cancer Registry and medical records at Hospital of Lithuanian University of Health Sciences in two stages:

- for Study I we obtained survival information for 64 GC patients and the study was closed on 5th of December, 2013;
- the updated survival information for Study II was obtained for 80 GC patients and the study was closed on 28th of February, 2017.

Patients for **Study III** came from previous projects of our research group [118, 119]. Blood samples from patients with gastric cancer (GC), high risk atrophic gastritis (HRAG) and control subjects were collected at 3 centres of gastroenterology in Lithuania (Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas), Latvia (Riga East University Hospital and Digestive Diseases Centre GASTRO, Riga) and Germany (Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg). Out-patients, who were referred for upper GI endoscopic examination regarding the dyspeptic symptoms, but without previous history of malignancy, surgery or other gastrointestinal disease, fell into control or HRAG groups. Patients for GC group came from out-patient and in-patient departments and had histological verification of stomach adenocarcinoma. In total the current study enrolled 995 individuals who were divided into national and clinicopathological groups as follows (see Table 3.2.1.):

Table 3.2.1. *Distribution of patients included in Study III.*

	Gastric cancer	**High risk atrophic gastritis**	**Control**
Lithuanian	118	81	146
Latvian	139	59	142
German	106	141	63
Total:	363	281	351

Histopathological assessment of gastric mucosa was carried out by clinicians for HRAG patients and controls according to the updated Sydney classification as well as serological anti-*H. pylori* IgG antibodies testing. Histological subtyping of gastric cancer patients was performed according to the Laurén classification. For more details regarding clinicopathological features of patients from Study III, please refer to publication *"Gene Polymorphisms of Micrornas in Helicobacter pylori-Induced High Risk Atrophic Gastritis and Gastric Cancer"* [120].

All patients included in **Studies I, II, III** were of European descent.

3.3. Study I: miR-137 expression and methylation analyses

3.3.1. Study I design

The aim of the Study I was to evaluate the alteration of miR-137 gene expression and promoter methylation status and its implication for gastric cancer and premalignant gastric lesions The study consisted of RNA and DNA isolation, miR-137 gene and its target genes expression analysis by quantitative real-time PCR, bisulfite conversion and miR-137 promoter methylation status analyses by pyrosequencing in the set of patients with cancerous/precancerous gastric diseases.

3.3.2. RNA and DNA extraction

RNA and DNA extractions from frozen tissue specimens were accomplished using two-steps approach. RNeasy Plus Universal Mini Kit (Qiagen, USA) was used for total RNA and miRNA isolation from frozen tissue samples. All the procedures followed the manufacturer's instructions with minor modifications and isolated RNA was stored at –80°C until analyses. DNA was isolated from the same samples, pretreated with QIAzol Lysis reagent and chloroform in accordance with the user-developed protocol (provided by Qiagen) and stored at –20°C until analyses. Spectrophotometric and electrophoretic examinations were used in order to evaluate the quality and quantity of RNA/DNA, and integrity of RNA.

3.3.3. MiR-137 and target genes expression analyses by qRT-PCR

For quantitative miR-137 expression analysis we used TaqMan® Assay (mmu-miR-137, 001129, Life Technologies) according to manufacturer's instruction. We chose housekeeping gene *RNU6b* as internal endogenous normalizer. For miR-137 target genes (*Cdc42* and *COX-2*) and housekeepping *β-actin* genes expression analyses we used SYBR Green method

according to recommendations of producer (Life Technologies). RNA samples were firstly reverse transcribed into cDNA, using miR-137 or *RNU6b* specific primers or random hexamers and TaqMan® MiRNA Reverse Transcription Kit (Life Technologies). QRT-PCR analyses were performed on BioRad CFX Cycler System (BioRad) in 96-well plates in technical duplicates. The relative expression analyses were performed using $2^{\Delta Ct}$ method. Please refer to the publication *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* for more details.

3.3.4. MiR-137 methylation analyses

Purified genomic DNA from tissues was used for miR-137 promoter region quantitative methylation status analysis. Conversion of unmethylated cytosine to uracil was performed using Cells-to-CpG™ Bisulfite Conversion Kit (Life Technologies) according to the manufacturer's instructions. Bisulfite converted DNA and specific biotin-labeled miR-137 primers (F: 5'-TGG ATT TTT TTT TAG GGA AAT-3'; R: 5'-biotin-CCA CCA AAA CTC TTA CTA CTC-3') were used for region of interest amplification using standard PCR procedure. PCR success was confirmed by agarose gel (1%) electrophoresis and no-template controls. Quantitative methylation status of miR-137 promoter region CpG sites was analysed by pyrosequencing. Briefly, biotin-labeled PCR products were firstly captured on streptavidin-coated magnetic beads and then underwent pyrosequencing procedure, using PyroMark® Gold Q96 reagents (Qiagen) on PyroMark Q96 ID pyro-sequencing system (Qiagen), according to manufacturer's recommendation (miR-137-pyro-seq primer ATT TTT TTT TAG GGA AAT). The methylated fraction of each analysed miR-137 CpG site was quantitatively (%) measured and the mean methylation level of each sample was defined. We used genomic DNA extracted from *Jurkat* (ATCC® CRL-2899™) cells as a control for bisulfite modification reaction and as positive control of high methylation level for pyrosequencing. The example pyrogram is shown in Fig. 3.3.4.1.

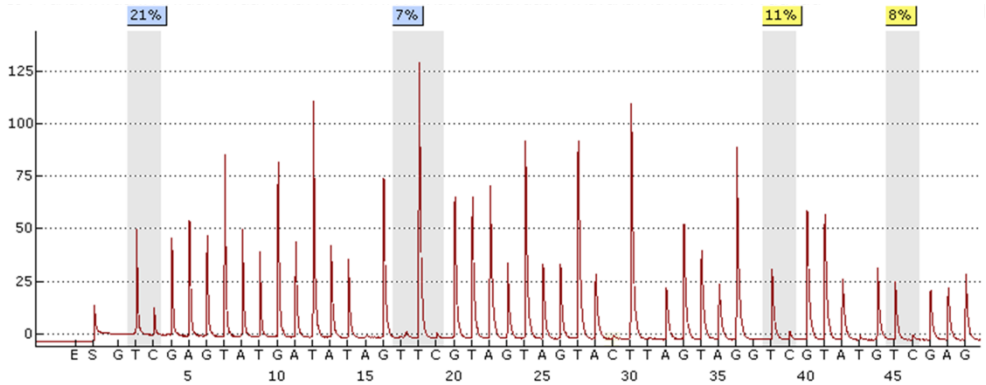

Fig. 3.3.4.1. *Pyrogram analysing 4 CpG sites spanning the promoter region of miR-137 coding gene.*

Methylation percentages (proportion of C at each CpG site after bisulfite modification) are marked within grey vertical boxes. Perfect calls are indicated in blue, minor deviations from the expected patterns are indicated in yellow. The X-axis represents the dispensation order; Y-axis shows the signal intensity in relative light units.

3.3.5. Statistical and survival analyses

All statistical analyses and graphical representation of the results were performed using GraphPad Prism 6.0 statistical software (San Diego, CA). For the applied statistical methods and criteria's please refer to the publication *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis".*

3.4. Study II: LINE-1 methylation analysis

3.4.1. Study II design

The aim of the Study II was to perform a comprehensive analysis on LINE-1 methylation level at different stages of gastric carcinogenesis and to assess its prognostic clinical potential. The study consisted of DNA extraction (the same samples and DNA extraction procedure as described in 3.3.2 section), bisulfite conversion (partly described in 3.3.4 section) and LINE-1 methylation status analyses by pyrosequencing. We performed comparative evaluation of LINE-1 methylation in the set of patients with cancerous/ precancerous gastric diseases and assessed its prognostic role based on survival analyses.

3.4.2. LINE-1 methylation analyses

For LINE-1 region of interest amplification by PCR we used biotin-labeled primers (F: 5'-TTT TGA GTT AGG TGT GGG ATA TA-3', R: 5'-biotin-AAA ATC AAA AAA TTC CCT TTC-3') and for pyrosequencing LINE-1-pyro-seq primer AGT TAG GTG TGG GAT ATA GT. Hereby we accessed LINE-1 103–249 bp region with mean of 4 CpG-sites (GenBank accession no. X58075).

3.4.3. Statistical and survival analyses

Statistical procedures were carried out on GraphPad Prism 6.0 statistical software, for detailed information please refer to publication "*LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis".*

3.5. Study III: genotyping of miRNA SNPs

3.5.1. Study III design

The aim of study III was to assess the distribution of single nucleotide polymorphisms in miRNA coding genes (miR-27a, miR-146a, miR-196a-2, miR-492 and miR-608) in a set of patients with gastric cancer and premalignant gastric lesions and to evaluate potential associations between investigated SNPs and the presence of gastric cancer or high risk atrophic gastritis. The study consisted of DNA extraction from blood samples and real-time PCR TaqMan® genotyping experiments.

3.5.2. DNA extraction

7-10 ml of venous blood was sampled for DNA isolation. Genomic DNA from peripheral blood was extracted using 2 different approaches. For samples from German group QIAamp DNA blood kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. For Lithuanian and Latvian blood samples we used salting-out procedure. Quantitative and qualitative testing of isolated DNA samples was carried out spectrophotometrically using Biophotometer (Eppendorf, Germany) or NanoDrop 2000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). DNA samples were aliquoted and stored at –20°C until analysis.

3.5.3. SNPs selection and genotyping

Five single nucleotide polymorphisms (SNPs) in miRNA coding genes were selected for the present study on the basis of the previously proposed association with cancer risks [121–124]. For SNPs of *miR-27a* C>T (rs895819), *miR-146a* C>G (rs2910164), *miR-196a-2* C>T (rs11614913), *miR-492* C>G (rs2289030) and *miR-608* C>G (rs4919510) genotyping we applied predesigned TaqMan® assays according to the manufacturer's instructions. The procedure was accomplished on 7500™ Fast real-time cycler (Life Technologies, CA, USA). For SNPs genotyping, 93 DNA samples were assembled in a 96 well plate according to a predesigned plate layout (see Fig. 3.5.3.1.).

	1	2	3	4	5	6	7	8	9	10	11	12
A	L2	L7	L14	L15	L16	L17	L18	L20	L21	L22	L25	L47
B	L30	L34	L46	L43	L64	L73	L79	L80	L83	L100	L101	L103
C	L105	L109	L116	L120	L128	L129	L131	L132	L133	L134	L142	L144
D	L145	NTC	L6	L8	L10	L11	L12	L27	L37	L36	L50	L54
E	L56	L57	L60	L66	L68	L69	L72	L76	L82	L85	L88	L89
F	L94	L97	L98	L107	L108	L111	L118	L119	L121	L122	L123	L124
G	L126	L130	L140	L143	L146	NTC	L5	L32	L42	L49	L55	L59
H	L62	L63	L70	L102	L93	L115	L96	L125	L137	L31	L78	NTC

Fig. 3.5.3.1. *Plate design.*

Wells D2, G6 and H12 were used as negative no template controls (NTC) to assess the possible contamination risk.

The distribution of genotypes was represented as a dot-plot in the SDS 2.0.5 software (see Fig. 3.5.3.2.). The automatic genotype assignments were verified manually. For more information regarding genotyping experiments, please refer to publication "*Gene Polymorphisms of Microrna*s *in Helicobacter pylori-Induced High Risk Atrophic Gastritis and Gastric Cancer".*

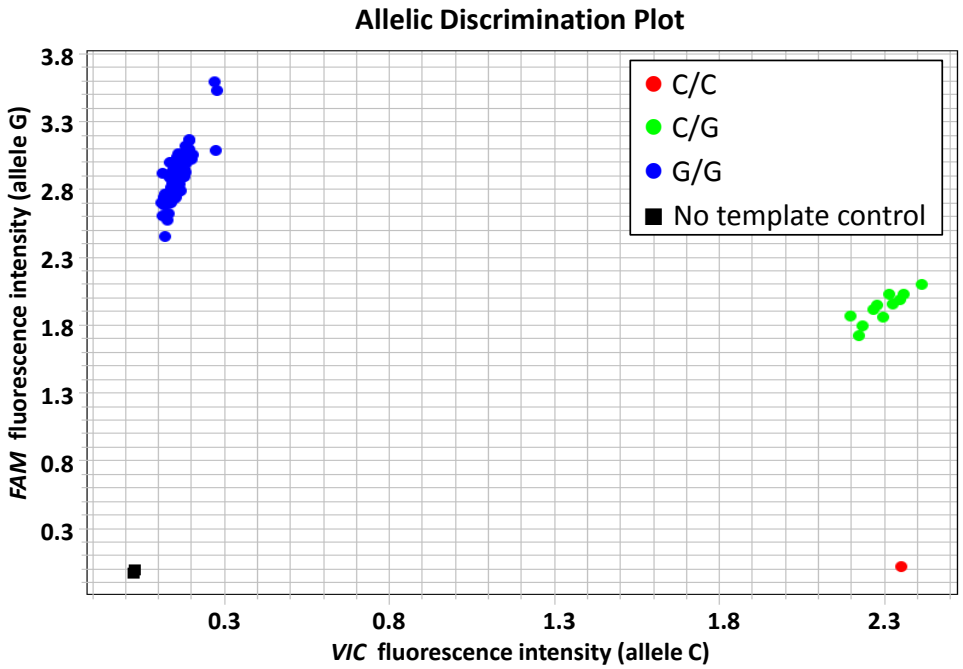

Fig. 3.5.3.2. *Allelic discrimination plot.*
Blue and red dots indicate homozygous (C/C or G/G) individuals, while green dots indicate heterozygous genotype (C/G). Black squares – no template control.

3.5.4. Statistical analysis

Statistical calculations and quality assessments were carried out on PLINK software version 1.07. For detailed information, regarding all statistical procedures applied for the data analysis, please refer to publication "*Gene Polymorphisms of Micrornas in Helicobacter pylori-Induced High Risk Atrophic Gastritis and Gastric Cancer".*

4. RESULTS

4.1. Study I

4.1.1. MiR-137 is epigenetically silenced in CRC and GC

On the basis, that miR-137 methylation is a pronounced event in colorectal carcinogenesis [125, 126], we firstly performed quantitative miR-137 methylation analysis in a cohort of paired primary CRC tissues (T-CRC) with corresponding adjacent non-tumorous colonic mucosa (N-CRC) by pyrosequencing. We investigated methylation of 4 CpG sites within the promoter region of miR-137 gene and calculated the average value of methylation level. In correlation with previous reports, statistically significant difference (P=0.0038) in miR-137 methylation level was found when compared T-CRC group (29.8±3.1%) to matched N-CRC group (15.4±3.5%, n=17 pairs) (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Fig. 1A'), suggesting that there is an increase in miR-137 methylation level during colon carcinogenesis.

We next used pyrosequencing approach to analyze miR-137 methylation level differences in 77 primary GC tissues (T-GC) with matching adjacent normal gastric mucosa (N-GC). We found statistically significant (P=0.045) increase in methylation level in T-GC tissue group (21.1±1.6%) compared to N-GC tissue group (16.9±1.3%) (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Fig. 1B').

4.1.2. MiR-137 methylation increases along with the Correa's cascade

To determine whether miR-137 methylation level in gastric tissue differs along with the severity from premalignant to malignant lesions, we subsequently examined the methylation level in normal gastric tissue from controls (N, n=19) and in tissues from patients with CG/AG with or without IM (CG, n=22). Gradual increase in miR-137 methylation was seen in correlation with Correa's cascade from N to CG and to N-GC (7.5±0.9%; 10.7±0.9%; 16.9±1.3%, respectively, P<0.0001). T-GC reached the highest rate of miR-137 methylation (21.1±1.6%), as mentioned before (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Fig. 2A').

On the basis of the quantitative output of the pyrosequencing data, we were able to analyze miR-137 methylation data as a categorical variable. The samples were defined as methylated based on the miR-137 methylation

cut-off, which was defined as follows: mean miR-137 methylation level for normal gastric mucosa from controls (N) plus 2 x standard deviations (SD) (cut-off equals 15%). In this analysis methylation of the miR-137 CpG sites was more frequent in tumorous (T-CRC 75%; T-GC 46.8%) compared to non-tumorous conditions (N-CRC 15.8%; N-GC 42.3%) and higher in CRC than in GC. In comparison to N-GC, miR-137 methylation was very low in N (5%) and CG (18.2%) tissues (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Fig. 2B').

Based on clinicopathological characteristics of GC patients we next performed miR-137 methylation analyses in GC subgroups. Subgroups were defined according to *H. pylori* status, tumour location in the stomach, TNM stage, differentiation level and histological tumour subtype based on Laurén classification. Subgroup analyses of GC revealed a trend for higher miR-137 methylation in tumours localized in *antrum* compared to *cardia* and *corpus* (P=0.07). The methylation level of miR-137 was significantly higher in intestinal compared to diffuse-type GC (P=0.03). No correlations were found for TNM stage, tumour differentiation or preexisting *H. pylori* infection (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Suppl. Fig. S2 A-G'). *H. pylori* status was available only for a small portion of GC patients (25 of 81); therefore, the *H. pylori* mediated miR-137 methylation in GC carcinogenesis was not evaluated.

4.1.3. MiR-137 expression in colon and gastric mucosa

We performed miR-137 expression analysis in T-CRC and N-CRC sample groups. To normalize miR-137 expression, ΔCt values were calculated using *RNU6b* as endogenous control. Based on our results, miR-137 was expressed at significantly lower level in T-CRC tissue samples compared with N-CRC (P=0.0014). Further miR-137 expression analysis was performed for T-GC and N-GC samples groups. We did not find statistically significant difference in miR-137 expression level between these two groups (P>0.05). Subsequently we tested miR-137 expression status in N, CG and AG/IM tissues. MiR-137 expression level was found to be unchanged along with Correa's cascade from N to CG or T-GC (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Fig. 5'). However, although there were no differences in miR-137 expression in gastric mucosa tissues along to Correa's cascade, we found an inverse correlation between miR-137 methylation and expression in gastric mucosa tissues (r= –0.397, P<0.0001) (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Fig. 4 B-C').

To further explore miR-137 expression variation among normal colonic and gastric tissues, we analyzed normal gastric tissues from controls (N), tumour adjacent normal gastric mucosa from patients with GC (N-GC) and tumour adjacent normal colonic mucosa from patients with CRC (N-CRC). Interestingly, we found substantially higher expression of miR-137 in normal colon tissue compared with normal gastric mucosa from controls and from patients with GC ($P<0.001$).

4.1.4. Correlation between miR-137 and its putative target-genes

The potential relation of miR-137 and two previously identified target-genes *Cdc42* [127] and *COX-2* [128] was investigated by correlating their mRNA expression level with miR-137 promoter methylation and expression status. The mRNA expression level of *COX-2* differed significantly in N, N-GC and T-GC in increasing manner, however *Cdc42* showed decreasing expression in the N, N-GC and T-GC cascade. Correlation analyses did not reveal the link between miR-137 promoter methylation or expression level and its target-genes mRNA expression level (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Suppl. Fig. S3 A-F').

4.1.5. MiR-137 methylation and expression association with GC patient survival

Overall survival of GC patient was defined as the time interval between the date of GC onset and the date of death. We had available survival data for 64 GC patients. The average survival time after diagnosis was estimated to be 520.2 days (range 9–1267 days). The patients, who were still alive at the moment of data collection, were censored for death date of 5th December, 2013. According to miR-137 methylation level, samples were divided into unmethylated and methylated groups based on miR-137 methylation cut-off 15%. Kaplan-Meier estimate revealed that overall survival of miR-137 methylated GC patients was slightly, but not statistically significantly worse than those with unmethylated miR-137 CpG island. For more accurate miR-137 methylation and overall survival association analysis, we sectioned our GC cohort into 2 subgroups of diffuse and non-diffuse GC. Kaplan-Meier survival curves showed slight differentiation of survival time in diffuse GC type between miR-137 methylated and unmethylated groups, although it did not reach statistically significant value ($P=0.065$). In the non-diffuse GC group no difference was seen between methylated and unmethylated group. To test whether overall survival is

associated with miR-137 expression, we chose mean value of miR-137 expression as the cut-off point for separating tumours with low or high expression level. Statistical analysis showed a similar overall mortality rate between these two groups (see *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis"* 'Fig. 6A-D').

4.1.6. More results of Study I

For more detailed information regarding results of Study I, please refer to publication *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis".*

4.2. Study II

4.2.1. LINE-1 methylation in colorectal and gastric cancer

Methylation status in LINE-1 has been widely investigated in CRC; therefore, we included a small cohort of patients with CRC for direct comparison. We carried out quantitative methylation analysis of LINE-1 103–249 bp region in a cohort of paired primary CRC tissues (T-CRC) with corresponding adjacent non-tumorous colonic mucosa (N-CRC). Decreased methylation in LINE-1 was found in T-CRC compared to N-CRC (mean±SD: 61.15±6.38% *vs.* 67.17±4.84%, respectively, P=0.0005) (see *"LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis"* 'Fig. 1A'). In GC patients group, decreased LINE-1 methylation level was also found in T-GC tissues compared to adjacent non-tumorous N-GC (62.48±8.15% *vs*. 65.73±4.56%, respectively, P=0.002) (see *"LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis"* 'Fig. 1B').

In the further analysis we performed calculations comparing the differences in LINE-1 methylation status between tumorous and adjacent non-tumorous tissue both for GC and CRC samples (LINE-1% methylation in T-CRC minus N-CRC and T-GC minus N-GC). Figures 1C and 1D in *"LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis"* illustrate that 69.6% of T-CRC samples showed lower methylation in LINE-1 compared to N-CRC and the same trend was noted for T-GC, where decreased methylation in comparison to N-GC was detected in larger proportion (53.8%) of tissue samples.

4.2.2. LINE-1 methylation may not occur in early stages of gastric carcinogenesis

One of the main purposes of current investigation was to evaluate the state of LINE-1 methylation in various stages of gastric carcinogenesis. We performed pyrosequencing analysis in a cohort of subjects with normal gastric mucosa without *H. pylori* infection (control group) and in patients with chronic/atrophic gastritis (CG/AG). We evaluated that methylation level at LINE-1 did not differ significantly between normal tissues (N), chronic/atrophic gastritis group (CG/AG) and tumour-adjacent (N-GC) gastric mucosa (mean±SD: 64.48±2.93%, 65.08±3.37%, 65.75±4.56%, P>0.05), suggesting that field defect might not be present at early stages of Correa's cascade in gastric carcinogenesis. Comparison between N, N-GC and N-CRC showed that methylation levels of LINE-1 were similar, suggesting that LINE-1 methylation levels might be similar in different anatomical sites of gastrointestinal tract (see "*LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis"* 'Fig 2A-B').

4.2.3. LINE-1 methylation levels in stratified groups of GC

LINE-1 methylation levels among GC samples bearing different clinical and pathological features are represented in "*LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis"* 'Fig. 4 A-H'. Methylation levels of LINE-1 were similar in tumours that originated from different gastric anatomical sites – *cardia*, *corpus* and *antrum* (P>0.05). Further additional analyses did not reveal significant differences between more and less advanced stages of GC (P>0.05). T-GC tissues of low (G1) and medium differentiation (G2) grade were characterized by similar levels of LINE-1 methylation as poorly differentiated (G3) tumours (P>0.05). The level of LINE-1 methylation was also similar between Laurén histological subtypes of GC – intestinal and diffuse (mean±SD: 61.84±7.97% and 63.32±7.78%, respectively, P>0.05). No differences were found concerning gender or *H. pylori* infection, but these sub-analyses were limited as clinical data was not available for some of the patients within the study.

4.2.4. LINE-1 methylation and GC survival

Data for 80 GC patients was available for survival analysis**.** The average survival time after diagnosis was estimated to be 1015 days (range 9–2451

days). The patients, who were still alive at the moment of data collection, were censored for death date of 28th February, 2017. We selected methylation level of 60% in T-GC sample as a cut-off value in order to separate individuals with low and high LINE-1 methylation. In general, we estimated the statistically significant survival difference with respect to UICC stage in our cohort (P=0.046). However, we did not observe significant differences in survival between the patients having low and high methylation levels at LINE-1 by Kaplan-Meier analysis (P=0.59). Survival analyses categorized according to the histological GC subtype also did not reveal significant differences in survival of the patients with low and high LINE-1 methylation levels (see "*LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis"* 'Fig. 5 B-C, E-F').

4.2.5. More results of Study II

For more detailed information regarding results of Study II, please refer to publication *"LINE-1 hypomethylation is not a common event in pre-neoplastic stages of gastric carcinogenesis".*

4.3. Study III

4.3.1. Characteristics of the study population

The control group consisted of 351 individuals – 94 males (26.8%) and 257 females (73.2%) with the mean age of 60.4±13.9 years. The group of HRAG consisted of 281 subjects – 106 males (37.7%) and 175 females (62.3%) with the mean age of 63.3±10.4 years. The GC group consisted of 363 patients – 231 males (63.6%) and 132 females (36.4%) with the mean age of 65.3±12.5 years. We evaluated statistically significant difference between groups with respect to age and gender distribution (P<0.001). *H. pylori* positive rates accounted for 47.3%, 42.7% and 52.6% in control, HRAG and GC groups respectively, however in approximately 27% GC patients *H. pylori* status could not be obtained. For more detailed study group representation please refer to publication "*Gene Polymorphisms of Micrornas in Helicobacter pylori-Induced High Risk Atrophic Gastritis and Gastric Cancer".*

4.3.2. MiRNA SNPs relation to risk of GC and HRAG

The observed distribution of genotypes for all studied SNPs in the control group corresponded to Hardy-Weinberg equilibrium: rs895819 (P=0.94); rs2910164 (P=0.44); rs11614913 (P=0.95); rs2289030 (P=0.44); rs4919510

(P=0.39). Overall we did not find any significant association between studied polymorphisms and the presence of HRAG or GC. We used corrected significance threshold (α=0.01 (0.05/5)), that was calculated to adjust for multiple testing. We found a tendency of miR-196a-2 (rs11614913) heterozygous CT genotype being associated with higher risk of gastric cancer to compare with CC genotype, but not statistically significantly (OR: 1.46, 95% CI: 1.03–2.07, P=0.032).We also found the higher incidence of miR-608 (rs4919510) GG genotype in gastric cancer group compared to control, but the difference did not reach the significance threshold (OR: 2.34, 95% CI: 1.08–5.04, P=0.029). Genotype comparison in recessive model of miR-608 CC + CG *vs*. GG resulted in OR: 2.44 (95% CI: 1.14–5.22), but significance remained marginal (P=0.021). None of the studied polymorphisms tended to be associated with the presence of HRAG. The results of all comparisons are presented in the paper "*Gene Polymorphisms of Micrornas in Helicobacter pylori-Induced High Risk Atrophic Gastritis and Gastric Cancer"*' 'Table 2'. The graphical representation of genotypes frequencies in control, HRAG and GC groups is shown in the Fig. 4.3.2.1.

4.3.3. MiRNA SNPs relation to risk of histological subtypes of GC

For more precise miRNA SNPs and the presence of GC association assessment we performed genotypes and alleles distribution comparison between two histological subtypes of GC – diffuse *vs*. intestinal. We found that dominant model for miR-27a revealed a trend for T allele to be associated with higher risk of diffuse-type GC (OR: 2.07, 95% CI: 1.13–3.79), however the significance remained marginal (P=0.018). The similar tendency was also observed for miR-492 where GC genotype showed increased risk for diffuse-type GC (OR: 1.95, 95% CI: 1.01–3.75), nevertheless P value remained insignificant (P=0.046). All genotype and allele frequencies and results of all comparisons are presented in the publication *"Gene Polymorphisms of Micrornas in Helicobacter pylori-Induced High Risk Atrophic Gastritis and Gastric Cancer"* 'Table 3'.

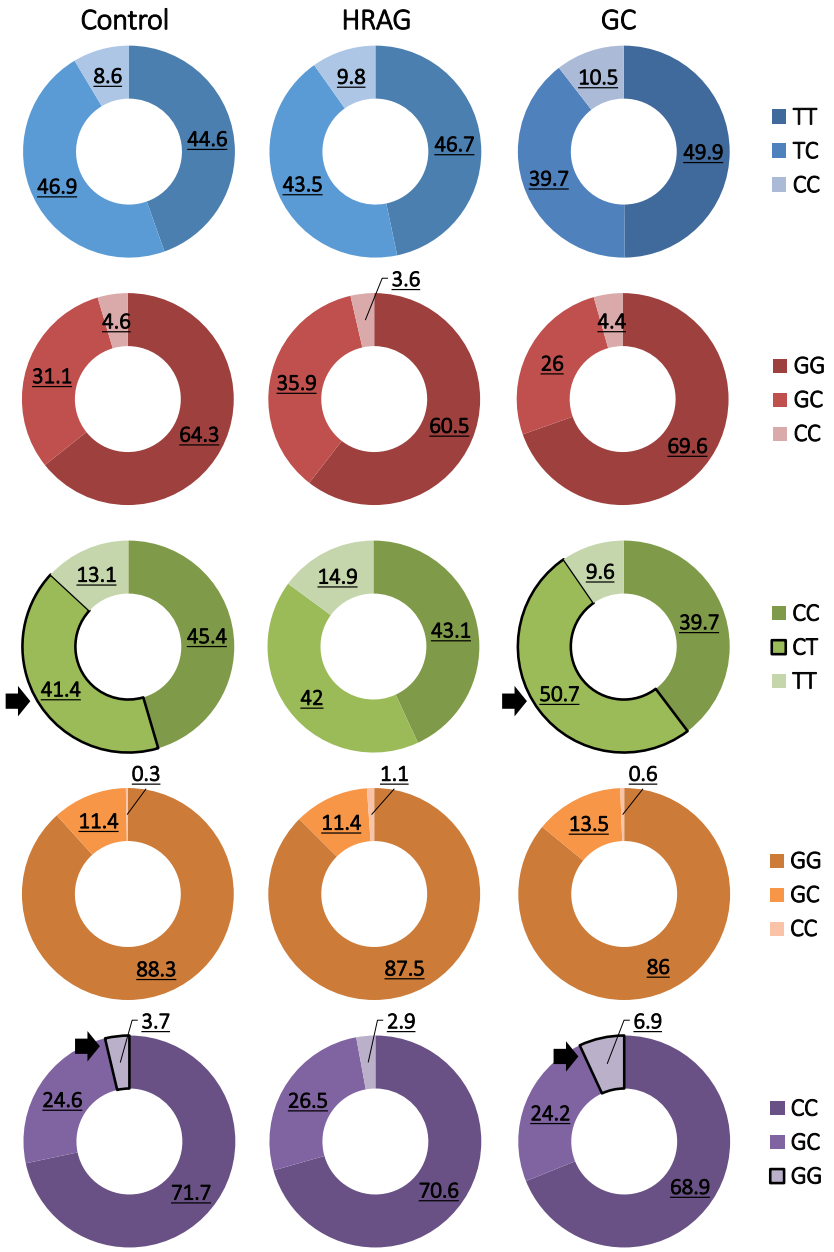

Fig. 4.3.2.1. *The prevalence of genotypes in controls, high risk atrophic gastritis (HRAG) and gastric cancer (GC) patients.*

From top to bottom: miR-27a *C/T (rs895819) (blue),* miR-146a *C/G (rs2910164) (red),* miR-196a-2 *C/T (rs11614913) (green),* miR-492 *C/G (rs2289030) (orange) and* miR-608 *C/G (rs4919510) (purple). The numbers indicate %. Marginal differences are presented in black arrows.*

5. DISCUSSION

5.1. Study I

In **Study I** we demonstrated that epigenetic silencing of miR-137 is a frequent event in GC and CRC. A step-wise increment in methylation of miR-137 along with severity of lesions was observed from normal gastric mucosa, chronic gastritis to cancerous tissues suggesting that it is an early and progressive event in gastric carcinogenesis. Our study is the first to show higher miR-137 expression levels in histologically normal colon tissue compared with normal gastric mucosa, which might suggest the different functional role of miR-137 in separate parts of digestive tract. Our data also clearly showed inverse correlation between CpG island methylation and relative level of miR-137 expression both in CRC and GC, underlying the importance of these mechanisms in gastrointestinal oncogenesis. Eventually, our results suggested the trends that diffuse type GC patients with methylated miR-137 promoter may have worse survival prognosis. The comprehensive discussion regarding our study results is provided in the publication *"Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis".* Some general information about miR-137 and updated findings regarding current issue in the context of existing literature will be discussed in subsequent paragraphs.

MiR-137 gene is mapped to human chromosome 1p21.3 within a long non-coding host gene *MIR137HG* and was for the first time identified in mice during tissue-specific small RNA profiling analysis [129, 130]. High evolutionary conservation of 86 bp hairpin sequence within pre-miR-137 and several key regulatory elements surrounding miR-137 across mammalian and non-mammalian vertebrates suggest its critical role in vertebrates biology [131]. The expression level of mature miR-137 may be affected by epigenetic and genomic variations, because miR-137 gene is directly overlapped by CpG island which hypermethylation supresses the gene expression level. Furthermore, variable tandem repeat (VNTR) domain of 15 nucleotide (5′-TAGCAGCGGCAGCGG-3′) is situated six bases upstream of the 5′ pre-miR-137 region and the length extension of the current VNTR changes its secondary structure and impairs the efficiency of miR-137 maturation [132, 133].

MiR-137 is expressed not only in tissues of gastrointestinal tract, which is the subject of current thesis, but also it is abundant in lung [134], kidney [135], pancreas [136], breast [137] and it is highly enriched in brain [138]. Recent studies revealed, that miR-137 is a significant network regulator of neurodevelopmental processes and of the pathogenesis of human cancer

[138]. Up to date, over 1000 putative target-genes of miR-137 have been identified using bioinformatic approaches [139], however only a small portion of them have been functionally validated yet in cell lines using expression profiling assays, luciferase expression reporter systems or Western immunoblotting. It is now known, that deregulation of miR-137 expression and subsequent deregulation of its target-genes or genetic variations within miR-137 gene itself drives such processes as human psychiatric disorders and various human malignancies [138]. In our study we also focused on 2 previously identified target-genes of miR-137, *Cdc42* [127] and *COX-2* [128]. Overexpression of *Cdc42* has been found to be oncogenic and responsible for such cellular processes as cell polarity, proliferation, migration, cytoskeleton remoulding, etc. [140]. Furthermore, overexpression of *COX-2* is involved in carcinogenesis by promoting cell proliferation, inducing vessel formation as well as enhancing metastasis and immunosuppression [141]. As both of the aforementioned genes are oncogenes and they are direct targets for tumour suppressor miR-137, we expected to find the correlation between miR-137 methylation/expression level and the expression level of *Cdc42* and *COX-2* in gastric cancer. However, no correlation was found, underlining the importance of some other unknown mechanisms or co-factors, which role should be elucidated in the process of gastric carcinogenesis.

Generally, there are only several researches referring the role of miR-137 in gastric cancer. Downregulation of miR-137 expression in gastric cancer tissues or human gastric cancer cell lines has been assessed by series of recent papers [142–146], while no updated information regarding miR-137 methylation status in GC could be provided yet. Our study is one of three studies, investigating miR-137 methylation status in GC published up to date [147, 148]. The expression level of miR-137 as a potential negative prognostic predictor for patients with GC was proposed, showing that low miR-137 expression in GC tissues is associated with shorter overall patient survival, advanced TNM stage and high grade tumours [143–146]. In our study, however, we did not reveal any significant associations between miR-137 expression or methylation level and GC patient's survival time or malignancy grade, only several minor trends were assessed, as discussed in our paper. The differences between our results and other published data may be partly explained by the origin of the study cohort. Our study is the only one performed with GC patients of European descent, while all other available studies were conducted with Asian GC subjects.

Several target-genes for miR-137 have been identified, that confirm the role of miR-137 as tumour suppressor in gastric carcinogenesis. Despite that in our study we did not reveal the correlation between *COX-2* expression

and miR-137 expression or methylation level in GC, Cheng *et al*. found functional correlation between *COX-2* and miR-137 expression in GC, showing that COX-2 is directly suppressed by miR-137, which in turn inhibits COX-2 mediated PI3K/AKT signalling pathway, which is usually overactive in human cancers, consequently suppressing apoptosis and activating cells proliferation [142]. Zheng *et al.* in their study demonstrated that *CDK6*, a key regulator of cell cycle, which suppression partly inhibits GC cells proliferation and tumour growth, is also the target of miR-137 [146]. *AKT2*, a crucial factor of PI3K/AKT signalling pathway, was also suggested as a direct target-gene for miR-137 [145]. Wu *et al*. in their experiments showed that downregulation of miR-137 in GC cell lines inhibits the suppression of *AKT2*, while the restoration of *AKT2* leads to an activation of invasion and an inhibition of apoptosis. *CUL4A*, as a target-gene for miR-9/miR-137 was proposed by Deng *et al*. [149]. They demonstrated that miR-9 together with miR-137 suppress 3′-UTR of *CUL4A* mRNA, which in turn indirectly regulates downstream Hippo-YAP signalling pathway in GC that is responsible for cells proliferation and invasion. Du *et al*. also displayed miR-137 tumour-suppressing properties by targeting *KLF12* and *MYO1C* in GC cell lines [144].

Taken all findings together we can conclude, that miR-137 is an important player in GC carcinogenesis and our data is a useful contribution to the common database on this issue, which will help us to better understand the regulatory mechanism of miR-137 in gastric carcinogenesis.

5.2. Study II

Study II aimed to provide a detailed characterization of LINE-1 methylation status across different stages of gastric carcinogenesis by employing pyrosequencing approach. In our study we showed that global hypomethylation determined in LINE-1 repetitive elements is characteristic feature of tumorous GC and CRC tissues. Contrary to some previous reports, our study revealed that LINE-1 hypomethylation might not be an early event in gastric carcinogenesis. LINE-1 methylation status did not differ significantly in normal gastric mucosa, chronic/atrophic gastritis and tumour-free adjacent gastric tissues suggesting the absence of field effect in gastric carcinogenesis. Furthermore, our data suggest that LINE-1 methylation status in GC tissue does not predict overall survival of GC patients. The comprehensive discussion regarding our study results is provided in the publication "*LINE-1 hypomethylation is not a common event in pre-neoplastic stages of gastric carcinogenesis"*. The article also presents a summary of analogous studies carried out by other research groups in 2009-

2016 in various sets of gastric cancer tissues employing different approaches of LINE-1 methylation level measurement. Bearing in mind, that our paper was published in the middle of 2017, not many updated findings regarding LINE-1 methylation in gastric cancer tissues were published yet. Min *et al*. in their recent study used two collections of GC tissue specimens, FFPE and frozen samples and methylation of four CpG sites in LINE-1 sequence was examined by pyrosequencing [150]. They demonstrated that LINE-1 methylation level was significantly lower in frozen GC tissues compared to their adjacent tumour-free gastric mucosa, which is in line with our data. They also associated LINE-1 methylation level differences with patients' clinicopathological characteristics – gender, tumour differentiation level and venous and lymphatic invasion in frozen samples, and with tumour location and venous invasion in FFPE. A short report published by Hong *et al.* also demonstrated significant hypo-methylation of LINE-1 in GC tissues compared with matched tumour-free mucosa [151]. Authors also declared the significant association of LINE-1 methylation level and such clinicopathological factors as Laurén histological tumour type, tumour differentiation level and the presence of background intestinal metaplasia. However, no new data regarding LINE-1 methylation alterations in preneoplastic lesions of the stomach was published so far.

Summarizing all data together we can conclude that our results are valuable in the context of the subjects' origin, while most of the studies of LINE-1 methylation were conducted on Asian patients, frequently not involving the set of patients with premalignant lesions. Given the existing inconsistency between the data, the potential of LINE-1 methylation employment as a biomarker for GC still must be taken with caution; however, there are no doubts that alterations of LINE-1 methylation level are relevant to stepwise development of GC.

5.3. Study III

In **Study III** we performed genetic association analysis of SNPs in miR-27a (rs895819), miR-146a (rs2910164), miR-196a-2 (rs11614913), miR-492 (rs2289030) and miR-608 (rs4919510) and the presence of gastric cancer or premalignant gastric lesions within the frame of case-control study design. Since the numerous studies implicated the importance of miRNA molecules for various processes of gastric carcinogenesis [152, 153], we supported the hypothesis that SNPs in miRNA coding genes may also be associated with the risk to develop gastric cancer. Contradictory results were published with regard to before-mentioned SNPs and the risk of GC or atrophic gastritis mostly for the subjects of Asian descent. Our results did

not reveal associations between miR-27a, miR-146a, miR-196a-2, miR-492 and miR-608 SNPs and the presence of GC or HRAG in European population. The comprehensive discussion on present issue with its strengths and limitations is provided in the publication *"Gene Polymorphisms of Micrornas in Helicobacter pylori-Induced High Risk Atrophic Gastritis and Gastric Cancer"*. However, taking into account that current study was published in early 2014, many complementary studies and meta-analyses have been reported since then, therefore some updated information, published in the interim of 2014-2018 will be provided in the subsequent section.

The SNP in miR-27a (**rs895819**) is located in the coding region of the pre-mir-27a hairpin in the stem-loop. Because of its location it may be important in the process of pre-miRNA maturation therefore it is suggested that rs895819 is a functional SNP affecting mature miR-27a function [154]. Its relation to the risk of various types of cancers, including GC have been studied widely, however the current available data are contradictory [155]. A recently published meta-analysis that summarizes data from different case-control studies during the period of 2010-2016 including various types of cancers (gastric, breast, colorectal, lung, etc.) indicated that rs895819 is not associated with the risk of GC [156]. Besides present study, up to date there are two more studies of Asian origin investigating the association of rs895819 and the risk for atrophic gastritis, one of which found a significant link [124, 154]. In our study we did not find significant association between rs895819 and the GC or HRAG risk in a cohort of European descent.

The C allele of SNP in miR-146a (**rs2910164**), located in miR-146a precursor affects the amount of production of mature miR-146a sequence compared to ancestral G allele and subsequently reduce the inhibition of multiple mRNA targets [157, 158]. MiR-146a is involved in such processes as cell proliferation and metastasis in various cancer types, therefore its deregulation may be important for carcinogenesis [159]. Up to date there were many studies published concerning the possible association between rs2910164 and various types of cancer, but the results are inconclusive though. With regard to gastric cancer, the meta-analysis by Xie *et al.* revealed the association for risk of GC and rs2910164, in particular in Caucasian populations [160], however the association was not confirmed by subsequent meta-analyses [157, 161]. Our study did not reveal a statistically significant association or a tendency of rs2910164 being linked with GC or HRAG either.

The C/T SNP in miR-196a2 (**rs11614913**) is located in the premature sequence of miR-196a2 and is announced to play a role in miRNA processing, has a positive effect on its expression and modulates the risk of tumour formation, because of being involved in cell proliferation, differ-

entiation, apoptosis, migration and invasion [162, 163]. The association of rs11614913 and the risk of cancer have been extensively investigated, however discrepancy of conclusions from individual studies are retaining, because of the different ethnicities of study participants and the effects of limited sample size in one study. With regard to gastric cancer, several recently published meta-analyses failed to reveal the possible association between rs11614913 and susceptibility to GC [161, 164, 165], while others indicated decreased risk to GC in Asians population [166] or the association under particular genetic models [93, 167]. In our study we indicated only a tendency of miR-196a-2 CT genotype to be associated with the risk of gastric cancer.

With regard to miR-492 (**rs2289030**) polymorphism and the risk to GC, no updated information can be provided yet. Our research team revealed no association between this SNP and the risk of CRC [168], while later on the group of Yu *et al*. indicated the significant association between rs2289030 and overall survival of patients with hepatocellular carcinoma [169]. All in all, to date there is little information about the clinical relevance of the current SNP in cancer patients.

The SNP of miR-608 (**rs4919510**) is located within the mature miR-608, but outside the seed sequence [170]. The C/G base substitution may contribute a different free energy to its binding site of target genes and therefore influence the expression of target proteins and modulate susceptibility to cancer [171]. There were only several papers published, investigating the relation between rs4919510 and the presence of GC. Jiang *et al*. found no association with GC susceptibility or prognosis in a large cohort of Asian subjects [172], while Wu *et al*. in their meta-analysis indicated a borderline significant association with increased risk of GC [173]. Subsequent meta-analysis by Li *et al*. investigated the potential association between rs4919510 and susceptibility to overall digestive system cancers (DSC); however no significant findings were made for all population, but importance for DSCs development was identified for Caucasian group [174].

Generally, the results of all combined studies must be taken with caution. The inconsistency between different studies and findings may originate from different genetic backgrounds of cancer patients, as gastric cancer is a heterogeneous multifactorial and polygenic disease. Therefore the statement that a certain SNP poses cancer risk is not appropriate – the effect of the SNP on the risk of cancer may be concealed by the imbalance of some other unknown causal genes that influence the process of carcinogenesis. Moreover, as a single SNP may affect the susceptibility to cancer marginally, the interplay of multiple SNPs may increase the effect to some extent.

Furthermore, in most case-control studies and further meta-analyses the considerable portion of study groups consisted of Asian subjects and the effect on Caucasians has been investigated marginally. The differences between Asian and Caucasian populations may be influenced not only by the ethnicity, but also by other co-factors, such as living areas, dietary habits, socio-economic status, that may impact the origin of cancer, but was beyond the control of the analysis. Therefore it is important to take into account such confounding factors as gene-environment interactions in the development of gastric cancer.

CONCLUSIONS

1. Methylation of miR-137 increases in a step-wise fashion in Correa's cascade from normal gastric mucosa to chronic gastritis and gastric cancer tissues. The methylation level of miR-137 is significantly higher in intestinal and mixed type compared to diffuse-type gastric cancer. Our data showed inverse correlation between CpG island methylation and relative level of miR-137 expression.

2. A reduced level of LINE-1 methylation was detected in cancerous gastric mucosa tissues compared to their paired adjacent non-cancerous tissues. The level of LINE-1 methylation did not differ in premalignant gastric mucosa – from normal gastric mucosa to chronic/atrophic gastritis and to adjacent tumour-free gastric tissue. LINE-1 methylation status in gastric cancer tissue does not predict overall survival of gastric cancer patients.

3. Single nucleotide polymorphisms in miR-27a, miR-146a, miR-196a-2, miR-492 and miR-608 genes were not associated with the risk of gastric cancer or high risk atrophic gastritis in the European subjects.

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PUBLICATIONS

Thesis publications:

1. Kupcinskas J, **Steponaitiene R**, Langner C, Smailyte G, Skieceviciene J, Kupcinskas L, Malfertheiner P, Link A. LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis. *Scientific Reports*. 2017, vol. 7, no. 1, p. 4828**. IF: 4.122** (2017/2018).
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Scientific conferences

The results of the thesis were presented in scientific conferences:

1. Kupčinskas, Juozas; **Steponaitienė, Rūta**; Langner, Cosima; Skiecevičienė, Jurgita; Kupčinskas, Limas; Malfertheiner, Peter; Link, Alexander. LINE-1 Hypomethylation is a rare event in preneoplastic stages of gastric carcinogenesis. Helicobacter: Special Issue: XXIXth International Workshop on Helicobacter and Microbiota in Inflammation on & Cancer: September 15–17, 2016, Magdeburg, Germany.
2. **Steponaitienė, Rūta**; Kupčinskas, Juozas; Langner, Cosima; Smailytė, Giedrė; Skiecevičienė, Jurgita; Kupčinskas, Limas; Malfertheiner, Peter; Link, Alexander. LINE-1 metilinimo lygmens tyrimas įvairiuose skrandžio kancerogenezės proceso etapuose. 2016 m. jaunųjų mokslininkų konferencija „BIOATEITIS: gamtos ir gyvybės mokslų perspektyvos“. December 7, 2016, Vilnius, Lithuania.
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1. Boehm, E., Langner, C., Kupcinskas, J., **Steponaitiene, R.**, Urba, M., Skiecevciene, J., Canbay, A., Malfertheiner, P., Link, A. The clinicopathological relevance of Fusobacterium nucleatum in gastric cancer patients. XXXIst International Workshop on Helicobacter & Microbiota in Inflammation & Cancer: September 14–15, 2018, Kaunas, Lithuania.
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Epigenetic Silencing of miR-137 is a Frequent Event in Gastric Carcinogenesis

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MicroRNAs (miRNA) are involved in posttranscriptional regulation of gene expression and are dysregulated during carcinogenesis. CpG island methylation of miR-137 is a common event in different cancers; however, the role of miR-137 in gastric cancer (GC) remains largely unexplored. In this study we aimed to characterize the epigenetic alterations of miR-137 in gastric carcinogenesis. We analyzed total 295 tissues including paired primary gastric cancer (T-GC) with corresponding adjacent gastric mucosa (N-GC), paired primary colorectal cancer (CRC) tissues with corresponding non-tumorous mucosa, gastric tissues from controls (N), and patients with chronic/atrophic gastritis (CG) with and without *Helicobacter pylori* infection. Bisulfite pyrosequencing and TaqMan RT-PCR were used to analyze miR-137 methylation and expression, respectively. Survival differences were evaluated using Kaplan-Meier analyses. miR-137 CpG island methylation was more frequent in tumorous compared to non-tumorous conditions and higher in CRC than in GC. In comparison to N-GC, miR 137 methylation level was lower in N and CG tissues, which correlates with Correas cascade. MiR-137 methylation inversely correlates with global LINE-1 methylation and miR-137 expression. miR-137 methylation was higher in intestinal type GC compared to diffuse one, and higher in antrum compared to cardia and corpus, however, miR-137 methylation was associated with worse prognosis in diffuse, but not in intestinal type of GC. The expression in colon was significantly higher compared to any gastric tissues suggesting functional difference. In summary, miR-137 methylation is a frequent event in gastrointestinal cancers which occurs early in stepwise manner during gastric carcinogenesis and inversely correlates with global methylation.

Key words: microRNA; miR-137; methylation; gastric cancer; gastritis; colorectal cancer

INTRODUCTION

Carcinogenesis is a consequence of multiple events that results in genetic and epigenetic alterations. In particular, DNA methylation and microRNA (miRNAs) expression have received marked attention for the functional relevance in carcinogenesis. MiRNAs are small noncoding molecules that, being part of so called RNA-induced silencing complex (RISC), regulate gene expression through post-transcriptional regulation [1,2]. Dependent on the target gene and background conditions, miRNAs may function as either tumor suppressor or as oncogene. In this way they play an essential role in almost every fundamental biological process such as cell proliferation, apoptosis and metastasis etc. [3].

MiRNA expression profiling studies revealed a broad spectrum of alterations in miRNA expression that are specific for different tumor types and even subtypes [4,5]. Despite the large number of miRNAs with elevated expression in cancer, there are also multiple miRNAs displaying reduced expression [6–10]. The precise mechanism responsible for miRNA expression alterations is not fully understood. One of the best explained and widely tested mechanisms is the aberrant DNA methylation silencing of the

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miRNA gene promoter. Similar to protein coding genes, CpG rich promoter regions of miRNA genes become methylated by DNA methyltransferases (DNMTs). These enzymes induce changes in 3-dimensional configuration of the DNA and prevent the interaction with transcription factor thereby causing gene silencing [3,11]. In addition, structural alterations of chromosomes or histone modification can be also involved in miRNA expression regulation [11,12]. Understanding of the mechanism responsible for cancer associated alterations may help to improve diagnostic and therapeutic strategies in cancer management [13].

Gastric cancer (GC) remains one of the most common malignancies, which is the third leading cause of cancer related deaths worldwide [14]. Pathogenesis of GC is complex and results from interaction of environmental and host factors [15]. *Helicobacter pylori* (*H.pylori*) infection is probably the most recognized risk factor for GC development. According to the widely accepted Correas cascade hypothesis, infection with *H. pylori* induces chronic inflammation of the mucosa, which can progress to mucosal atrophy, intestinal metaplasia, dysplasia, and finally cancer which is further dependent on various co-factors [16]. However, not all subtypes of GC can be explained by the hypothesis and the understanding gap in gastric carcinogenesis remains unclosed [15,17]. Similar to other malignancies, epigenetic alterations such as global and gene specific DNA methylation changes and miRNA expression alterations, received increasing attention and are believed to be a crucial step in gastric carcinogenesis [15,18].

MiR-137 is located on the chromosome 1p22 within the noncoding RNA gene AK094607 [19] and is embedded in a CpG island [20]. Because of these features, miR-137 is frequently in focus of multiple diseases and has been found down-regulated in bladder cancer [21], glioblastoma multiforme [22], lung cancer [23] and colorectal cancer (CRC) [20,24,25]. The exposure to DNMT-inhibitors causes reversal of CpG promoter methylation and miR-137 re-expression in CRC cells in vitro [20,26]. Functional analyses using miR-137 precursors showed reduction of cell proliferations [20,27] suggesting that miR-137 acts as a tumor-suppressor. Until now, multiple target genes such as KDM1A [28] LSD-1 [20], HMGA1 [29] have been validated for miR-137 and new targets are still to come. At the same time clinical relevance of those alterations is still not clear. While miR-137 has gained a lot of attention in glioblastoma multiforme and CRC, there are only very few high quality data in gastric cancer and its preneoplastic precursor conditions [30].

In the present study, we evaluate the clinical and translational significance of miR-137 promoter methylation and miR-137 expression in gastric carcinogenesis and compare it to CRC. We show that, similar to CRC, miR-137 methylation is a frequent event in GC and increasing methylation occurs early in preneoplastic conditions. However, alterations of miR-137 are mostly seen at the methylation level and induce only marginal miR-137 expression changes. Furthermore, miR-137 methylation is more frequent in distal GC of intestinal subtype according to Lauren's classification and shows trend for the worse prognosis in diffuse type GC.

MATERIALS AND METHODS

Tissue Samples

Tissue specimens were prospectively collected in Departments of Gastroenterology and Surgery, Hospital of Lithuanian University of Health Sciences (Kaunas, Lithuania) and Department of Gastroenterology, Hepatology and Infectious Diseases Otto-von-Guericke University Magdeburg, (Germany) as part of the ERA-Net PathoGenoMics project. The study protocol was approved by Bioethics Committee of Lithuanian University of Health Sciences (Protocol Nr. 2/2008) and by the Institutional Review Board of Otto-von-Guericke University Magdeburg (Protocol Nr. 80/2011). The study was performed according to principles of the Declaration of Helsinki and written informed consent was obtained from all patients.

Study Design

For this study we obtained the following materials: tissue specimen collection (biopsies or surgical material) from 81 GC patient tumor tissues (T-GC) with corresponding adjacent normal gastric mucosa (N-GC)(age: mean $\pm$ SD 65.8 ± 11.6), normal gastric mucosa tissue from 31 control subjects (N)(age 50.3 ± 17.5), 44 gastric tissue samples from patients with chronic/atrophic gastritis $\pm$ intestinal metaplasia (CG)(age 58.2 ± 13.0) and 28 primary CRC tumor tissues (T-CRC) with corresponding adjacent normal colonic mucosa (N-CRC)(age 68.2 ± 8.5). Samples from N and CG were prospectively collected during upper GI endoscopy, and high quality characterization of the samples was performed histologically and according to *H.pylori* status using microbiology, serology and histological evaluation, as previously described [31]. The histological typing and grading of gastritis was performed according to the updated Sydney classification [32]. The histological classification of gastric cancer patients was performed using International Classification of Diseases for Oncology and Lauren criteria. The antrum biopsies for molecular analyses were immediately snap-frozed in liquid nitrogen and stored at -80°C until usage. *H.pylori* status was assessed in GC samples using ELISA IgG test (Virion\Serion GmbH, Germany). Clinical and demographical data are shown in Table 1.

Survival Analyses

The survival data were obtained using the data of Lithuanian Cancer Registry and medical records at Hospital of Lithuanian University of Health Sciences.

Table 1. Clinicopathological Features of Gastric and Colorectal Cancer Patients Analyzed for miR-137 Methylation

	Gastric cancer				Colorectal cancer
	Whole cohort ($n=81$)	miR-137 unmethylated ($n=36$)*	miR-137 methylated ($n=43$)*	*P* value	Whole cohort ($n=28$)†,‡
Age					
mean (SD)	65.8 (11.6)	64.3 (10.9)	66.4 (11.7)	ns	68.2 (8.5)
Gender					
Female, *n* (%)	34 (42.0)	17 (47.2)	15 (34.9)	ns	13 (46.4)
Male, *n* (%)	47 (58.0)	19 (52.8)	28 (65.1)		15 (53.6)
Tumor localization					
Cardia, *n* (%)	8 (9.9)	5 (13.9)	3 (7.0)	ns	–
Corpus, *n* (%)	45 (55.5)	20 (55.6)	23 (53.5)		–
Antrum, *n* (%)	28 (34.6)	11 (30.5)	17 (39.5)		–
Proximal colon, *n* (%)	–	–	–		10 (35.7)
Distal colon, *n* (%)	–	–	–		18 (64.3)
TNM staging					
I, *n* (%)	16 (19.8)	6 (16.7)	8 (18.6)	ns	3 (10.7)
II, *n* (%)	21 (25.9)	10 (27.8)	11 (25.6)		12 (42.9)
III, *n* (%)	36 (44.4)	17 (47.2)	19 (44.2)		7 (25.0)
IV, *n* (%)	8 (9.9)	3 (8.3)	5 (11.6)		4 (14.3)
Unknown, *n* (%)	–	–	–		2 (7.1)
T					
1/2	18 (22.2)	7 (19.4)	9 (20.9)	ns	4 (15.4)
3	36 (44.4)	16 (44.4)	20 (46.5)		20 (76.9)
4	27 (33.3)	13 (36.1)	14 (32.6)		2 (7.7)
N					
0	30 (37.0)	13 (36.1)	15 (34.9)	ns	16 (61.5)
1	15 (18.5)	6 (16.7)	9 (21.0)		8 (30.8)
2	13 (16.0)	5 (13.9)	8 (18.6)		2 (7.7)
3	23 (28.4)	12 (33.3)	11 (25.5)		–
M					
0	73 (90.1)	33 (91.7)	38 (88.4)	ns	22 (84.6)
1	8 (9.9)	3 (8.3)	5 (11.6)		4 (15.4)
G					
1/2	32 (39.5)	12 (33.3)	18 (41.9)	ns	22 (88.0)
3	49 (60.5)	24 (66.7)	25 (58.1)		3 (12.0)
Lauren classification					
Diffuse, *n* (%)	44 (54.3)	25 (69.4)	19 (44.2)	$P=0.0085$	–
Intestinal, *n* (%)	26 (32.1)	8 (22.2)	16 (37.2)		–
Mixed, *n* (%)	7 (8.6)	–	7 (16.3)		–
Unknown, *n* (%)	4 (4.9)	3 (8.3)	1 (2.3)		–
H. pylori infection					
Positive, *n* (%)	17 (21.0)	9 (25.0)	8 (18.6)	ns	–
Negative, *n* (%)	8 (9.9)	2 (5.6)	6 (14.0)		–
Unknown, *n* (%)	56 (69.1)	25 (69.4)	29 (67.4)		–

*methylation results referred to 79 patients.
†TNM data referred to 26 patients.
‡G data referred to 25 patients.

Overall survival of GC patients was defined as the time interval between the date of GC onset and the date of death. The survival data was available for 64 GC patients. The patients, who were still alive at the moment of data collection, were censored for death date of 5th December, 2013. Samples were analyzed based on the miR-137 methylation (cutoff methylation of 15%). Survival analyses were further performed based on the miR-137 expression (above and below the mean). Kaplan–Meier survival analyses were performed for patients with available data sets and subgroups with diffuse and intestinal GC.

RNA and DNA Isolation

Total RNA (including miRNA) was extracted from samples using RNeasy Plus Universal Mini Kit (QIAGEN, Valencia, CA) according to the manufacturers instructions with slight modifications. Briefly, 20–30 mg of frozen tissue specimen was lysed with 900 μL QIAzol Lysis reagent (QIAGEN). Subsequently,

RNA was precipitated after homogenization with 180 μL chloroform and the upper aqueous phase was mixed with 1.5 volume of 100% ethanol. Further RNA washing and elution steps were accomplished using spin columns and buffers, included in the kit. DNA was isolated from the same tissue samples treated with QIAzol Lysis reagent and chloroform, using the interphase, according to user-developed protocol (provided by QIAGEN). Qualitative and quantitative testing of extracted RNA and DNA samples was performed spectrophotometrically using Biophotometer (Eppendorf, Germany). Qualitative examination of RNA integrity was performed by electrophoresis on agarose gel (1%).

MiR-137 Expression Analysis

Quantitative miR-137 expression analysis was performed using TaqMan® assay (Assay Name mmu-miR-137, 001129, Life Technologies, Inc., Carlsbad, CA) according to manufacturer's instructions. Housekeeping *RNU6b* gene was used as internal endogenous normalizer and expression level was analyzed using SYBR Green method according to producer's recommendations (Life Technologies). Approximately 20 ng of total RNA were reverse transcribed into cDNA, using miR-137 or *RNU6b* specific primers and TaqMan® MiRNA RT Kit (Life Technologies). Quantitative real-time PCR analyses were completed using BioRad CFX Cycler System (BioRad, CA) in duplicates. Quality of experiment was confirmed by no-template controls and reference samples. Relative expression was analyzed using $2^{\Delta Ct}$ method.

Real time PCR for Target Genes

Approximately 1 μg of total RNA were reverse transcribed to cDNA using random hexamers. SYBR Green method was used according to manufacturer's recommendations (Life Technologies). Primer sequences for Cdc42 were as followed: F- CATCGGAATATGTACCGACTGTT; R- GTGGATAACTCAGCGGTCGT; product size 135 bp and annealing temperature 60°C. Primer sequences for COX-2 were: F- GCCATGGGGTGGACTTAAA, R- CAGCAAACCGTAGATGCTCA; product size 186 bp and annealing temperature 60°C. Housekeeping β-actin gene was used for normalization. Relative expression was analyzed using $2^{\Delta Ct}$ method.

DNA Methylation Analyses

Purified genomic DNA was bisulfite modified using Cells-to-CpG™ Bisulfite Conversion Kit (Life Technologies) according to the manufacturers protocol. Bisulfite converted DNA was used for miR-137 CpG island and long interspersed nucleotide element-1 (LINE-1) methylation analyses as previously described [20,33]. Following PCR using biotin-labeled primers, the effectivity of reaction was confirmed using agarose gel (1%) electrophoresis and no-template controls. For quantitative methylation analyses we used bisulfite pyrosequencing of miR-137 CpG promoter and LINE-1. Pyrosequencing was performed on PyroMark Q96 ID (QIAGEN) using PyroMark® Gold Q96 reagents (QIAGEN) according to manufacturer's instructions. Mean methylation level of analyzed CpG sites was used for the further analyses. Samples with poor DNA quality and/or insufficient bisulfite conversion were excluded from the analyses.

Statistical Analysis

The statistical analyses were performed using GraphPad Prism 6.0 statistical software (San Diego, CA). Data are presented as mean % methylation or mean expression ± standard deviation (SD). Qualitative analyses were performed using χ^2 test or Fishers exact test. Quantitative variables for nonparametric analyses were performed using Wilcoxon text for paired and Mann-Whitney U test for unpaired analyses. For multivariate analyses we used Kruskal–Wallis test with Dunns multiple comparison post test. Correlation analyses were performed using Spearmans Test. Log-rank (Mantel–Cox) test was used to compare survival curves. Two-sided *P*-values of <0.05 were considered statistically significant in all tests.

RESULTS

MiR-137 CpG Methylation in CRC and GC

Until now, miR-137 CpG island methylation has been most deeply studied in CRC, therefore we included in our analyses a cohort of CRC patients for direct comparison. To determine the quantitative differences in miR-137 methylation in GC we used bisulfite pyrosequencing. First, we performed quantitative miR-137 methylation analysis in cohort of paired primary CRC tissues (T-CRC) with corresponding adjacent tumor-free colonic mucosa (N-CRC). In correlation with previous data, we confirmed increased methylation of miR-137 CpG island in T-CRC compared to N-CRC (mean ± SD: 29.8 ± 3.1% vs. 15.4 ± 3.5%, respectively, $P = 0.0038$) (Figure 1A). In patients with GC, miR-137 CpG methylation level was also higher in T-GC tissues compared to adjacent N-GC (21.1 ± 1.6% vs. 16.9 ± 1.3%, respectively, $P = 0.045$) (Figure 1B). Interestingly, in direct comparison between T-CRC and T-GC the methylation level of miR-137 was higher in CRC compared to GC ($P = 0.0105$), but not in N-CRC compared to N-GC ($P > 0.05$), suggesting that probably miR-137 CpG methylation may differ between different locations of GI tract. Next we performed paired analyses showing the difference in miR-137 methylation between tumorous and adjacent normal tissues (miR-137% methylation in T-CRC or T-GC minus N-CRC or N-GC, respectively). As shown in the Figure 1C and D, 76.5% of T-CRC showed miR-137 hypermethylation

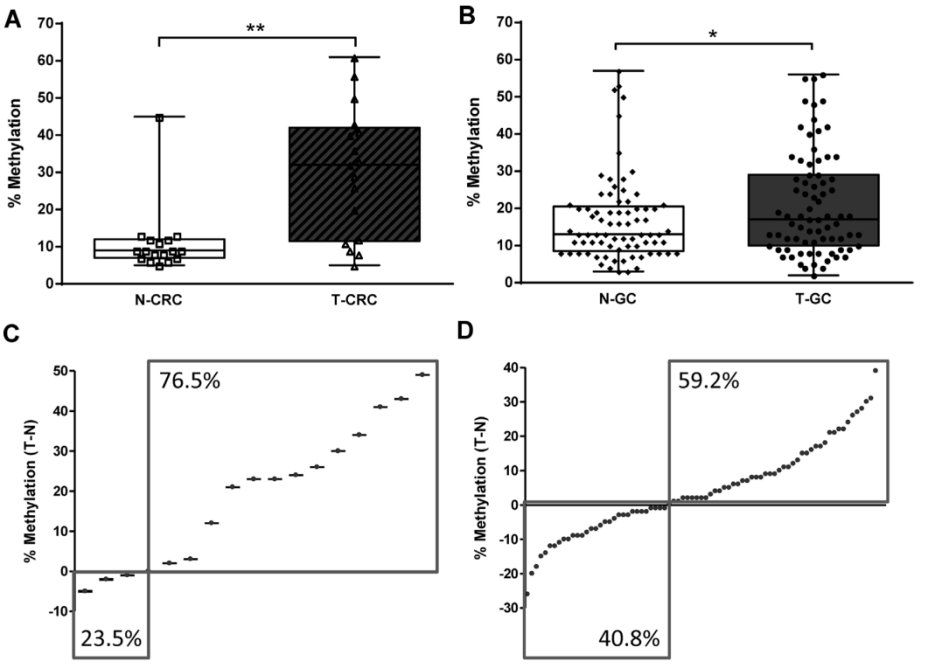

Figure 1. Quantitative miR-137 CpG islands methylation analyses in paired colorectal (CRC) and gastric cancer (GC) tissues. (A) miR-137 methylation in paired T-CRC and adjacent N-CRC tissues ($n = 17$). (B) miR-137 methylation level in T-GC and adjacent N-GC tissues ($n = 77$). (C and D) Difference between miR-137 methylation in (C) T-CRC and N-CRC, and (D) T-GC and N-GC tissues. The number refereed to percent of samples with higher and lower methylation in matching analyses. Wilcoxon test has been used for paired analyses.

compared to N-CRC, while 59.2% of T-GC showed higher methylation compared to N-GC suggesting a potential difference in miR-137 methylation in CRC and GC and arguing for more prominent field defect in stomach as observed in colon.

MiR-137 Methylation Occurs Early During the Process of Carcinogenesis

To determine the role of miR-137 CpG methylation in gastric carcinogenesis, we further analyzed gastric samples from patients with inflammatory and premalignant conditions such as atrophic gastritis and compared them to histologically confirmed *H.pylori*-negative gastric mucosa. We observed gradual increase in miR-137 CpG methylation in correlation with Correas cascade from N to CG/AG and to N-GC ($7.5 \pm 0.9\%$; $10.7 \pm 0.9\%$; $16.9 \pm 1.3\%$, respectively, $P < 0.05$), while T-GC reached the highest rate of miR-137 CpG methylation ($21.1 \pm 1.6\%$), as mentioned above (Figure 2A). In addition to the mostly used mean percent methylation level of miR-137 promoter sequence, we also included the CpG site specific data as shown in Supplemental Figure 1.

CpG methylation is frequently described as a categorical variable that may be helpful to define the patient subgroups based on the presence or absence of CpG island methylation. First, having obtained the quantitative values using pyrosequencing, we determined methylation cutoff based on the average miR-137 methylation level in normal gastric mucosa (N) plus 2 standard deviations (SD). The miR-137 methylation cutoff level was 15% and was similar to cutoff level previously defined for colon mucosa [20]. As shown in the Figure 2B, miR-137 methylation was present in 5.3% (1 of 19) of N, 18.2% (4 of 22) of CG/AG, 42.3% (33 of 78) of N-GC and 46.8% (37 of 79) of T-GC. For comparison, miR-137 methylation was found in 15.8% (3 of 19) N-CRC and 75% (18 of 24) of T-CRC. The subgroup analyses based on the clinicopathological characteristics revealed significantly higher miR-137 methylation in GC with intestinal and mixed type, but not in diffuse type tumors (Table 1). This correlated with qualitative methylation data (Suppl. Figure S2), where methylation level of miR-137 was lower in diffuse type GC compared to intestinal and mixed type ($P = 0.03$).

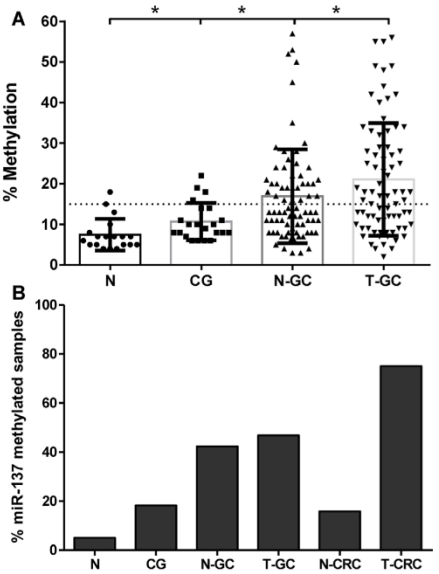

Figure 2. Quantitative and qualitative miR-137 methylation analyses in gastric tissue. (A) miR-137 methylation values were obtained using bisulfite pyrosequencing in gastric tissues from controls (N), patients with chronic gastritis (CG), normal adjacent tissues (N-GC) and gastric cancer tumor tissues (T-GC). Dotted line shows miR-137 methylation cut-off for qualitative miR-137 methylation variable. (B) Qualitative methylation frequency based on the methylation cut-off defined in normal gastric tissues. N-CRC and T-CRC were included for comparison purposes. Statistical analyses where performed using Mann–Whitney test.

Although, we observed a trend for increasing miR-137 methylation from cardia to corpus to antrum (Suppl. Figure S2), the difference using qualitative variables did not reveal significant differences (Table 1). We found further no correlation for gender, TNM stages, tumor differentiation or preexisting *H. pylori* infection although the last was limited by availability of the data.

Correlation Between miR-137 CpG and Global Genome Methylation

Global hypomethylation and gene specific promoter methylation are distinct and frequent events in carcinogenesis. Whether global hypomethylation may correlate with miRNA expression has not been studied yet. Having shown increasing miR-137 methylation in GC and CRC, we questioned if miR-137 CpG methylation may correlate with global genome-wide hypomethylation and its surrogate biomarker LINE-1 methylation specifically. We therefore performed matching comparison of miR-137 and LINE-1 methylation for T-CRC ($n=24$) and T-GC ($n=79$). Both CRC and GC showed inverse correlation between miR-137 and LINE-1% methylation for CRC ($r=-0.53$ for CRC $P=0.0073$) and GC ($r=-0.241$, $P=0.0327$), although the correlation was more pronounced for CRC compared to GC (Figure 3A and B).

Correlation Between miR-137 Methylation and Expression

Next, we aimed to evaluate the functional consequences of miR-137 methylation by analyzing the miR-137 expression. In similar fashion to LINE-1, we found an inverse correlation between miR-137 methylation and miR-137 expression for CRC ($r=-0.524$, $P=0.0003$) and for GC ($r=-0.397$, $P<0.0001$), respectively, (Figure 4A and B). To confirm those results, we divided our cohort of GC patients into three approximately equal subgroups according to miR-137 methylation status, that is, low ($\leq$12.5%), medium (12.6–24.9%) and high ($\geq$25%) methylation level (Figure 4C), and compared miR-137 expression between the subgroups. We found stepwise decrease in expression of miR-137 in subgroups of samples with low to moderate and high miR-137 methylation level ($P=0.0032$); thereby, confirming that miR-137 CpG methylation correlates with decreased miR-137 expression in gastric tissues.

MiR-137 Expression in CRC and GC

MiR-137 CpG methylation is increasing in both CRC and GC during the carcinogenesis. To evaluate if miR-137 expression shows similar, but inverse pattern, we performed expression analyses. In correlation with previous results, the expression of miR-137 was almost 10-fold higher in N-CRC compared to T-CRC (Figure 5). In GC, the expression level of miR-137 was higher in N-GC, but it did not reach statistically significant difference compared to T-GC. Overall, miR-137 expression was similar between various gastric conditions (N, GC, AG/IM, N-GC, T-GC) and T-CRC (Figure 5), but each significantly different to N-CRC ($P<0.0001$). Interestingly, there was a trend for higher miR-137 expression in N-GC compared to N ($P=0.0938$, Mann–Whitney test), but only in direct comparison.

Correlation Between miR-137 and Potential Target Genes (COX-2 and Cdc42)

Several studies have previously identified Cdc42 [23,26], COX-2 [22], LSD-1 [20] and several other genes as potential target genes of miR-137. Having shown the difference in miR-137 methylation, we questioned if those changes may inversely correlate with the expression of potential target genes. For this purpose, we selected two mostly studied genes Cdc42 and COX-2 and evaluated the gene expression alterations on the mRNA level. As shown in Suppl. Figure S3 A and B, we found significant expression difference for both Cdc42 and COX-2; however, while Cdc42 expression was rather decreasing in the cascade from N to N-GC to T-GC

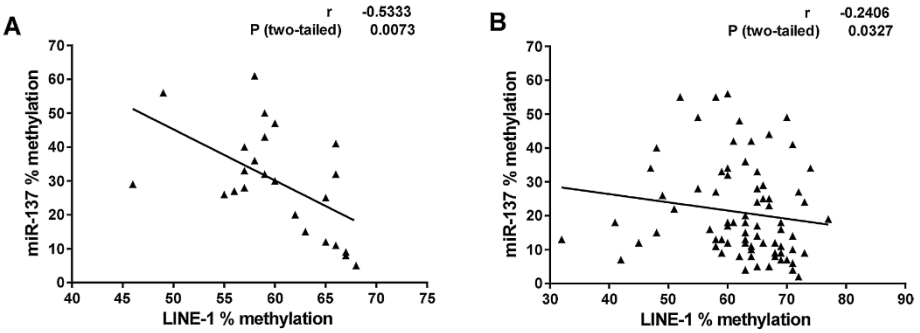

Figure 3. Correlation between miR-137 hypermethylation and LINE-1 hypomethylation. MiR-137 methylation obtained using bisulfite pyrosequencing were correlated with LINE-1 methylation level for (A) CRC and (B) GC tissues. Statistical analyses were performed using Spearman's test.

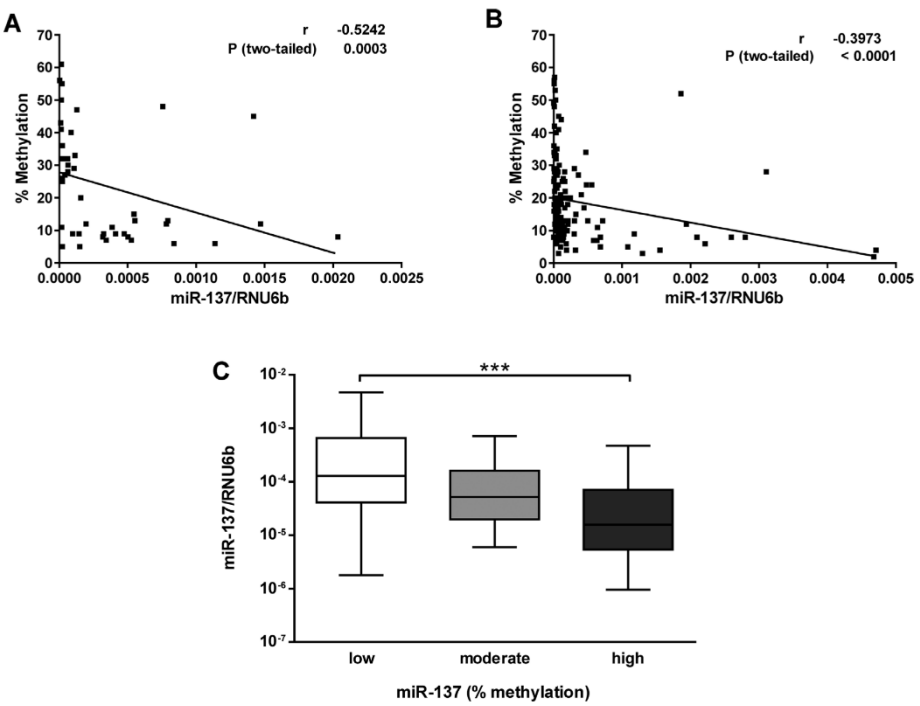

Figure 4. Correlation between miR-137 DNA methylation and expression. Quantitative miR-137 methylation was correlated with miR-137 expression for (A) colon and (B) gastric mucosa independently. Statistical analyses were performed using Spearman's test. (C) Three similar cohorts were build based on the miR-137 methylation values [<12.5% low ($n = 25$), and >25% with high methylation ($n = 24$), and n = 21 with intermediate, $P = 0.0006$]. Statistical comparison was performed using Kruskal–Wallis test with Dunns posttest ($P < 0.05$ for low vs. high).

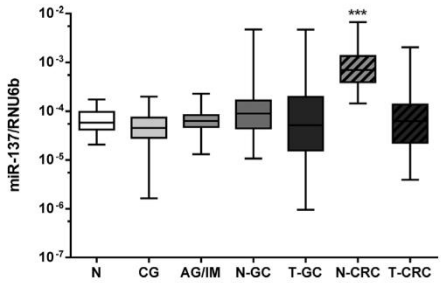

Figure 5. MiR-137 expression pattern in colonic and gastric tissues. qRT-PCR analysis revealed statistically significant difference between high expression of miR-137 in adjacent non-tumor-CRC (N-CRC) tissue samples compared to tumor CRC tissues (T-CRC) or any gastric tissue subgroup including controls. Statistical comparison was performed using Kruskal-Wallis test with Dunns post test.

($P = 0.001$, N vs. N-GC and T-GC each with $P < 0.001$, Dunns multiple comparison text), COX-2 showed, as expected, increasing expression ($P < 0.0001$, N vs. T-GC and N-GC vs. T-GC each $P < 0.0001$). Next, we performed correlation analyses between both miR-137 promoter methylation, expression and the Cdc42 and COX-2 expression. Interestingly, none of the genes showed an inverse correlation to miR-137 methylation or expression in all (Suppl. Figure S3 C–F) as well as in only T-GC samples (data not shown).

MiR-137 Alterations and Survival

There are still very limited data available on the impact of miR-137 methylation and expression alterations on clinical outcome of GC patients. We obtained survival data for 64 patients with GC. The average overall survival time after disease onset was estimated to be 520.2 d (range 9–1267 d). First, we divided our samples in two groups based on the miR-137 methylation status (methylated vs. unmethylated; miR-137 methylation cut-off 15%). Kaplan–Meier analysis revealed that overall survival of patients with GC with miR-137 methylation was slightly worse than those without CpG methylation, but the difference was not statistically significant (Figure 6A). Similarly, patients with low expression had also slightly worse overall survival than those with high miR-137 expression (below vs. above the mean miR-137 expression as cutoff, respectively). Remarkably, in GC patients with diffuse type there was a trend for a worse overall survival if miR-137 methylation was detected ($P = 0.065$), while no differences were found for non-diffuse type of GC tissue (Figure 6C and D).

DISCUSSION

In this study, we show that epigenetic silencing of miR-137 is a frequent and early event in gastric and colon carcinogenesis. Our subsequent analyses revealed increasing miR-137 CpG methylation from normal tissues to chronic gastritis, and to non-tumor and tumor tissues from GC patients, suggesting stepwise alteration during oncogenesis &in particular during Correas cascade. CpG island methylation of miR-137 is inversely correlated with LINE-1, a marker for global DNA methylation, and miR-137 expression. However, the miR-137 expression level in colon tissue was significantly higher compared to T-CRC, as well as to normal gastric mucosa and to CG/AG/N-GC or T-GC, suggesting potential difference in miRNA functional role between gastric and colon tissues of GI tract. Furthermore, in correlation with low miR-137 expression in gastric tissues, we did not find any direct inverse correlation between miR-137 expression and some potential targets such as Cdc42 and COX-2. Finally, we found some evidence for the worse prognosis in patients with miR-137 CpG methylation and low miR-137 expression, although the trend to difference in overall survival was mainly found in diffuse type gastric cancer.

In this study, we used bisulfite pyrosequencing, which is the well-established method for quantitative methylation analyses. First, the data from our study confirm that miR-137 epigenetic silencing by methylation is frequent and marked event in colon carcinogenesis, which causes significant reduction of miR-137 expression as previously described [20]. Systematic data analyses regarding miR-137 methylation and expression in GC is, however, still lacking. In one of the earlier studies, miR-137 CpG methylation has been observed in vitro in GC cell lines and several gastric cancer tumor tissues using methylation-specific PCR [26]. Those results were further confirmed and extended in another study where miR-137 methylation was evaluated using bisulfite-DHPLC and bisulfite clone-sequencing, which is a quantitative methylation assay [30]. The authors demonstrated, in comparison to Chen et al. that miR-137 methylation was found not only in GC tumor tissue (92.3%), but also in normal control tissues (38.5%). While the data from Du et al. come from Asian population, our results are the first to provide the quantitative data to miR-137 methylation in GC for European population. In our cohort of samples, mean miR-137 CpG methylation was 21.1% in T-GC, 16.9% for adjacent normal gastric tissues and 7.5% in healthy control stomach tissues. Based on our observations, we can suggest that the pattern of miR-137 CpG promoter methylation correlates with progression of Correa's cascade [34] from normal mucosa to premalignant precursor gastric conditions to GC and the earliest changes are present at earliest inflammatory state of mucosa as the most of the samples with chronic gastritis were from patients with *H.pylori* infection.

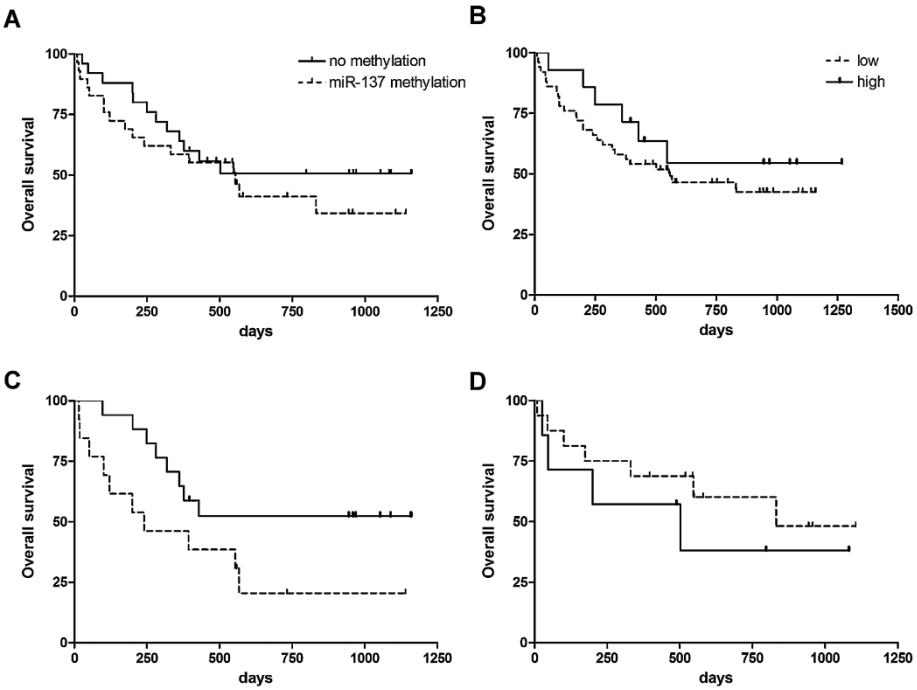

Figure 6. Overall survival analyses of patients with GC based on miR-137 methylation/expression. (A) Kaplan–Meier analyses of patients with and without miR-137 methylation defined by 15% cut-off value. (B) Kaplan–Meier analyses based on the miR-137 expression. Patients were defined as low and high expression based on the mean miR-137 expression value as cut-off. (C) Kaplan–Meier survival analyses in patients with diffuse-type GC (cut-off methylation 15%). (D) Kaplan-Meier survival analyses in patients with intestinal type GC (cut-off methylation 15%). Statistical comparison between curves was performed with Log-rank (Mantel–Cox) test.

Qualitative methylation status of CpG island has been frequently used to define categorical methylation state. In similar fashion to previously described method [20], we have therefore defined miR-137 CpG methylation status in gastric mucosa based on the methylation level in normal tissue, which was 15% and was equal to the level of miR-137 methylation for colon tissues [20]. In correlation to quantitative values of miR-137 methylation, we observed increasing frequency of miR-137 methylation in patients with CG, N-GC and T-GC. High methylation level of miR-137 in adjacent normal tissues has been described for CRC previously and was explained by the field defect hypothesis [20]. Interestingly, the difference between adjacent normal tissues from the stomach and normal control tissues in GC was much higher and more frequent (7.74% vs. 16.9% for N and N-GC for quantitative values and 5% vs. 42.3% for qualitative miR-137 values, respectively) compared to N-CRC or the data from Balaguer et al. [20]. This observation could be linked to the more prominent epigenetic field defect of gastric local tissues during carcinogenesis in regard of chronic broad inflammatory alterations for example related to *H.pylori* infection. At present a number of studies have shown existence of epigenetic alterations in GC patients both for tumor-suppressor genes as for miRNAs. For example, RASGRF1 methylation was found in non-tumor gastric mucosa of GC patients [35]; miR-34b/c gene methylation was involved in epigenetic field defect alterations in *H. pylori*-dependent manner [36]. Unfortunately, *H.pylori* status was known only in a subset of patients and direct link between the effect of *H.pylori* and miR-137 methylation could not be provided, yet. Nevertheless, *H.pylori* status was positive in majority of patients with chronic gastritis and may indirectly correlate with *H. pylori*-related alterations in miR-137 methylation and support the association with *H.pylori*-induced inflammatory cascade.

Global hypomethylation and gene-specific promoter hypermethylation are common events in multistep process of carcinogenesis which may frequently be found simultaneously in solid tumors,

including gastric cancer [37]. LINE-1 comprises a substantial portion of human genome and therefore is frequently used as a valuable surrogate marker of global DNA methylation [38]. Recently, positive link between CpG hypermethylation and LINE-1 hypomethylation was shown in *H. pylori*-triggered gastritis [39]. Despite of increasing knowledge in miRNA promoter silencing through DNA methylation, including miR-137, the correlation to LINE-1 has not been shown, yet. We show that in tumor tissues from both CRC and GC there is an inverse correlation between miR-137 and LINE-1 methylation, suggesting that those mechanism may depend on each other and be a part of two sided global epigenetic event in carcinogenesis.

Our data, showing inverse correlation between CpG island methylation and miR-137 expression both in CRC and GC, underline the importance of these mechanisms in gastrointestinal carcinogenesis. However, overall expression of miR-137 was almost 10-fold higher in normal CRC mucosa of our study group compared to any type of gastric mucosa or T-CRC. This observation is novel and surprising as the methylation pattern in colon mucosa shows similar methylation pattern as in stomach [20]. Although speculative, the high expression level of miR-137 may mirror the specific functional role of miR-137 in colon in context of molecular cellular status in comparison to gastric mucosa. The expression of miR-137 in normal gastric tissues was similar to chronic gastritis and preneoplastic precursor conditions, further questioning the functional role of miR-137 methylation.

Although multiple in vitro studies clearly support tumor-suppressor function of miR-137 [24,29], the clinical significance of miR-137 in particular CpG methylation is still not fully clear. In CRC miR-137 methylation was associated with presence of KRAS mutation [20]. In GC it was shown that miR-137 methylation is more common in non-cardia tumor and patients with miR-137 methylation had shorter overall survival [30]. However, the data are partly limited by the small number of patients without miR-137 methylation (nine subjects from Asian population). We confirmed that miR-137 methylation was lower in cardia tumor and the highest level was found in tumor localized in antrum. Furthermore, diffuse type GC had lower miR-137 methylation level compared to intestinal or mixed type, but no difference was found between TNM staging and grading (Suppl. Figure S2). Although, the overall survival was slightly higher for subjects without miR-137 methylation (cutoff 15%) and subjects with higher miR-137 expression, the difference did not reach statistical significance in comparison to Du et al. [30]. Interestingly, the trend for worse overall survival was found only for diffuse but not intestinal type GC, however, whether this finding is of the clinical significance needs further evaluation in larger cohort of patients in the future. It is important to mention that our study and the study from Du et al. [30] have several methodological (different methylation analyses assay) and analytical differences (different miR-137 methylation cut-off) that at least partially, explain the differences in some of results.

Our study provides an important insight into translational meaning of miR-137 methylation in GC, however, it has several limitations. We performed systematic multiple level high quality analyses for a miR-137, though many other interesting miRNA-targets may be of the greater value. Next, survival data and *H.pylori* status for GC patients were available not for the complete cohort. Furthermore, based on the low variation in miR-137 expression in GI diseases, we concluded that miR-137 methylation rather than miR-137 expression may play more important role in gastric carcinogenesis. To this support, in an additional set of experiments, we show that miR-137 promoter methylation and miR-137 expression did not correlate with previously identified miR-137 targets (Cdc42 and COX-2). Although, we cannot explain this observation at present, it is possible that functional role of a single microRNA is not always easy to access in biopsies or surgical specimens because of heterogeneous cell population. At the same time, the missing correlation indeed could also be an indirect evidence for the limited functional role of miR-137 that needs to be clarified in further systematic patients-based studies. And finally, additional analyses have to address if miR-137 methylation could be some epiphenomena which correlates with early process of gastric carcinogenesis, as it is inversely correlates with one of the most frequent hallmarks of carcinogenesis, global hypomethylation and the function output in gastric tissue, such as decreased miR-137 expression, is rather moderate.

In summary, this study demonstrates that miR-137 is frequently silenced in GC and CRC through CpG island promoter hypermethylation. Methylation of miR-137 increases in a step-wise fashion from normal gastric mucosa to chronic gastritis and GC tissues, indicating that it is an early and progressive event in gastric carcinogenesis. Despite the correlation between miR-137 methylation and miR-137 expression, we suggest that miR-137 may be an epigenetic phenomenon that inversely correlates with global hypomethylation. Differences in miR-137 expression between gastric and colon mucosa are suggestive for unique functional differences between GI regions that needs further evaluation.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

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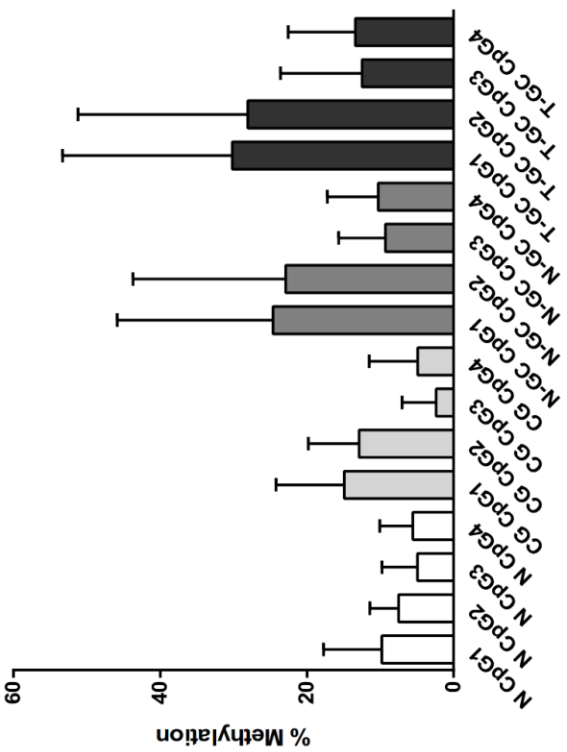
Suppl. Figure S1



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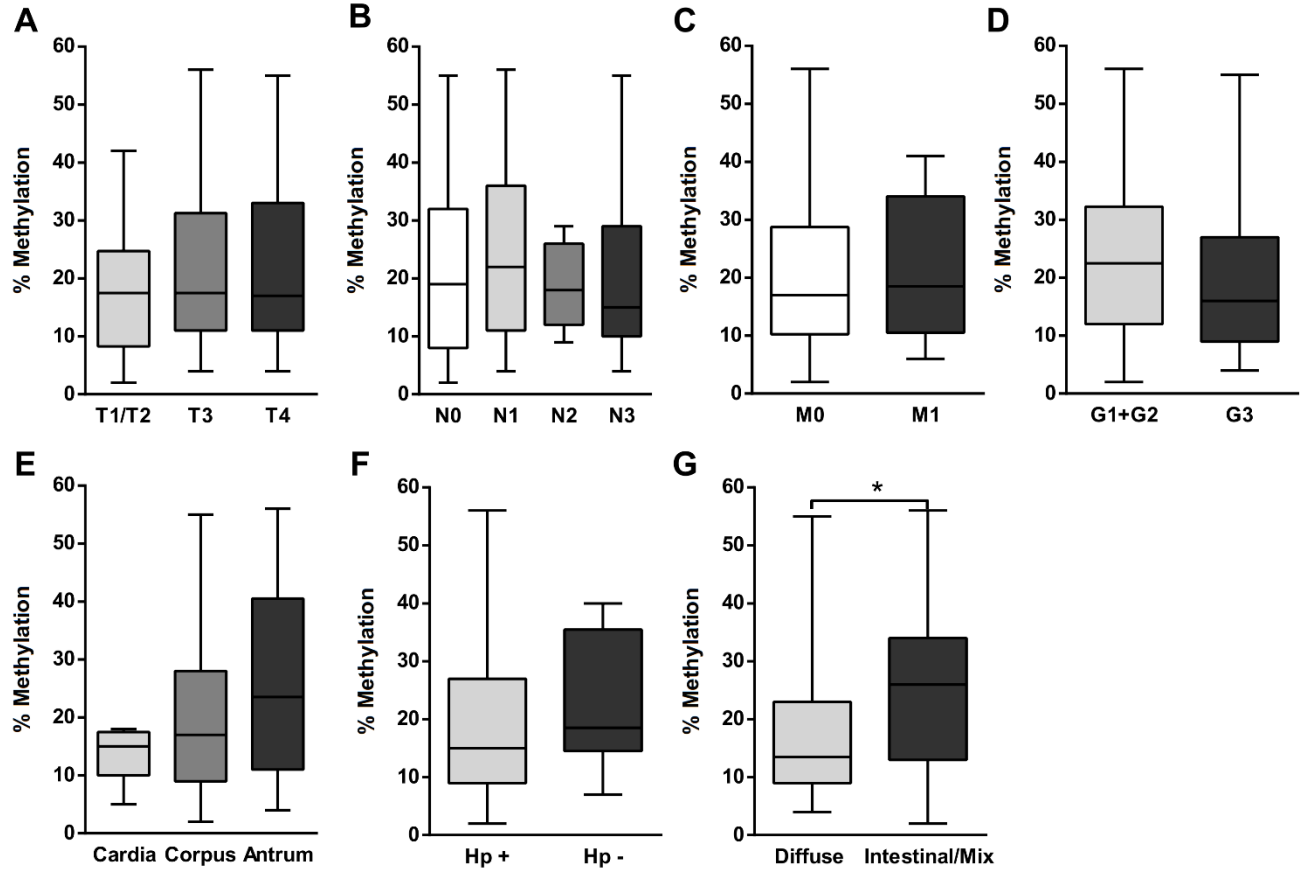

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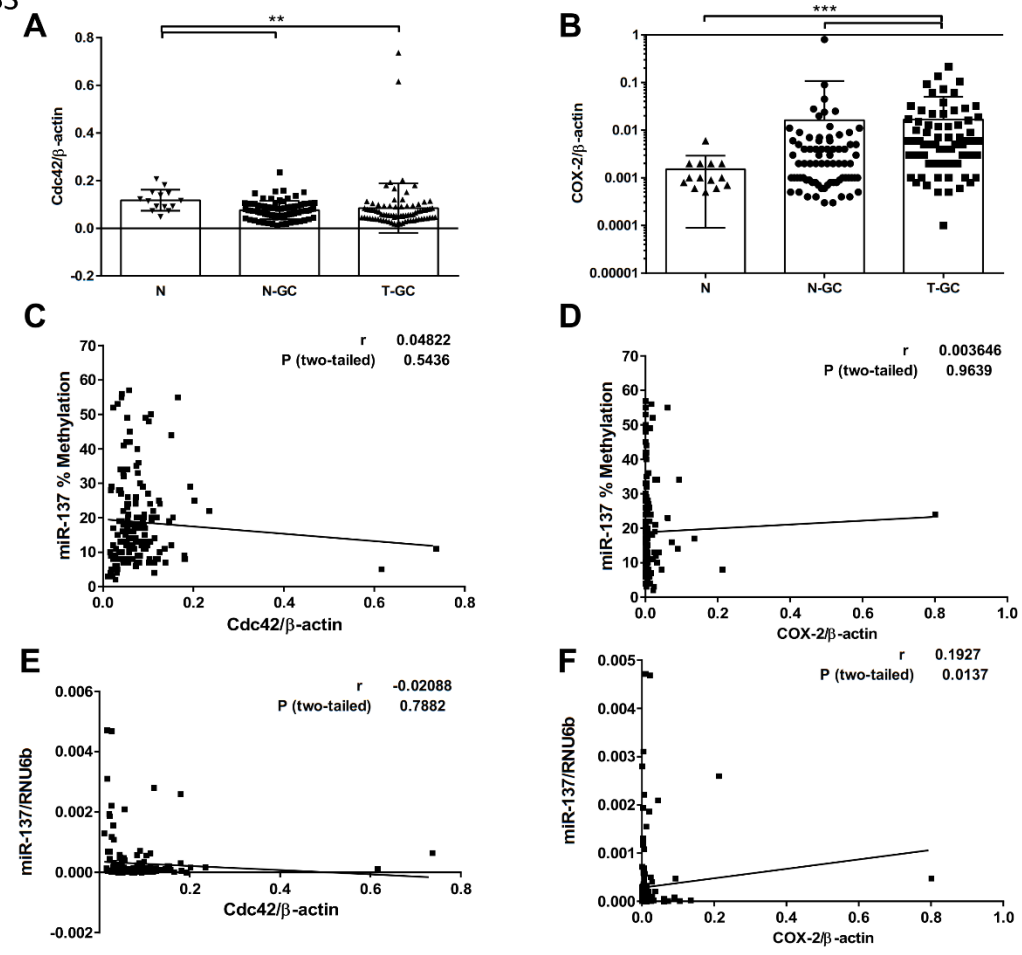

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LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis

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LINE-1 hypomethylation is widely accepted as marker for global genomic DNA hypomethylation, which is a frequent event in cancer. The aim of the study was to evaluate LINE-1 methylation status at different stages of gastric carcinogenesis and evaluate its prognostic potential in clinical settings. LINE-1 methylation was analyzed in 267 tissue samples by bisulfite pyrosequencing including primary colorectal cancer tissues (T-CRC) with corresponding adjacent colon mucosa (N-CRC), gastric cancer tissues (T-GC) with corresponding gastric mucosa (N-GC), normal gastric tissues (N), chronic non-atrophic and atrophic gastritis (CG). LINE-1 methylation level was lower in both T-GC and T-CRC when compared to paired adjacent tissues. No difference was observed for LINE-1 methylation status between patients with normal gastric mucosa, CG and N-GC. LINE-1 methylation in T-GC but not N-GC tended to correlate with age. Subgroup stratification analysis did not reveal significant differences in LINE-1 methylation status according to tumor stage, anatomical location, histological subtype, differentiation grade. We observed similar overall survival data between patients with high or low LINE-1 levels. In summary, LINE-1 hypomethylation is a characteristic feature in GC but not very common in early preneoplastic stages of gastric carcinogenesis. Prognostic role of LINE-1 hypomethylation in GC patients could not be confirmed in this cohort.

Gastric cancer (GC) remains a major healthcare burden across the globe and ranks as the second most common cause of cancer-related mortality[1]. The disease becomes clinically apparent mostly in advanced stages leading to the poor patients' outcomes[2]. Gastric carcinogenesis results from the accumulation of multiple factors and characterized by a step-wise process from *Helicobacter pylori* (*H. pylori*) induced chronic active gastritis, to atrophic gastritis with intestinal metaplasia, dysplasia and adenocarcinoma[3]. Underlying molecular alterations that progress from gastritis to gastric cancer have been explored, but the exact mechanisms and interactions with risk factors remain unclear. Identification and description of carcinogenesis-related biological processes across all stages of gastric carcinogenesis are highly desirable for translational purposes in order to improve diagnostic and therapeutic strategies[4].

Epigenetics is a crucial element involved in regulation of genetic stability in different malignancies[5]. DNA methylation is the most extensively studied epigenetic phenomenon in a wide range of diseases[6]. Global hypomethylation refers to decrease in DNA methylation across the entire genome and is linked with genetic instability and procarcinogenic events[7]. Hypomethylation of the entire genome partially results from demethylation in repetitive elements that account for about a half of the human genome. This is essential in gene regulation and genomic stability[8]. Long Interspersed Nucleotide Element 1 (LINE-1) is one of the major genetic elements which constitute ~17% of the genome[8]. CpG sites located within LINE-1 and their methylation levels correlate with the global genomic DNA methylation status[9]. Therefore, LINE-1 status is frequently used as surrogate marker for estimation of global DNA hypomethylation[10]. LINE-1 hypomethylation has been frequently reported in different types of cancer especially colorectal cancer (CRC)[5,11,12]. Furthermore, LINE-1 methylation status has been suggested as a potential biomarker for cancer detection and disease outcomes[11,13,14].

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Total		Gastric cancer n(%) n = 80	Colorectal cancer n(%) n = 24	Chronic/atrophic gastritis n(%) n = 37	Controls n(%) n = 19
Age	mean (SD)	65.9 (11.7)	69.3 (8.8)	57.3 (13.0)	49.1 (14.9)
Gender	Female	33 (41.3)	11 (45.8)	25 (67.6)	12 (63.2)
	Male	47 (58.7)	13 (54.2)	12 (32.4)	7 (36.8)
Tumor localization	Cardia	8 (10.0)	—	—	—
	Corpus	44 (55.0)	—	—	—
	Antrum	28 (35.0)	—	—	—
	Proximal colon	—	9 (37.5)	—	—
	Distal colon	—	15 (62.5)	—	—
TNM staging	I	15 (18.7)	2 (8.3)	—	—
	II	21 (26.3)	10 (41.7)	—	—
	III	36 (45.0)	6 (25.0)	—	—
	IV	8 (10.0)	4 (16.7)	—	—
	Unknown	—	2 (8.3)	—	—
T	1/2	17 (21.3)	2 (8.3)	—	—
	3	36 (45.0)	18 (75.0)	—	—
	4	27 (33.7)	1 (4.2)	—	—
	Unknown	—	3 (12.5)		
N	0	28 (35.0)	13 (54.2)	—	—
	1	15 (18.7)	7 (29.2)	—	—
	2	13 (16.3)	2 (8.3)	—	—
	3	22 (27.5)	—	—	—
	Unknown	2 (2.5)	2 (8.3)		
M	0	72 (90.0)	8(33.3)	—	—
	1	8 (10.0)	4 (16.6)	—	—
	Unknown		12 (50.0)		
G	1 and 2	31 (38.8)	18 (75.0)	—	—
	3	49 (61.2)	3 (12.5)	—	—
	Unknown	—	3 (12.5)	—	—
Lauren's classification	Diffuse	44 (55.0)	—	—	—
	Intestinal	25 (31.3)	—	—	—
	Mixed	7 (8.7)	—	—	—
	Unknown	4 (5.0)	—	—	—
H. pylori infection	Positive	17 (21.3)	—	25 (67.6)	—
	Negative	8 (10.0)	—	12 (32.4)	19 (100)
	Unknown	55 (68.7)	—	—	—

Table 1. Characteristics of patients included in the LINE-1 methylation analysis: controls, gastritis, gastric cancer and colorectal cancer patients.

LINE-1 methylation levels in tissues and blood samples of gastric cancer patients have been analyzed in several studies previously suggesting lower LINE-1 methylation as a characteristic event in GC[12,14–17]. Of the relevance was the finding that LINE-1 hypomethylation may be associated with poor survival in Asian patients with GC[12,18]. The analysis of LINE-1 methylation levels in DNA samples derived from blood of GC patients suggests furthermore potential diagnostic implications[16,17]. To date, there have been several attempts to define LINE-1 methylation levels in premalignant lesions. Some of them showed a gradual hypomethylation across preneoplastic stages with gradual progression during gastric carcinogenesis[18–20]. The data on LINE-1 methylation status across different stages of gastric carcinogenesis are still limited. Most of reported studies have been conducted on Asian GC patients, while data on LINE-1 methylation levels in European subjects with GC and premalignant gastric lesions is largely unexplored[14]. It is also worth pointing out that several studies have already employed pyrosequencing method for LINE-1 analysis in GC, which is considered very robust technical modality used for LINE-1 methylation analyses[21,22].

The aim of the present study was to perform a comparison analysis on LINE-1 methylation level in gastric carcinogenesis. First, we compare LINE-1 methylation in tumor and non-tumor tissues in GC and CRC patients. Further, we elucidate the changes in preneoplastic conditions and compared them to methylation in normal mucosa. For the evaluation of the prognostic role of LINE-1 methylation, we performed survival analyses.

First Author	Year	Tissue origin	Tissues	Micro-dissection	Gastric cancer	Paired samples	Healthy controls	Preneoplastic/-cancerous stages	*H. pylori* status	Methods	LINE-1 in GC	Survival analysis	Ref.
Kupcinskas *et al.*	2017	Europe (mix)	fresh-frozen	no	80	yes	19	37	yes	PyroSeq	↓ in GC	↔	
Song *et al.*	2016	Korea	FFPE	yes	434	no	no	no	no	PyroSeq		↓	34
Kim *et al.*	2016	Korea	FFPE	yes	no	no	no	89	yes	COBRA	↓ in HGIEN		40
Kosumi *et al.*	2015	Japan	FFPE	yes	87	yes	17	20	yes	PyroSeq	↓ in GC		41
Yang *et al.*	2014	Korea	FFPE, fresh-frozen	yes	88/115	no	22	39	yes	PyroSeq	↓ in GC		42
Shigaki *et al.*	2013	Japan	FFPE	yes	203	yes	no	no	no	PyroSeq	↓ in GC	↓	12
Saito *et al.*	2012	Japan	fresh-frozen	no	101	yes	83	82	yes	qMSP	↓ in GC		43
Pavicic *et al.*	2012	Europe (Finland)	FFPE	yes	58	yes	no	no	no	COBRA MS-MLPA	↓ in GC		15
Bae *et al.*	2012	Korea	FFPE	yes	198/59	no	no	190	yes	PyroSeq	↓ in GC/ adenoma	↓	18
Balassiano *et al.*	2011	Europe (mix)	FFPE	no	98/20	yes	15	no	no	PyroSeq	↓ in GC		44
Lee *et al.*	2011	Korea	FFPE	yes	53	yes	no	79	yes	COBRA	↓ in GC/ HGIEN		20
Yoshida *et al.*	2011	Japan	fresh-frozen	no	52	no	34	76	yes	PyroSeq	↓ in GC		45
Park *et al.*	2009	Korea	FFPE	yes	59	no	no	143	yes	COBRA	↓ in GC		19

Table 2. Summary of the studies related to LINE-1 methylation in gastric cancer patients. qMSP: quantitative MSP (real-time); NA: non-available; FFPE: formalin-fixed paraffin-embedded tissue; PyroSeq: pyrosequencing; GC: gastric cancer; HGIEN: high-grade intraepithelial neoplasia; Ref: references.

Results

LINE-1 methylation in CRC and GC. Methylation status in LINE-1 has been extensively studied in CRC; therefore, we included a group of patients with CRC for comparative analysis (Table 1). We performed quantitative LINE-1 methylation analysis in a cohort of paired primary CRC tissues (T-CRC) with corresponding adjacent tumor-free colonic mucosa (N-CRC). Lower LINE-1 methylation was found in T-CRC compared to N-CRC (mean ± SD: 61.15 ± 6.38% vs. 67.17 ± 4.84%, respectively, $p = 0.0005$; Fig. 1A). In patients with GC, LINE-1 methylation level was also lower in T-GC tissues compared to adjacent N-GC (62.48 ± 8.15% vs. 65.73 ± 4.56%, respectively, $p = 0.002$; Fig. 1B). Absolute number of tissues with lower LINE-1 methylation in tumorous tissue compared to non-tumorous was higher in CRC compared to GC (69.6% vs. 53.8%, respectively) (Fig. 1C and D).

LINE-1 methylation in preneoplastic gastric mucosa. One of the major aims of our study was to evaluate LINE-1 methylation status at different stages of gastric carcinogenesis. For this reason, we performed LINE-1 methylation analysis in individuals with normal gastric mucosa without *H. pylori* infection and in patients with chronic atrophic gastritis. We found that methylation of LINE-1 did not differ significantly between normal tissues (N), chronic gastritis group (CG/AG) and tumor-adjacent (N-GC) gastric mucosa (mean ± SD: 64.48 ± 2.93%, 65.08 ± 3.37%, 65.75 ± 4.56% ($p > 0.05$), respectively) (Fig. 2A). Furthermore, we compared the LINE-1 methylation level between chronic atrophic gastritis (AG) with intestinal metaplasia and CG but no significant difference was found (data not shown), suggesting that LINE-1 methylation is rather a rare event in early stages of Correa's cascade in gastric carcinogenesis (Fig. 2A). The only significant difference among gastric tissues with respect to LINE-1 methylation status was observed between N-GC and T-GC samples as described above (Fig. 2A). Analysis of LINE-1 methylation between N, N-GC and N-CRC revealed similar level, suggesting that LINE-1 may have relatively stable methylation pattern in GI tract in non-malignant tissues (Fig. 2B).

LINE-1 methylation correlation analysis. We further analyzed whether lower LINE-1 hypomethylation occurs simultaneously in tumorous and tumor-adjacent tissues. Analysis for LINE-1 methylation status in GC and CRC revealed no significant correlation between N-GC and T-GC ($r = 0.16$, $p = 0.15$, Fig. 3A) and between N-CRC and T-CRC ($r = 0.19$, $p = 0.37$, Fig. 3B), suggesting that global hypomethylation might be a focal tumor-specific event of cancerous tissues. In the next step, we evaluated the link between methylation level and patients' age. A trend towards significant correlation was observed between patient's age and LINE-1 methylation levels in T-GC tissue (r - 0.1918; $p = 0.0884$; Fig. 3C) and difference in LINE-1 methylation between T-GC and N-GC tissues (r - 0.1954; $p = 0.084$; Fig. 3D), however, the results did not reach the level of statistical significance.

LINE-1 methylation in GC subgroups. LINE-1 methylation in GC samples with different clinical and pathological characteristics are presented in Fig. 4. Hypomethylation level were similar in tumors arising from different anatomical sites of stomach such as cardia, corpus and antrum (Fig. 4A, $p = 0.41$). No difference was found between more and less advanced stages of GC (Fig. 4B–D; T- ($p = 0.20$), N- ($p = 0.11$) and M-tumor

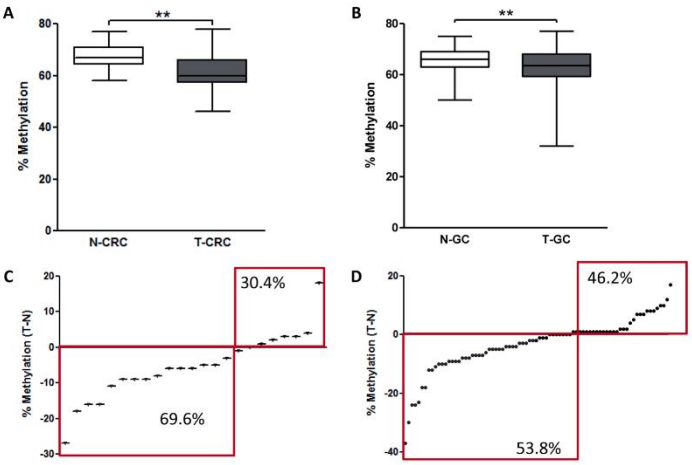

Figure 1. Quantitative LINE-1 methylation analyses in paired colorectal (CRC) and gastric cancer (GC) tissues. (**A**) LINE-1 methylation in paired T-CRC and adjacent N-CRC tissues (n = 24) (p = 0.0005). (**B**) LINE-1 methylation level in T-GC and adjacent N-GC tissues (n = 80) (p = 0.002). (**C** and **D**) Absolute difference between LINE-1 methylation in matching (**C**) T-CRC and N-CRC, and (**D**) T-GC and N-GC tissues. Wilcoxon test has been used for paired analyses **P < 0.005.

staging (p = 0.17) or tissues with low/medium (G1/2) and poorly differentiated (G3) tumors (p = 0.26; Fig. 4E). LINE-1 methylation status was similar between histological subtypes of GC – intestinal vs. diffuse (61.84 ± 7.97% and 63.32 ± 7.78%, respectively, p = 0.39, Fig. 4F). We also found no differences with respect to gender (p = 0.83) or preexisting *H. pylori* infection (p = 0.70), but these sub-analyses were limited by availability of the clinical/serological data for patients with GC within the study (Fig. 4G and H).

LINE-1 methylation and overall survival. Survival data for all 80 GC patients were available for analysis. The average overall survival time after disease onset was estimated to be 1015 days (range 9–2451 days). 60% cut-off value was selected to discriminate patients with high and low LINE-1 methylation based on the previous publications and the LINE-1 methylation distribution in our set of data (21.5% of samples with low methylation)[23,24] (Fig. 5A). Overall, there was a significant survival difference dependent on UICC stage (Fig. 5B), confirming the validity of the survival data in our cohort. We found no differences in survival between the patients with low compared to high LINE-1 methylation (Fig. 5C, p = 0.59). This was also true if we used the more stringent cut-off of 55 creating three groups with low, middle and high LINE-1 methylation groups (Fig. 5D). Survival analyses stratified by histological GC subtype also revealed no differences in survival (Fig. 5E and F).

Discussion

Findings of our study provide a detailed characterization of LINE-1 methylation status across preneoplastic and neoplastic stages of gastric carcinogenesis. LINE-1 hypomethylation did not differ significantly between normal gastric mucosa, chronic gastritis and tumor-adjacent tissues and was rare in preneoplastic mucosa, suggesting LINE-1 methylation predominantly as a late event in gastric carcinogenesis. More importantly, patients with low LINE-1 methylation in GC tissues showed no difference in overall survival compared to patients with high LINE-1 methylation.

Global DNA hypomethylation and CpG island promoter hypermethylation are characteristic features of various tumors[13,25]. For instance, we have previously reported site-specific CpG island promoter hypermethylation of miR-137 in GC tissue samples, which was inversely correlated with LINE-1 methylation status[25]. In the present study, in the line with the previous reports, we confirmed decreased methylation of LINE-1 in T-CRC compared to N-CRC[26,27]. In concordance to the results of other groups, similar observation was made for T-GC in comparison to LINE-1 methylation in N-GC (Table 2). For instance, in sporadic GC, both microsatellite stable (MSS) and unstable (MSI) GC tumors had lower LINE-1 methylation levels in tumorous tissues when compared to normal healthy mucosa[15]. Two larger studies from Japan and South Korea also showed lower LINE-1 methylation level in gastric cancer compared to matched non-tumorous gastric mucosa[12,18]. Although the absolute LINE-1 methylation levels differed between above mentioned studies, the difference may be explained by potential confounding factors including methodological approach, sample bias or region where the study was performed[14].

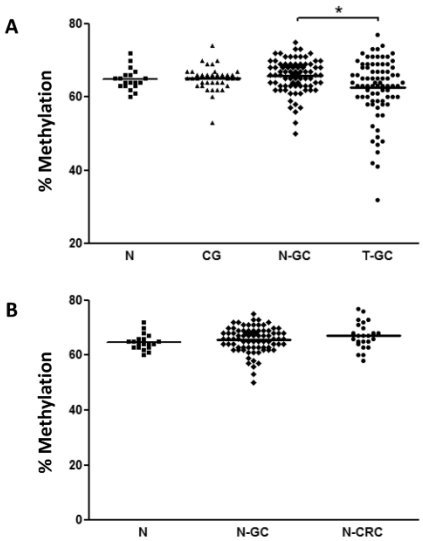

Figure 2. Quantitative LINE-1 methylation analyses in gastric and colon tissues. (**A**) LINE-1 methylation values were obtained using bisulfite pyrosequencing in gastric tissues from controls (N), patients with chronic gastritis (CG), adjacent non-tumor tissues (N-GC) and gastric cancer tumor tissues (T-GC) ($p > 0.05$). (**B**) LINE-1 methylation level comparison between normal gastric (N), adjacent non-tumor gastric tissues (N-GC) and non-tumoral adjacent colon tissue (N-CRC) ($p = 0.2$). Statistical analyses where performed using Mann-Whitney test $^*P < 0.05$.

Contrary to previous reports, our study has revealed that LINE-1 methylation level did not differ significantly between normal gastric tissue, CG and N-GC. Using a COBRA method, Park *et al.* found lower LINE-1 methylation already in preneoplastic lesion including CG[19]. A study by Bae *et al.* reported that LINE-1 methylation decreased during the transition from intestinal metaplasia to gastric adenoma while no further decrease occurred during the transition from gastric adenoma to GC as determined by pyrosequencing technique[18]. High-grade dysplasia had significantly lower LINE-1 methylation level compared to low-grade dysplasia and this difference was associated with high diagnostic sensitivity and specificity[20]. Unfortunately, histologically confirmed adenoma or dysplasia of the stomach are quite rare in European countries, therefore we could not address this issue in our work. Because of this limitation, we cannot exclude certain degree of LINE-1 methylation changes in early neoplastic stages. Furthermore, it is also possible that with larger number of samples with preneoplastic conditions we could potentially identify smaller changes, however, the fact that LINE-1 methylation levels were similar in N, CG, AG and N-GC and the range of methylation was quite constant this rather supports our conclusions. Another factor that needed to be taken into consideration is the difference in confounding factors (exp. diet) between Asian and European cohorts. This may contribute to pronounced alterations in LINE-1 during the earlier stages of gastric carcinogenesis[28]. It is important to mention that in our cohort we had a quite a large number of diffuse-type GC cases (55%). Diffuse histological sub-type of GC may often arise from normal gastric mucosa in the absence of premalignant gastric conditions[29] and direct comparison to molecular alterations in premalignant gastric lesions might be flawed. At this stage we can solely speculate for the difference in epigenetic "field defect" between diffuse and intestinal subtypes of GG.

In subgroup analysis, LINE-1 methylation analyses revealed no significant differences among analyzed subtypes including GC with different anatomical styles, various stages of GC, differentiation level. Furthermore, LINE-1 methylation status was similar between intestinal and diffuse subtypes of GC. Our results are supported by the study by Shigaki *et al.*, where the authors also found no difference while others did show the difference[12,18]. Because of this heterogeneity, the biological implication is probably questionable, although additional large studies may be needed to identify potential confounding factors.

Two studies in Asian population analyzed LINE-1 methylation level in regard of *H. pylori* infection where no association could be identified[18,19]. Our results confirm those data, showing missing association for both tumoral and non-tumoral tissues, which is also in concordance with our results to gastritis/preneoplastic conditions. On the other hand, Yamamoto *et al.* showed significantly reduced level of LINE-1 methylation in gastric mucosa of

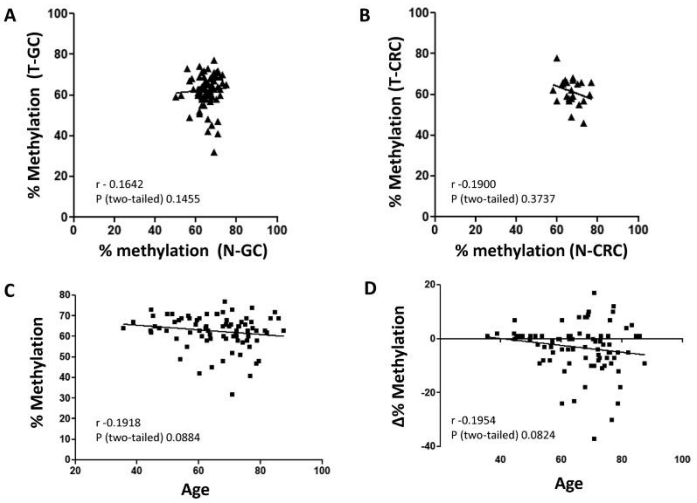

Figure 3. Correlation between LINE-1 methylation status in tumorous and adjacent non-tumorous tissue. LINE-1 methylation obtained using bisulfite pyrosequencing did not correlate between (**A**) N-GC and T-GC and (**B**) N-CRC and T-CRC tissues. (**C** and **D**) Correlation analysis between patient's age and (**C**) absolute LINE-1 methylation in T-GC; and (**D**) difference in LINE-1 methylation between T-GC and N-GC tissues. Analyses were performed using Spearman's test.

patients with enlarged-fold gastritis, which is strongly associated with *H. pylori* infection[30]. Taking together, the direct impact of *H. pylori* infection is still not fully understood. For instances, the strong infiltration of inflammatory cells of the mucosa due *H. pylori* could have an impact on global LINE-1 methylation while being different in damaged preneoplastic mucosa.

In our study, we observed a trend towards negative correlation between patient's age and LINE-1 hypomethylation in GC tissue. Bae *et al.* have reported a similar negative correlation between LINE-1 methylation level of GC and the patient age in male but not in female patients[18]. Another study assessing age-dependent hypomethylation suggested that age was negatively associated with methylation levels of Alu, but not LINE-1[31]. Since the highest risk of GC is in older population, we speculate that age-associated global hypomethylation may contribute to gastric carcinogenesis, however, this point need to be addressed in specifically designed studies.

Hypomethylation has been linked to the worse overall survival of the patients with multiple tumors including CRC, liver, lung and ovarian cancers[32]. Nevertheless, the exact mechanism is not fully understood. Opposite correlation has been, however, demonstrated in melanoma where LINE-1 hypomethylation was associated with a favorable outcome[33]. In our cohort of patients, we observed no differences in overall survival between the patients with low or high LINE-1 methylation. This was also true for different GC subtypes. Our results do not support existing data to prognostic role of LINE-1 in GC patients. For instance, LINE-1 hypomethylation was significantly associated with shorter overall survival in large cohort of GC patients from Japan[12] and South Korea[18]. Higher proportion of patients with diffuse GC according to Lauren's classification could be one of the explanation. Another explanation may be the difference in tumor biology between tumor in Europe and Asia. Majority of previously published papers come from Asian countries with predominantly intestinal type GC patients included in the studies ranging from 39% to 64% in study populations[12,18]. Large number (55% of all cancer cases) of GC cases in our study were diffuse-type according to Lauren's classification. At least applicable for our European population, our results do not support the prognostic value of LINE-1 methylation in GC patients.

LINE-1 methylation status in gastric cancer and premalignant gastric conditions among European subjects remains poorly investigated and our study provides valuable insights for perception of stepwise development of GC. Here, we performed a systematic analysis of the literature to the topic of LINE-1 methylation in gastric cancer. Table 2 summarizes the differences between various studies including tissue origin, performance of microdissection, applied methods and main output. While three studies show an association between LINE-1 methylation and worse prognosis, in our European cohort we failed to confirm those results. Although, this could be related to specific tumor biology, there is also several other factors that need to be mentioned. In comparison to survival studies from Asia[12,18,34], we analyzed surgically- or endoscopically-obtained samples without prior microdissection; therefore, we could not evaluate the purity of the tumor. This limitation does not allow direct comparison to

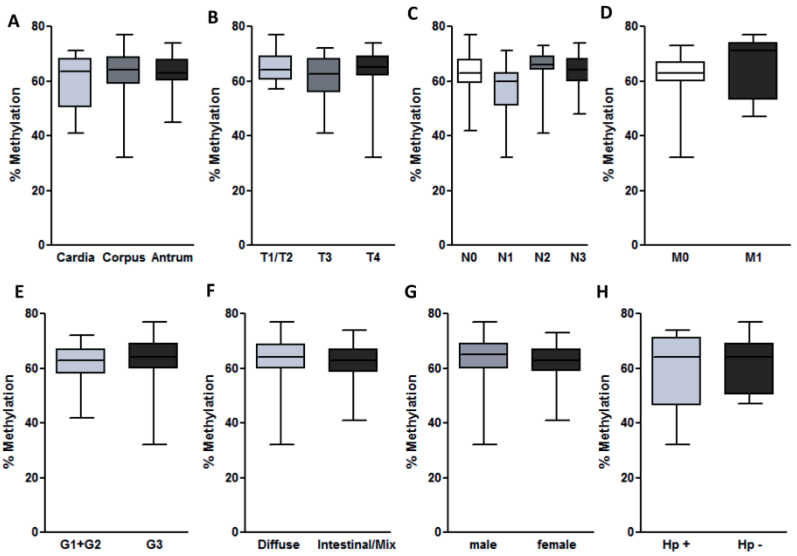

Figure 4. Subgroup analyses of LINE-1 methylation in gastric cancer patients according to clinicopathological data. LINE-1 methylation analyses based on (**A**) anatomical tumour localization ($p = 0.41$), (**B**) T- ($p = 0.20$), (**C**) N-, ($p = 0.11$) and (**D**) M-tumor staging ($p = 0.17$). (**E**) LINE-1 methylation differences in patients with low and high-grade tumors ($p = 0.26$). LINE-1 methylation differences in GC patients according to (**F**) Lauren's classification of GC type ($p = 0.39$), (**G**) gender ($p = 0.83$) and (**H**) *H. pylori* status ($p = 0.70$). Statistical analyses were performed using Mann-Whitney for two and Kruskal-Wallis test with Dunn's posttest for multiple comparison analyses.

existing studies since the proportion of tumor cells (in particular in diffuse gastric cancer cells) may be variable. In similar fashion, we did not perform microdissection of epithelial cells in preneoplastic and the small amount of LINE-1 hypomethylation is still possible. Nevertheless, our results are important from the translational point of view highlighting the potential limitation of LINE-1 methylation analysis in everyday clinical praxis.

Overall, our results confirm that LINE-1 hypomethylation is characteristic feature in GC tissues. Since only marginal difference in LINE-1 hypomethylation was observed in preneoplastic tissues, we conclude that the global hypomethylation may be rather an end stage event in gastric carcinogenesis. In this European cohort of patients, LINE-1 methylation showed no association to an overall survival of GC patients.

Materials and Methods

Tissue samples. Tissue samples were collected at two clinical centers: Department of Gastroenterology and Surgery, Hospital of Lithuanian University of Health Sciences (Kaunas, Lithuania) and Department of Gastroenterology, Hepatology and Infectious Diseases Otto-von-Guericke University (Magdeburg, Germany) under the frame of the ERA-Net PathoGenoMics project. The study protocol was approved by Kaunas Regional Biomedical Research Ethics Committee (Protocol Nr. 8/2011) and by the Institutional Review Board of Otto-von-Guericke University Magdeburg (Protocol Nr. 80/2011). The study was performed according to the guidelines of Declaration of Helsinki. All patients participating in the study have signed an informed consent form.

Study design. Study design and tissue collection protocol has been partly described in the previous study[25,35]. For the LINE-1 methylation analyses, we had available 267 tissue specimens (biopsies and surgical material) including: 80 GC tumor tissues (T-GC) with corresponding adjacent non-tumorous gastric mucosa (N-GC) from GC patients; normal gastric mucosa tissue from 19 controls (N); 37 gastric antrum tissues from patients with chronic non-atrophic and atrophic gastritis with/-out intestinal metaplasia (CG); 24 primary CRC tumor tissues (T-CRC) with corresponding adjacent non-tumorous colonic mucosa (N-CRC). N and CG samples were obtained during upper GI endoscopy and were characterized histologically according to the updated Sydney classification[36]; the presence of *H. pylori* was additionally investigated by serology (ELISA IgG test, Virion\Serion

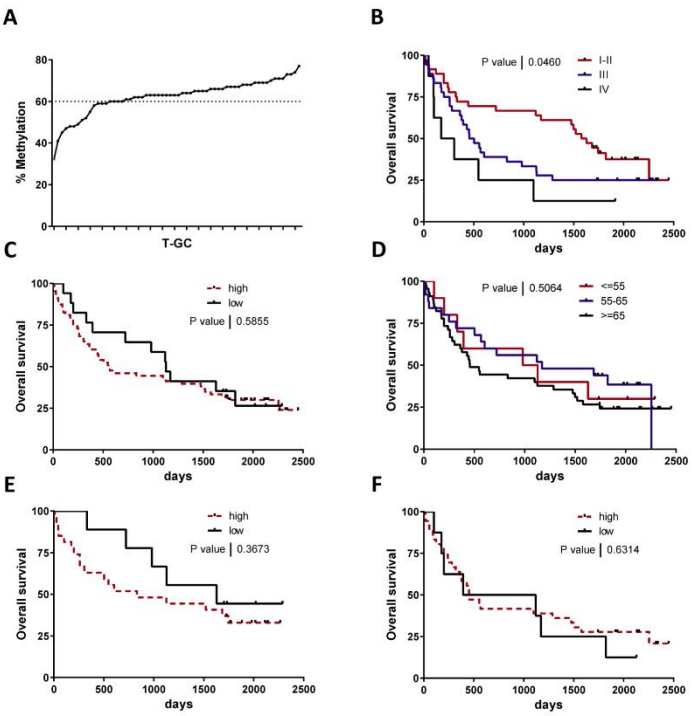

Figure 5. Overall survival analyses of patients with GC based on LINE-1 methylation. (**A**) Patients with GC with high and low LINE-1 methylation status defined by cut-off 60% based on LINE-1 methylation in T-GC sample (low LINE-1 methylation 21.5%). (**B**) Kaplan-Meier analyses based on UICC tumor stage with significant surivival difference among the groups. (**D**) Kaplan-Meier analyses based on high (n = 63) and low (n = 17) LINE-1 methylation status (cut-off methylation 60%) (p = 0.59). (**D**) Kaplan-Meier analyses based on high ≥ 65% (n = 45), middle > 55% and <65% (n = 25) and low ≤55% (n = 10) LINE-1 methylation status (p = 0.51). (**E**) Kaplan-Meier analyses of survival difference based on LINE-1 methylation status in intestinal/mixed type GC (p = 0.36) and in (**F**) diffuse type GC (p = 0.63). Statistical comparison between curves was performed with Log-rank (Mantel-Cox) test.

GmbH, Germany or Helicobacter pylori IgG ELISA Kit Biohi, Helsinki, Finland) and microbiological analysis as reported previously[37]. Histological subtypes of GC patients were determined using Lauren's criteria. All tissue samples were histologically examined and it was confirmed as non-tumorous or tumorous tissue. Biopsies from the patients N/CG/AG were obtained during endoscopy and same region samples were used for histological evaluation and methylation analysis. Tissue samples for methylation studies were immediately snap-frozed in liquid nitrogen and stored at −80 °C until analyses. Detailed characteristics of the subjects included in the study are presented in Table 1.

Survival analyses. The survival data of 80 GC patients were retrieved from Lithuanian Cancer Registry and medical records at Hospital of Lithuanian University of Health Sciences. The time interval between the date of GC onset and the date of death was defined as overall survival of GC patients. The patients, who were still alive at the moment of data collection, were censored as dead as for 28th February, 2017. For survival analysis we used a cut-off value of 60% at LINE-1 (high *vs.* low methylation levels). This selection was based on the observation of several previously published papers where cut-off of 55/65% has been suggested as appropriate to define the subjects with global hypomethylation[23,24]. Survival data were analyzed using Kaplan-Meier survival curves.

DNA isolation. DNA for methylation analyses from tissue samples was extracted as described previously[25]. Briefly, DNA was isolated with QIAzol Lysis reagent and chloroform, using the interphase, according to user-developed protocol (QIAGEN, Valencia, CA, USA). Qualitative and quantitative testing of extracted DNA samples was performed spectrophotometrically using Biophotometer (Eppendorf, Germany).

DNA methylation analyses. Bisulfite conversion of purified genomic DNA was performed using Cells-to-CpG™ Bisulfite Conversion Kit (Life Technologies) according to the manufacturer's protocol. After PCR using biotin-labeled LINE-1 region primers, the success of reaction was verified in agarose gel (1%) electrophoresis and no-template controls. For quantitative methylation analyses we used bisulfite pyrosequencing of LINE-1, which was performed on PyroMark Q96 ID (QIAGEN) using PyroMark® Gold Q96 reagents (QIAGEN) according to manufacturer's instructions. As previously described, we accessed LINE-1 X58075 103–249 bp region with mean of 4 CpG-sites[38,39]. LINE-1 primers: forward TTTTGAGTTAGGTGTGGGATATA, reverse 5′-biotin-AAAATCAAAAAATTCCCTTTC and pyrosequencing AGTTAGGTGTGGGATATAGT. Briefly, biotin-labeled PCR products were first captured on streptavidin-coated magnetic beads and then underwent pyrosequencing procedure. Mean methylation level of 4 measured CpG sites was used for the further analyses. Samples with poor DNA quality and/or repeatedly insufficient bisulfite conversion were excluded from further analyses.

Statistical analysis. The statistical analyses were performed using GraphPad Prism 6.0 statistical software (San Diego, CA, USA). Data were presented as mean % methylation ± standard deviation (mean ± SD) and absolute numbers with proportions (n, %) where appropriate. Quantitative variables for nonparametric analyses were performed using Wilcoxon text for paired and Mann-Whitney U test for unpaired analyses. For multivariate analyses, we used Kruskal-Wallis test with Dunn's multiple comparison *post test*. Correlation analyses were performed using Spearman's Test, and Log-rank (Mantel-Cox) test was used to compare survival curves. Two-sided p-values of <0.05 were considered statistically significant in all tests.

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Author Contributions

A.L., J.K., L.K., P.M.: study concept and design; obtaining funding; J.K., R.S., A.L.: analyses and interpretation of data, drafting of manuscript; G.S.: data collection and analysis; J.K., J.S., A.L., P.M.: provided clinical materials; R.S., C.L.: performed the experiments; all authors approved the final version of the manuscript.

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Gene Polymorphisms of Micrornas in *Helicobacter pylori*-Induced High Risk Atrophic Gastritis and Gastric Cancer

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Abstract

Background and aims: MicroRNAs (miRNAs) are known for their function as translational regulators of tumor suppressor or oncogenes. Single nucleotide polymorphisms (SNPs) in miRNAs related genes have been shown to affect the regulatory capacity of miRNAs and were linked with gastric cancer (GC) and premalignant gastric conditions. The purpose of this study was to evaluate potential associations between miRNA-related gene polymorphisms (*miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608*) and the presence of GC or high risk atrophic gastritis (HRAG) in European population.

Methods: Gene polymorphisms were analyzed in 995 subjects (controls: n = 351; GC: n = 363; HRAG: n = 281) of European descent. *MiR-27a* T>C (rs895819), *miR-146a* G>C (rs2910164), *miR-196a-2* C>T (rs11614913), *miR-492* G>C (rs2289030) and *miR-608* C>G (rs4919510) SNPs were genotyped by RT-PCR.

Results: Overall, SNPs of miRNAs were not associated with the presence of GC or HRAG. We observed a tendency for *miR-196a-2* CT genotype to be associated with higher risk of GC when compared to CC genotype, however, the difference did not reach the adjusted *P*-value (odds ratio (OR) - 1.46, 95% confidence interval (CI) 1.03-2.07, $P = 0.032$). *MiR-608* GG genotype was more frequent in GC when compared to controls (OR −2.34, 95% CI 1.08–5.04), but significance remained marginal ($P = 0.029$). A similar tendency was observed in a recessive model for *miR-608*, where CC + CG vs GG genotype comparison showed a tendency for increased risk of GC with OR of 2.44 (95% CI 1.14–5.22, $P = 0.021$). The genotypes and alleles of *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* SNPs had similar distribution between histological subtypes of GC and were not linked with the presence of diffuse or intestinal-type GC.

Conclusions: Gene polymorphisms of *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492*, *miR-492a* and *miR-608* were not associated with the presence of HRAG, GC or different histological subtypes of GC in European subjects.

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Introduction

Despite decreasing incidence of gastric cancer (GC) in most developed countries, GC accounted for a total of 989,600 new cases and 738,000 deaths in 2008 worldwide [1]. *Helicobacter pylori* (*H. pylori*) infection in gastric mucosa may lead to the development of atrophic gastritis (AG) and intestinal metaplasia (IM) and is a cardinal risk factor for development of GC [2]. Nevertheless, the development of gastric adenocarcinoma cannot be explained by the presence of *H. pylori* alone. Synergistic effects of environmental, host, nutritional and bacterial factors are believed to trigger gastric carcinogenesis, however, the mechanisms and interaction are still poorly understood [3]. A number of gene alterations have been implicated in gastric carcinogenesis, but none of them has been yet transferred to daily clinical practice due to the lack of association strength [4]. Recent data suggest a potential influence of single nucleotide polymorphisms (SNPs) of microRNA-related genes (miRNAs) for the risk of cancer development [5].

MiRNAs are only ~22 bp long, and because of its unique biogenesis are highly stable in different tissues or specimens, making them an attractive target in biomarker research field [6,7]. A growing number of functional studies suggest that miRNAs may

be involved in different stages of gastric carcinogenesis [8]. This concordance is also reflected in miRNA profiling studies that revealed specific alterations in expression pattern in mucosa of GC patients. Furthermore, miRNA expression changes are already detectable in early stages of gastric carcinogenesis including *H. pylori* induced AG [8].

Increasing understanding of functional role of miRNAs has opened a new capitel in gene polymorphism research in cancer [7,9]. Single miRNA is capable of targeting multiple genes; therefore, the significance of the SNPs in miRNA gene sequence may potentially be associated with remarkable alterations in regulation of gene expression and modification of the risk to development of certain human diseases, including GC [8]. Different studies have shown that certain SNPs of miRNA-encoding genes may alter miRNA expression or its functional role and in this way influence the risk of cancer development or progression [10,11]. Growing number of case-control studies have shown association between the polymorphisms of the genes encoding miRNAs and the risk of different malignancies [5]. Gene polymorphisms of five miRNA-related genes, *miR-27a* T>C (rs895819), *miR-146a* G>C (rs2910164), *miR-196a-2* C>T (rs11614913), *miR-492* G>C (rs2289030) and *miR-608* C>G (rs4919510), have been chosen for the present study because of previously suggested association with cancer risks [5,12–14].

The above mentioned miRNAs have been implicated in various cancer-related pathways. Liu et al. showed that *miR-27a* is up-regulated in GC and acts as an oncogene by targeting prohibitin [15]. SNP of *miR-27a* (rs895819) contributes to GC susceptibility through affecting the expression of *miR-27a* and targets gene zinc finger and BTB domain containing 10 (*ZBTB10*) [16]. *MiR-146a* was shown to modulate *H. pylori* induced inflammatory response in human gastric epithelial cells by targeting IL-1 associated kinase 1 (*IRAK1*) and TNF receptor associated factor 6 (*TRAF6*) [17]. Furthermore, *miR-146a* SNP (rs2910164) has been associated with AG in Japanese subjects [18]. Previous studies have demonstrated aberrant over expression of *miR-196a-2* and consequent down regulation of p27 (kip1) in GC [16]. Another study has shown that gene polymorphism of *miR-196a-2* (rs11614913) was associated with increased risk for GC [19,20] while a study by Ahn et al. (2012) could not confirm this association, but this SNP was linked with survival in GC patients [21]. *MiR-492* was identified to play an important role in the progression of malignant embryonic liver tumors [22] and is deregulated in colorectal cancer when compared to normal colon mucosa [23]. A report in Chinese population showed that SNP of *miR-608* (rs4919510) may influence HER2-positive breast cancer risk and tumor proliferation [14].

Most of the currently published genotyping studies related to miRNA genes in GC were performed only in Asian subjects, are limited by small sample sizes and report partially conflicting results. Furthermore, very few studies have previously addressed the role of these miRNA-related SNPs in premalignant gastric conditions. Based on the evidence provided above, we performed systematic genotyping analysis for *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* SNPs in patients with GC, high risk atrophic gastritis and controls using three groups of patients from Germany, Lithuania and Latvia of European descent.

Methods

Ethics statement

The study was approved by the Ethics Committees of the Otto-von-Guericke University Magdeburg, Lithuanian University of Health Sciences and Central Medical Ethics Committee of of Latvia. All patients have signed an informed consent form to participate in the study.

Study population

Subjects included in the study come from our previous research projects on SNPs in GC and premalignant gastric conditions [24,25]. Patients and controls were recruited during the years 2005–2012 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). Patients with HRAG and controls were included from the out-patient departments, who were referred for upper endoscopy because of dyspeptic symptoms. The inclusion criteria of HRAG and controls were no history of malignancy, gastrointestinal disease or surgery. GC patients had histological verification of gastric adenocarcinoma and were recruited from out-patient and stationary departments. In total, 995 individuals, for whom material with appropriate DNA quality was available (351 controls, 281 HRAG and 363 GC), were included in the study. There were 310 subjects from German group (63 controls, 106 GC and 141 HRAG), 340 subjects from Latvian group (142 controls, 139 GC and 59 HRAG) and 345 subjects from Lithuanian group (146 controls, 118 GC and 81 HRAG). All patients were of European descent.

Histological analysis and *H. pylori* status

Detailed histological evaluation of gastric mucosa was performed in controls and HRAG groups according to the modified Sydney classification [26]. HRAG was defined as pan-gastritis (similar inflammatory scores in antrum and corpus), corpus-predominant gastritis with or without the presence of gastric atrophy, and IM either in antrum or corpus of the stomach [27]. *H. pylori* status was determined by testing for anti-*H. pylori* IgG antibodies in sera. Histological subtyping of GC was carried out according to the Laurén classification into intestinal and diffuse-types [28].

DNA extraction and genotyping

Genomic DNA from German group was extracted from peripheral blood mononuclear cells using the QIAamp DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA from samples of Lithuanian and Latvian groups was extracted using phenol-chloroform extraction method from peripheral blood mononuclear cells. DNA samples were stored at −20°C until analysis. SNPs of *miR-27a* C>G (rs895819), *miR-146a* C>G (rs2910164), *miR-196a-2* C>T (rs11614913), *miR-492* C>G (rs2289030) and *miR-608* C>G (rs4919510) were genotyped by using predesigned TaqMan® assays with a 7500^{TM} real-time cycler, in accordance with the manufacturer's instructions (Life Technologies, CA, USA). Genotype assignments were manually confirmed by visual inspection with the SDS 2.0.5 software compatible with the TaqMan® system.

Genotyping quality control

Quality control procedures were applied throughout genotyping of the samples. After genotyping, approximately 10% of samples in each genotype group were selected for repetitive analysis with 100% concordance rate. Dubious samples had triplet repetitive analysis. All parties involved in genotyping were blinded to the

case or control status of the samples. Samples that failed to genotype were recorded as undetermined.

Statistical analysis

All subjects were classified into three study groups: controls (n = 351), HRAG (n = 281), GC (n = 363). Age is shown as means and standard deviations, and was compared using ANOVA and unpaired Student's t-test. Categorical data (e.g. gender, *H. pylori* infection, histological GC subtypes, distribution of genotypes or alleles) are presented as frequencies; comparisons were performed using the Chi-square test.

Quality assessments and statistical analysis of the genotyping data were performed using PLINK software version 1.07 [29]. Individuals with more than 10% missing genotypes and SNPs with a call-rate below 90% or deviation from Hardy-Weinberg equilibrium (HWE) in the controls ($P<0.05$) were excluded from further analysis. The average genotyping rate across all samples was 99.6%. Differences in allele frequencies between cases and controls were calculated in the combined German, Lithuanian and Latvian study sample, using the Breslow-Day test for heterogeneity of ORs. Only one SNP (*miR-608* C>G rs4919510) showed heterogeneity of ORs between the three GC study groups ($P_{BD}<0.05$) and, therefore, the country of birth was included as the covariate in further analysis. Association between HRAG and GC with gene polymorphisms was calculated using logistic regression analysis with adjustment for age, gender and country of birth with 95% confidence intervals (CI). The relative risks for mutations were studied using recessive and dominant model that led to a comparison between wild type + heterozygous vs. homozygous and wild type *vs.* heterozygous + homozygous, respectively. To adjust for multiple testing we calculated a corrected significance threshold $\alpha = 0.01$ (0.05/5).

Results

Characteristics of the subjects

The characteristics of control, GC and HRAG groups are presented in **Table 1**. In accordance with real-life age, subjects differed significantly according to age and gender distribution between the groups. As expected, males accounted for 63.6% in GC group, while in control and HRAG groups this gender constituted 26.8% and 37.7%, respectively. Control subjects were significantly younger than HRAG group (3 years) and GC group (5 years). 52.6% of patients were positive for *H. pylori* in GC group, however, in around 27% of individuals in GC group *H. pylori* IgG status could not be obtained. Over 47% of subjects in control group and 42.7% in HRAG group were *H. pylori* positive. Histological classification for diffuse and intestinal GC types was retrieved for 62.0% of GC patients (**Table 1**).

Associations of miRNA SNPs and risk of GC and HRAG

Genotype distribution for all five polymorphisms in the study were similar to those expected for Hardy-Weinberg Equilibrium: rs895819 (p = 0.94); rs2910164 (p = 0.44); rs11614913 (p = 0.95); rs2289030 (p = 0.44); rs4919510 (p = 0.39). Genotype and allele distributions for *miR-27a* C>G (rs895819), *miR-146a* C>G (rs2910164), *miR-196a-2* C>T (rs11614913), *miR-492* C>G (rs2289030) and *miR-608* C>G (rs4919510) gene polymorphisms in GC and HRAG study groups are presented in **Table 2**. No significant associations were observed for diseases under study following correction for multiple testing. We observed a tendency for *miR-196a-2* CT genotype to be associated with higher risk of GC when compared to CC genotype, however, the difference did not reach the adjusted significance threshold (OR - 1.46, 95% CI 1.03–2.07, $P = 0.032$). *MiR-608* GG genotype was marginally associated with higher risk of GC when compared to CC genotype (OR - 2.34, 95% CI 1.08–5.04, $P = 0.029$). A similar tendency was observed in a recessive model for *miR-608*, where CC + CG vs GG genotype comparison resulted in an OR - 2.44 (95% CI 1.14–5.22), ($P = 0.021$).

Associations of miRNA SNPs and risk of intestinal and diffuse-type GC

MiR-27a, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* SNP associations were tested with two histological types of GC (diffuse or intestinal type-GC). Genotype and allele frequencies for the study groups are presented in **Table 3.** Dominant model for *miR-27a* showed a tendency for allele T vs. C to be linked with higher risk of diffuse-type GC (OR - 2.07, 95% CI 1.13–3.79, $P = 0.018$). *MiR-196-a2* SNP showed a tendency towards reduced risk of intestinal type GC in recessive model (CC + CT vs. TT), however, the association strength did not reach statistical significance (OR – 0.47, 95% CI 0.20–1.08, $P = 0.077$). Similar observation was made in a dominant model for *miR-492* SNP (OR - 1.95, 95% CI 1.01–3.75) where GG vs. GC comparison led to an increased risk of diffuse-type GC, however the P value did not reach required significance level ($P = 0.046$). All the other comparisons between control and diffuse or intestinal-type GC groups did not reveal significant associations or trends for five SNPs of miRNAs.

Discussion

In the present study we performed a genotyping analysis for *miR-27a* (rs895819), *miR-146a* (rs2910164), *miR-196a-2* (rs11614913), *miR-492* (rs2289030) and *miR-608* (rs4919510) gene polymorphisms in a case control study including 351 controls, 281 HRAG and 363 GC patients from three European study groups. These polymorphisms have been associated with the risk of GC or overall cancer risks; however, reported data are partially conflicting or based on only few studies related to Asian groups. Despite some minor differences, our results do not support the link between *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* gene polymorphisms and the presence of GC or HRAG. Furthermore, we could not find an association between SNPs of miRNAs and the presence of different histological subtypes of GC. Since miRNAs have been shown to have profound effect in gastric carcinogenesis [8], we expected that these polymorphisms could modify the risk of GC or its precursors. To our best knowledge, this is the first study which investigated the associations between these miRNA polymorphisms and the risk of GC or HRAG in subjects of European descent. More than that, SNPs of *miR-492* (rs2289030) and *miR-608* (rs4919510) have not been studied in relation to GC or premalignant gastric conditions previously.

Gene polymorphism of miR-27a is at present one of the most studied SNPs in cancer related case-control studies. We expected that carriers of C allele for *miR-27a* SNP could have a reduced risk of GC as has been suggested by some research groups. A Chinese case-control study including 311 GC patients and 425 cancer-free controls found that minor allele C of rs895819 in the *miR-27a* significantly reduced risk of GC with OR - 0.77 [30]. Another study showed a significantly increased risk of GC (OR - 1.48) for *miR-27a* SNP and further functional analyses indicated that variant genotypes might be responsible for elevated *miR-27a* levels and reduced *ZBTB10* mRNA levels [16]. In contrast, a study including 2,380 participants with diverse gastric lesions did not find an increased risk of gastric IM or dysplasia for rs895819 [12]. A meta-analysis summarizing the findings of different case-control studies revealed that rs895819 polymorphism was significantly

Table 1. Characteristics of subject groups.

	Controls (n = 351)	GC (n = 363)[1]	HRAG (n = 281)[1]	ANOVA (Age)[2] Chi-squared test *P* value
Age				
Mean ± SD	60.4±13.9	65.3±12.5	63.3±10.4	<0.001
Gender				
Male	94 (26.8%)	231 (63.6%)	106 (37.7%)	<0.001
Female	257 (73.2%)	132 (36.4%)	175 (62.3%)	
H. pylori[1]				
Positive	166 (47.3%)	191 (52.6%)	120 (42.7%)	<0.001
Negative	184 (52.4%)	74 (20.4%)	161 (57.3%)	
Unknown	1 (0.3%)	98 (27.0%)		
GC Lauren type[1]				
Intestinal		136 (37.5)		
Diffuse		89 (24.5)		
Mixed		33 (9.1)		
Data unavailable		105 (28.9)		

[1]GC – gastric cancer; HRAG – high risk gastritis; *H. pylori* – *Helicobacter pylori*.
[2]Statistical analysis was performed globally for all three groups.
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associated with decreased risks of cancer in white, but not in Asians [31]. The results of our study do not support the possible protective effect of C allele for GC development in European subjects. Our study is the first which evaluated the role of *miR-27a* rs895819 in GC and HRAG in subjects of European descent, however, no significant associations have been observed.

Considering the existing evidence on *miR-146a* SNP (rs2910164) for cancer development we wanted to assess the possible association in our group of patients with GC and HRAG. Song et al. found that CC carriers of rs2910164 had a significantly increased risk of IM (OR - 1.42) and dysplasia (OR - 1.54) compared to GG carriers [12]. A similar observation was made in another study where combined effect of *miR-146a* (rs2910164) G/G and TLR4 SNP on the increased risk of severe AG among Japanese subjects was observed [18]. A paper by Okubo et al. [32] reported that carriage of rs2910164 CC genotype was associated with a significantly higher risk of GC when compared to non-cancer subjects (OR - 1.30). A meta-analysis by Wang et al. [33] on *miR-146a* rs2910164 including 19 case-control studies found that rs2910164 was linked with increased cancer susceptibility in Asians with overall OR - 1.18, but the same meta-analysis urges for further well-designed studies with large sample size for further risk identification. The results of our case-control study did not reveal a significant association between rs2910164 and the risk of GC or HRAG.

MiR-196a-2 C>T SNP (rs11614913) is one of the best studied miRNA SNPs in relation to different malignancies. A report by Wang et al. (2013) showed that CC genotype was associated with a significantly reduced risk of gastric cancer (OR - 0.78) compared with the CT and TT genotypes in a large case-control study [33]. A meta-analysis by Wang et al. (2013) found a significantly increased GC risk, but the association was observed only in homozygote comparison. This meta-analysis demonstrated that rs11614913 polymorphism is significantly associated with overall risk of gastrointestinal cancers [34]. Okubo et al. [32] did not find a link for this SNP and GC, however, in their study rs11614913 was associated with the degree of *H. pylori*-induced mononuclear cell infiltration. Interestingly, another meta-analysis on the same SNP did not determine relationship between rs11614913 and GC development [35]. The results of our study are in line with the conclusion of the latter meta-analysis as we did not observe significant association between SNP of *miR-196a-2* and GC risk.

Polymorphism of *miR-492* (rs2289030) is hypothesized to mediate cancer risk, but the data on possible associations for this SNP and cancer risk are still scarce. A study by Yoon et al. (2012) did not find an association between SNP of *miR-492* and the risk or survival in patients with non-small cell lung cancer [36]. Another study on colorectal cancer has demonstrated that progression-free survival of the patients with the combined *miR-492* CG and GG genotype was significantly worse than that of the patients with the *miR-492* CC genotype [13]. Our results indicate that rs2289030 of *miR-492* was not linked with the presence of GC or HRAG. To our best knowledge this is the first study which examines the relationship between *miR-492* SNP and premalignant gastric conditions or GC and, therefore, no comparison for our data can made.

SNP of *miR-608* (rs4919510) was not studied in relation to GC risks previously. Our study is the first to report null association between this polymorphism and the risk of GC or premalignant gastric conditions. In colorectal cancer patients rs4919510 was significantly linked with cancer recurrence and death [37]. Another case-control study on colorectal cancer patients did not find an association with cancer risk, but their results showed that GG genotype was associated with an increased risk of death in white population and reduced risk of death in African Americans [38]. Further larger scale studies might determine the role *miR-608* rs4919510 SNP in gastric and other malignancies.

Intestinal and diffuse-types of GC have been shown to have different pathogenetic pathways [3]. Some studies have found that miRNA polymorphisms might have different effects for histological subtypes of GC. After stratifying the patients in intestinal and diffuse-type GC groups, *miR-499* rs3746444 was shown to affect

Table 2. Genotype frequencies of *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492*, *miR-608* SNPs in controls, gastric cancer and high risk gastritis patients.

Genotype	Controls (n = 351)	GC[1] (n = 363)			HRAG[1] (n = 281)		
	n (%)	n (%)	aOR (95% CI)[1]	*P*	n (%)	aOR (95% CI)[1]	*P*
***miR-27a* (rs895819)[2]**							
TT	156 (44.6)	181 (49.9)	1 (Reference)		129 (46.7)	1 (Reference)	
TC	164 (46.9)	144 (39.7)	0.73 (0.52–1.03)	0.080	120 (43.5)	1.01 (0.61–1.67)	0.959
CC	30 (8.6)	38 (10.5)	0.97 (0.54–1.77)	0.944	27 (9.8)	3.52 (0.35–34.7)	0.280
TT vs TC + CC			0.77 (0.55–1.07)	0.123		1.07 (0.66–1.75)	0.765
TT + TC vs. CC			1.13 (0.64–2.00)	0.660		3.52 (0.35–34.6)	0.280
Allele T	476 (68.0)	506 (69.7)	0.88 (0.68–1.13)	0.318	387 (69.0)	1.13 (0.87–1.37)	0.462
Allele C	224 (32.0)	220 (30.3)			174 (31.0)		
***miR-146a* (rs2910164)[3]**							
GG	223 (64.3)	252 (69.6)	1 (Reference)		170 (60.5)	1 (Reference)	
GC	108 (31.1)	94 (26.0)	0.68 (0.47–0.99)	0.047	101 (35.9)	1.08 (0.76–1.53)	0.646
CC	16 (4.6)	16 (4.4)	0.87 (0.40–1.91)	0.742	10 (3.6)	1.18 (0.73–1.93)	0.484
GG vs GC +. CC			0.71 (0.50–1.01)	0.058		1.11 (0.80–1.53)	0.524
GG + GC vs CC			0.98 (0.45–2.11)	0.968		0.77 (0.34–1.74)	0.536
Allele G	554 (79.8)	598 (82.6)	0.79 (0.60–1.06)	0.119	441 (78.5)	1.08 (0.86–1.36)	0.462
Allele C	140 (20.2)	126 (17.4)			121 (21.5)		
***miR-196a-2* (rs11614913)[4]**							
CC	159 (45.4)	144 (39.7)	1 (Reference)		121 (43.1)	1 (Reference)	
CT	145 (41.4)	184 (50.7)	1.46 (1.03–2.07)	0.032	118 (42.0)	1.22 (0.86–1.72)	0.256
TT	46 (13.1)	35 (9.6)	0.95 (0.55–1.63)	0.851	42 (14.9)	0.83 (0.36–1.89)	0.660
CC vs. CT + TT			1.34 (0.96–1.87)	0.083		1.17 (0.84–1.63)	0.351
CC + CT vs. TT			0.78 (0.47–1.30)	0.339		0.77 (0.34–1.74)	0.536
Allele C	463 (66.1)	472 (65.0)	1.11 (0.87–1.41)	0.412	360 (64.1)	1.08 (0.82–1.44)	0.567
Allele T	237 (33.9)	254 (35.0)			202 (35.9)		
***miR-492* (rs2289030)**							
GG	310 (88.3)	312 (86.0)	1 (Reference)		246 (87.5)	1 (Reference)	
GC	40 (11.4)	49 (13.5)	1.19 (0.72–1.95)	0.492	32 (11.4)	0.85 (0.60–1.19)	0.353
CC	1 (0.3)	2 (0.6)	2.99 (0.21–41.1)	0.411	3 (1.1)	1.11 (0.62–1.98)	0.718
GG vs. GC + CC			1.22 (0.74–2.00)	0.416		0.89 (0.64–1.23)	0.485
GG + GC vs.CC			2.93 (0.21–40.2)	0.420		1.20 (0.69–2.09)	0.512
Allele G	660 (94.0)	673 (92.7)	1.25 (0.78–1.99)	0.356	524 (93.2)	0.97 (0.76–1.24)	0.804
Allele C	42 (6.0)	53 (7.3)			38 (6.8)		
***miR-608* (rs4919510)[5]**							
CC	251 (71.7)	250 (68.9)	1 (Reference)		197 (70.6)	1 (Reference)	
GC	86 (24.6)	88 (24.2)	0.84 (0.57–1.24)	0.395	74 (26.5)	1.07 (0.73–1.54)	0.719
GG	13 (3.7)	25 (6.9)	2.34 (1.08–5.04)	0.029	8 (2.9)	0.79 (0.32–1.98)	0.627
CC vs. CG + GG			1.01 (0.70–1.45)	0.938		1.03 (0.72–1.47)	0.851
CC + CG vs. GG			2.44 (1.14–5.22)	0.021		0.78 (0.31–1.94)	0.598
Allele C	588 (84.0)	588 (81.0)	1.15 (0.87–1.53)	0.334	468 (83.9)	0.99 (0.74–1.35)	0.985
Allele G	112 (16.0)	138 (19.0)			90 (16.1)		

[1]GC – gastric cancer; HRAG – high risk gastritis; aOR – adjusted odds ratio (age, sex, country); CI – confidence interval.
[2]six patients with missing values on rs895819 were excluded from analysis.
[3]five patients with missing values on rs2910164 were excluded from analysis.
[4]one patient with missing values on rs11614913 was excluded from analysis.
[5]three patients with missing values on rs4919510 were excluded from analysis.
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Table 3. Genotype frequencies of *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492*, *miR-608* SNPs in controls, intestinal and diffuse-type GC subjects.

Genotype	Controls (n = 351)	Intestinal GC[1] (n = 136)			Diffuse GC[1] (n = 89)		
	n (%)	n (%)	aOR (95% CI)[1]	*P*	n (%)	aOR (95% CI)[1]	*P*
***miR-27a* (rs895819)**							
TT	156 (44.6)	70 (51.9)	1 (Reference)		46 (51.7)	1 (Reference)	
TC	164 (46.9)	52 (38.5)	0.64 (0.40–1.02)	0.065	39 (43.8)	0.78 (0.47–1.29)	0.343
CC	30 (8.6)	13 (9.6)	0.89 (0.40–1.97)	0.780	4 (4.5)	0.44 (0.14–1.38)	0.163
TT vs. TC + CC			0.68 (0.44–1.06)	0.090		0.73 (0.45–1.19)	0.210
TT + TC vs. CC			1.10 (0.51–2.35)	0.804		0.50 (0.16–1.51)	0.223
Allele T	476 (68.0)	192 (71.1)	1.24 (0.67–2.29)	0.495	141 (75.0)	2.07 (1.13–3.79)	0.018
Allele C	224 (32.0)	78 (28.9)			47 (25.0)		
***miR-146a* (rs2910164)**							
GG	223 (64.3)	88 (65.2)	1 (Reference)		64 (71.9)	1 (Reference)	
GC	108 (31.1)	43 (31.9)	0.95 (0.59–1.53)	0.841	21 (23.6)	0.65 (0.37–1.15)	0.143
CC	16 (4.6)	4 (3.0)	0.77 (0.24–2.49)	0.673	4 (4.5)	0.83 (0.26–2.66)	0.760
GG vs GC +. CC			0.93 (0.58–1.47)	0.761		0.67 (0.39–1.15)	0.154
GG + GC vs CC			0.79 (0.24–2.51)	0.690		0.95 (0.30–3.00)	0.932
Allele G	554 (79.8)	219 (81.1)	0.99 (0.71–1.38)	0.945	149 (83.7)	1.09 (0.77–1.54)	0.618
Allele C	140 (20.2)	51 (18.9)			29 (16.3)		
miR-196a2 (rs11614913)							
CC	159 (45.4)	57 (41.9)	1 (Reference)		37 (41.6)	1 (Reference)	
CT	145 (41.4)	71 (52.2)	1.46 (0.92–2.32)	0.103	41 (46.1)	1.29 (0.76–2.18)	0.332
TT	46 (13.1)	8 (5.9)	0.57 (0.24–1.36)	0.211	11 (12.4)	1.05 (0.48–2.28)	0.898
CC vs. CT + TT			1.25 (0.80–1.95)	0.312		1.23 (0.75–2.01)	0.403
CC + CT vs. TT			0.47 (0.20–1.08)	0.077		0.92 (0.44–1.91)	0.835
Allele C	463 (66.1)	185 (68.0)	0.92 (0.63–1.37)	0.692	115 (64.6)	0.76 (0.48–1.19)	0.229
Allele T	237 (33.9)	87 (32.0)			63 (35.4)		
***miR-492* (rs2289030)**							
GG	310 (88.3)	114 (83.8)	1 (Reference)		71 (79.8)	1 (Reference)	
GC	40 (11.4)	22 (16.2)	1.28 (0.68–2.42)	0.428	17 (19.1)	1.95 (1.01–3.75)	0.046
CC	1 (0.3)	0 (0.0)	0.36 (0.09–0.54)	0.999	1 (1.1)	8.01 (0.48–132.1)	0.145
GG vs. GC + CC			1.26 (0.67–2.37)	0.456		2.05 (1.08–3.91)	0.028
GG + GC vs. CC			0.35 (0.07–0.68)	0.999		7.21 (0.43–118.6)	0.166
Allele G	660 (94.0)	250 (91.9)	0.81 (0.57–1.14)	0.226	159 (89.3)	0.73 (0.49–1.09)	0.128
Allele C	42 (6.0)	22 (8.1)			19 (10.7)		
***miR-608* (rs4919510)**							
CC	251 (71.7)	96 (70.6)	1 (Reference)		63 (70.8)	1 (Reference)	
GC	86 (24.6)	31 (22.8)	0.74 (0.43–1.26)	0.279	25 (28.1)	0.98 (0.56–1.72)	0.950
GG	13 (3.7)	9 (6.6)	2.03 (0.74–5.55)	0.164	1 (1.1)	0.44 (0.05–3.55)	0.447
CC vs. CG + GG			0.88 (0.54–1.44)	0.624		0.92 (0.53–1.60)	0.788
CC + CG vs. GG			2.18 (0.81–5.89)	0.122		0.44 (0.05–3.55)	0.448
Allele C	588 (84.0)	223 (82.0)	1.04 (0.70–1.53)	0.858	151 (84.8)	0.89 (0.55–1.44)	0.637
Allele G	112 (16.0)	49 (18.0)			27 (15.2)		

[1]GC – gastric cancer; HRAG – high risk gastritis; aOR – adjusted odds ratio (age, sex, country); CI – confidence interval.

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the risk of diffuse-type GC in Korean population [21]. Although the number of individuals within intestinal and diffuse-type GC groups was not very high in our study, analysis comparing these two distinct histological types of GC was performed. Overall, *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* SNPs were not linked with the presence of different histological subtypes of GC. Although some tendencies described in the results section have been observed between the histological subgroup of GC, the differences in genotype and allele frequencies did not reach required significance levels.

Results of our study clearly support the importance of careful evaluation of gene polymorphisms in different ethnical groups. Previously suggested miRNA-related SNPs did not show substantial GC related differences in our European population. Nevertheless, we admit that there are several limitations related to the design of our study. There were gender distribution differences between GC, HRAG and control groups; however, when performing statistical analysis we included gender as a covariate, thus minimizing the potential influence of gender for the outcome of our results. Here in this study we focused on histological changes rather than the presence of *H. pylori*; therefore, additional tests apart from serum IgG antibodies for detection this bacterium were not applied. Furthermore, it is well know that chronic atrophic gastritis is caused by *H. pylori* infection. We did not adjust OR for *H. pylori* IgG antibodies, *CagA* or *VacA* status because this information was not available for all the subjects. We could not perform association analysis for SNPs of miRNAs with respect to survival of GC patients, as the information was available only for a small proportion of subjects. Due to the same reason we also did not perform analysis according to the anatomic location of the in the stomach (proximal vs. distal). In this study we performed solely gene polymorphism analysis without addressing the miRNA expression differences in cancerous and non-cancerous tissues and, therefore, we cannot postulate if these polymorphisms could have a functional role. Further studies should evaluate the role of SNPs for miRNA levels in GC tissue and assess potential target genes in functional studies. The number of individuals within the subgroups in our study of intestinal and diffuse-type GC groups is relatively small and might be underpowered to detect specific associations. We found some differences for miR-196a-2 and miR-608 SNPs among the groups with $P<0.05$; however, we think that we cannot report a significant association, since they did not reach our adjusted P value. Multiple comparisons for genetic association tests have been applied in this paper and we believe that we should use the adjusted P value for drawing the conclusions.

Conclusions

Our study shows that gene polymorphisms of *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* are not associated with risk of GC and HRAG in subjects of European descent. These SNPs do not appear as potential biomarkers for identifying individuals with increased risk for GC.

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Author Contributions

Conccived and designed the expcrimcnts: JK TW LK PM. Performed the experiments: IB RS SJ UG. Analyzed the data: JK JS AL LK. Contributed reagents/materials/analysis tools: ML AI GA VP. Wrote the paper: JK JS AL.

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SANTRAUKA

Įvadas

Skrandžio vėžys (SV) pagal paplitimą pasaulyje užima penktąją vietą, o pagal mirštamumą nuo vėžio – trečiąją vietą visų onkologinių ligų tarpe. Kasmet pasaulyje diagnozuojama apie 1 milijonas naujų SV atvejų ir registruojama apie 750 tūkstančių mirčių nuo SV [1]. Ligos paplitimas ir mirštamumo dažnis labai skiriasi tarp skirtingų pasaulio regionų, tačiau didžiausi rodikliai nustatomi besivystančiose šalyse [2]. Lietuva priklauso labiausiai nuo SV kenčiančių Europos šalių grupei, kurioje fiksuojami gana aukšti SV dažniai (ligos paplitimas 29,5 atvejų/100 000 gyventojų, mirštamumo dažnis 24,4 atvejų/100 000 gyventojų 2018 metais abiems lytims) [3]. Skrandžio kancerogenezė yra daugiaveiksnis procesas, apimantis *Helicobacter pylori* infekciją, aplinkos veiksnius bei asmens paveldimus ir genetinius veiksnius [4]. Gerai žinoma patofiziologinė kaskada, kurią pasiūlė Kolumbijos patologas Pelayo Correa [5], yra kelias nuo skrandžio uždegimo iki žarninio tipo skrandžio adenokarcinomos. Ši kaskada apima tokius etapus: skrandžio gleivinės uždegimą, sukeltą *H. pylori* infekcijos, lėtinį gastritą, skrandžio gleivinės atrofiją, žarninę metaplaziją, skrandžio epitelio displaziją ir, galiausiai, žarninio tipo skrandžio adenokarcinomą. Kita vertus, difuzinio tipo skrandžio adenokarcinomai nėra būdingi šie ikivėžiniai pakitimai ir ji atsiranda skrandyje, kuriame nėra nustatyta atrofija ir žarninė metaplazija bei uždegimas [6]. Kadangi skrandžio vėžėjimo procesas trunka daugelį metų, dažniausiai SV yra diagnozuojamas vėlyvose stadijose, kuomet atsiranda klinikiniai simptomai. Vis dėlto, palyginti su daugeliu kitų vėžio formų, klinikinės SV patikros ir gydymo pažanga per pastaruosius dešimtmečius nepasistūmėjo toli į priekį [7]. Nors ankstyvesnis SV diagnozavimas padėtų pagerinti šia ligą sergančių pacientų gydymo ir išgyvenamumo rezultatus, reikalingas geresnis fundamentinių vėžio mechanizmų supratimas [8].

Proveržis molekulinės biologijos tyrimų srityje pastaraisiais dešimtmečiais parodė, jog didelė dalis žmogaus genetinės medžiagos yra sudaryta iš baltymų nekoduojančių DNR sekų [9]. Viena dalis DNR sekų yra transkribuojamos į baltymų nekoduojančias RNR molekules (miRNR, siRNR, piRNR, snoRNR ir kt.), kurios atlieka skirtingas funkcijas įvairiuose ląstelės procesuose [11]. Kita dalis sekų išlieka DNR pavidale kaip kartotiniai genomo elementai (LTR, LINE, SINE, minisatelitinė DNR, mikrosatelitinė DNR, satelitinė DNR) [10]. Nauji molekulinių mokslų atradimai atskleidžia

svarbius šių molekulių vaidmenis įvairių vėžio tipų, įskaitant ir SV, atsiradime ir vystymesi.

MikroRNR (miRNR) yra labai gausi trumpų nekoduojančių RNR molekulių klasė, kurios yra maždaug 22 nukleotidų ilgio ir pasižymi aukštu konservatyvumo lygmeniu. MiRNR post-transkripciniame lygmenyje nuslopina savo genus-taikinius, pilnai ar iš dalies komplementariai susijungdamos su jų iRNR, o tai nulemia iRNR degradaciją arba transliacijos užblokavimą [12]. Nuo tada, kai miRNR molekulės buvo atrastos 1993 metais, jos yra labai intensyviai tyrinėjamos [13–15]. Yra įrodyta, jog miRNR atlieka esminę funkciją ląstelių proliferacijoje, diferenciacijoje bei apoptozėje – pagrindiniuose ląstelės biologiniuose procesuose [16]. Po to kai 2002 metais pasirodė įrodymai, jog miRNR atlieka svarbias funkcijas vėžėjimo procesuose [17], buvo atlikta daugybė mokslinių tyrimų su įvairiais vėžio tipais. Nustatyta, jog skirtingos miRNR gali funkcionuoti kaip onkogenai arba kaip vėžio supresoriai, nors jų genai-taikiniai vis dar yra tyrinėjami [18]. Be to, buvo nustatyta, jog vieno nukleotido polimorfizmai miRNR koduojančiuose genuose, kurie pakeičia miRNR biogenezę ar jų genų-taikinių iRNR prisijungimo vietų struktūrą, taip pat gali daryti įtaką vėžėjimo procesui [19]. Be struktūrinių genetinių variacijų, miRNR koduojantys genai taip pat gali būti veikiami epigenetiniais pokyčiais, tokiais kaip DNR metilinimas ar histonų acetilinimo praradimas [20].

Epigenetiniai pokyčiai yra esminis elementas, užtikrinantis genetinį stabilumą. DNR metilinimo mechanizmas yra geriausiai išstudijuotas epigenetinis reiškinys, turintis realią biologinę prasmę įvairių ligų inicijavimui [21]. Globalus hipometilinimas – tai viso genomo DNR metilinimo lygio sumažėjimas, siejamas su bendru genetinio stabilumo praradimu (onkogenų, endoparazitinių DNR sričių aktyvavimu, chromosomų stabilumo netekimu), vedančiu prie vėžėjimo procesų inicijavimo [22]. Manoma, jog viso genomo genetinio stabilumo praradimo mechanizmas yra siejamas su kartotinių genomo elementų, sudarančių didelę dalį žmogaus genomo sekų ir atsakingų už genų reguliaciją, metilinimo lygio sumažėjimu [10]. Ilgi išsklaidyti nukleotidiniai elementai-1 (angl. *Long Interspersed Nucleotide Element 1*), arba LINE-1 yra vienas iš pagrindinių kartotinių genomo elementų, sudarančių apie ~17 proc. viso genomo DNR sekų [23]. CpG nukleotidų salos, lokalizuotos LINE-1 elementų viduje ir jų metilinimo lygis tiesiogiai koreliuoja su globaliu viso genomo DNR metilinimo lygiu ir dažnai LINE-1 metilinimo lygio statusas yra naudojamas kaip pakaitinis biožymuo, atspindintis globalų viso genomo DNR hipometilinimo lygį [24]. LINE-1 elementų hipometilinimo reiškinys buvo tirtas įvairių vėžinių susirgimų atvejais ir yra manoma, jog jis galėtų tarnauti kaip potencialus biologinis

žymuo vėžio nustatymui, išgyvenamumo prognozavimui bei tinkamiausios terapinės strategijos parinkimui [25].

Tikslas ir uždaviniai

Šio tyrimo **tikslas** – nustatyti epigenetinius ir genetinius baltymų nekoduojančių genomo struktūrų pokyčius ir jų svarbą skrandžio vėžio patogenezėje.

Uždaviniai:

1. Įvertinti miR-137 koduojančio geno raiškos ir promotoriaus metilinimo pakitimus ir jų reikšmę sergant skrandžio vėžiu ir ikivėžinėmis skrandžio ligomis.
2. Atlikti LINE-1 metilinimo lygmens analizę vėžio ir ikivėžiniuose skrandžio audiniuose ir įvertinti jo prognostinį potencialą.
3. Nustatyti vieno nukleotido polimorfizmų, esančių miR-27a, miR-146a, miR-196a-2, miR-492, miR-608 koduojančiuose genuose sąsajas su skrandžio vėžiu ir ikivėžinėmis skrandžio ligomis.

Darbo naujumas ir aktualumas

Šis tyrimas pateikia: 1) naujus įrodymus apie epigenetinius ir genetinius miR-137 pokyčius skrandžio vėžėjimo procese; 2) išsamią LINE-1 metilinimo analizę ir jos prognostinį potencialą SV pacientams; 3) naujas įžvalgas apie genetinių variantų miRNR genuose svarbą ir jų sąsajas su SV ir atrofiniu gastritu. Visi tyrimai buvo atlikti europiečių kilmės tiriamųjų grupėje, kuri, atsižvelgiant į visą egzistuojančios literatūros šiomis temomis kontekstą, pabrėžia SV patogenezės skirtumus, pagrįstus tiriamųjų etnine kilme. Be to, visose trijose studijose buvo įtraukta grupė pacientų, sergančių ikivėžinėmis skrandžio ligomis (lėtiniu/atrofiniu gastritu), kurios nebuvo dažnai tiriamos analogiškose studijose. Galiausiai, mūsų rezultatai prisideda prie bendros SV patogenezės molekulinės bazės ir gali būti naudojami kaip palyginamieji duomenys ateities moksliniuose tyrimuose.

Medžiagos ir metodai

Studija I. Į miR-137 koduojančio geno metilinimo ir raiškos analizės studiją buvo įtrauktas 81 SV pacientas, 28 storosios žarnos vėžio pacientai, 31 sveikas individas bei 44 pacientai, sergantys lėtiniu/atrofiniu gastritu su/be žarninės metaplazijos. Vėžiu sergantiems pacientams buvo tirti vėžiniai skrandžio arba storosios žarnos gleivinės audiniai (T-GC, T-CRC) bei šalia esantys histologiškai sveiki gleivinės audiniai (N-GC, N-CRC).

Ikivėžinių ligų (CG/AG/IM) bei sveikųjų kontrolinėje (N) grupėje buvo tirtos skrandžio gleivinės biopsijos. Tiriamųjų audiniai buvo homogenizuoti, bei, naudojant komercinį RNeasy Plus Universal Mini Kit rinkinį, išskirta visuminė RNR medžiaga. DNR buvo išskirta iš tų pačių mėginių, naudojant adaptuotą Qiagen protokolą. MiR-137 koduojančio geno raiškos analizei buvo pasitelkti gamykliškai validuoti TaqMan® pradmenys, RNU6b buvo naudotas kaip referentinis genas. MiR-137 genų-taikinių *Cdc42* ir *COX-2* bei referentinio *β-actin* geno raiškos nustatymui naudotas SYBR Green metodas. Tiriamųjų genų raiška įvertinta kiekybiniu tikro laiko PGR metodu. Kiekybiniam miR-137 koduojančio geno promotoriaus metilinimo nustatymui buvo naudotas pirosekoskaitos metodas. Tiriamosios DNR bisulfitinė modifikacija atlikta naudojant komercinį Cells-to-CpG™ Bisulfite Conversion Kit rinkinį. Standartinė PGR reakcija buvo atlikta norimo miR-137 specifinio regiono pagausinimui su biotinu žymėtais pradmenimis. MiR-137 koduojančio geno promotoriaus pirosekoskaitai naudotas komercinis PyroMark® Gold Q96 rinkinys. Statistiškai genų raiškos bei metilinimo duomenys buvo apdoroti GraphPad Prism 6.0 programa.

Studija II. LINE-1 metilinimo analizei buvo naudota ta pati tiriamųjų imtis kaip ir I studijoje. Išskirtai tiriamųjų DNR buvo atlikta bisulfitinė modifikacija, kaip aprašyta aukščiau, bei norimo pagausinti regiono amplifikavimui naudoti biotinu žymėti LINE-1 specifiniai pradmenys. Pagausinto LINE-1 103–249 bp regiono su 4 CpG vietomis metilinimo nustatymui atlikta pirosekoskaitos procedūra su PyroMark® Gold Q96 rinkiniu. Gauti metilinimo duomenys apdoroti GraphPad Prism 6.0 statistiniu paketu.

Studija III. Genetinių sąsajų tarp miRNR genų vieno nukleotido polimorfizmų ir SV bei atrofinio gastrito įvertinimui buvo naudojamas atvejo ir kontrolės tyrimo tipas. Tyrime dalyvavo lietuvių, latvių ir vokiečių kilmės tiriamieji, suskirstyti į grupes pagal diagnozę:

1 lentelė. *Pacientų, įtrauktų į III studiją, pasiskirstymas*

	Skrandžio vėžys	**Atrofinis gastritas**	**Kontrolė**
Lietuviai	118	81	146
Latviai	139	59	142
Vokiečiai	106	141	63
Iš viso:	363	281	351

Tiriamųjų asmenų DNR medžiaga buvo išskirta iš periferinio kraujo mononuklearinių ląstelių naudojant DNR išdruskinimo metodą arba komercinį QIAamp DNA blood kit rinkinį. MiRNR koduojančiuose genuose esančių vieno nukleotido polimorfizmų miR-27a C>T (rs895819), miR-146a C>G (rs2910164), miR-196a-2 C>T (rs11614913), miR-492 C>G

(rs2289030) ir miR-608 C>G (rs4919510) genotipavimas buvo atliktas tikro laiko PGR metodu naudojant gamykliškai validuotus TaqMan® genotipavimo rinkinius. Statistinė rezultatų analizė buvo atlikta naudojant PLINK 1.07 programinį paketą.

Rezultatai

Studija I. Mūsų studijos rezultatai parodė, jog miR-137 koduojančio geno promotoriaus metilinimo lygmuo yra padidėjęs vėžio apimtuose storosios žarnos gleivinės bei skrandžio gleivinės audiniuose, palyginti su šalia esančiais histologiškai sveikais audiniais (T-CRC *vs*. N-CRC 29,8 ± 3,1 proc. *vs*. 15,4 ± 3,5 proc., P = 0,0038; T-GC *vs*. N-GC 21,1 ± 1,6 proc. *vs*. 16,9 ± 1,3 proc., P = 0,045). Palyginus miR-137 koduojančio geno metilinimo lygmenį ikivėžinių skrandžio būklių gleivinės audiniuose, rastas laipsniškas metilinimo lygmens didėjimas pagal P. Correa klasifikaciją nuo sveikos gleivinės iki vėžinio audinio (N *vs*. CG *vs*. N-GC *vs*. T-GC 7,5 ± 0,9 proc.; 10,7 ± 0,9 proc.; 16,9 ± 1,3 proc.; 21,1 ± 1,6 proc., p < 0,0001). Išskirsčius SV tiriamųjų grupę į pogrupius pagal pacientų klinikinius parametrus nustatyta, jog miR-137 koduojančio geno promotoriaus metilinimo lygmuo buvo statistiškai patikimai didesnis žarninio ir mišraus tipo SV vėžiniame audinyje palyginti su difuzinio tipo (P = 0,03). Taip pat nustatyta tendencija jog miR-137 koduojančio geno metilinimo lygmuo yra aukštesnis SV vėžiniuose audiniuose, lokalizuotuose *antrum* skrandžio dalyje palyginti su *cardia* ir *corpus* (P = 0,07). Daugiau sąsajų tarp miR-137 koduojančio geno metilinimo lygmens bei kitų klinikinių charakteristikų (TNM stadijų, diferenciacijos lygmens, *H. pylori* infekcijos) nustatyta nebuvo.

MiR-137 koduojančio geno raiškos analizė parodė, jog miR-137 raiška buvo sumažėjusi storosios žarnos vėžio T-CRC audinyje palyginti su N-CRC (P = 0,0014). Skrandžio gleivinės audiniuose miR-137 koduojančio geno raiška išliko nepakitusi kaskadoje nuo sveiko iki vėžio apimto audinio (P > 0,05). Vis dėlto, nors raiškos skirtumų skrandžio gleivinės audiniuose nebuvo rasta, tačiau nustatyta atvirkštinė koreliacija tarp miR-137 koduojančio geno metilinimo ir raiškos skrandžio gleivinės audiniuose (r = –0,397, P < 0,0001).

Potenciali sąsaja tarp anksčiau identifikuotų miR-137 genų-taikinių *Cdc42* ir *COX-2* buvo įvertinta nustatant koreliaciją tarp šių genų iRNR raiškos bei miR-137 koduojančio geno raiškos bei promotoriaus metilinimo lygmens. *COX-2* iRNR raiška laipsniškai didėjo N *vs*. N-GC *vs*. T-GC kaskadoje, tuo tarpu *Cdc42* iRNR raiška laipsniškai mažėjo. Nebuvo nustatyta koreliacija tarp šių genų raiškos lygmens bei miR-137 koduojančio geno raiškos ar metilinimo lygmens.

Pacientų išgyvenamumo sąsajų su miR-137 koduojančio geno promotoriaus metilinimo lygmeniu analizė buvo atlikta išreiškus kiekybinius miR-137 metilinimo duomenis kaip kokybinį kintamąjį (nustatyta 15 proc. metilinimo lygmens riba, suskirstanti tiriamąją grupę į „metilintą“ ir „nemetilintą“). Statistinė analizė parodė, jog pacientų su nemetilintu miR-137 promotoriumi prognozė yra truputį geresnė nei su metilintu promotoriumi, tačiau skirtumai nėra statistiškai patikimi. Suskirsčius SV grupę į žarninio ir difuzinio SV prograpius, nustatyta tendencija, jog difuzinio tipo SV pacientų su nemetilintu miR-137 promotoriumi prognozė yra šiek tiek geresnė, nei su metilintu (P = 0,065). Pacientų išgyvenamumo sąsajų su miR-137 raiškos lygmeniu nustatyta nebuvo.

Studija II. Šio tyrimo rezultatai parodė, jog LINE-1 metilinimo lygmuo yra sumažėjęs vėžio apimtuose storosios žarnos gleivinės bei skrandžio gleivinės audiniuose, palyginti su šalia esančiais histologiškai sveikais audiniais (T-CRC vs. N-CRC 61,15 ± 6,38 proc. *vs.* 67,17 ± 4,84 proc., P = 0,0005; T-GC *vs.* N-GC 62,48 ± 8,15 proc. *vs.* 65,73 ± 4,56 proc., P = 0,002). Bendrai paėmus, 69,6 proc. T-CRC mėginių pasižymėjo žemesniu LINE-1 metilinimo lygmeniu, palyginti su šalia esančiu poriniu N-CRC mėginiu; SV atveju žemesnis LINE-1 metilinimo lygmuo nustatytas 53,8 proc. T-GC mėginių palyginti su jų poriniais N-GC mėginiais. Palyginus LINE-1 metilinimo lygmenį ikivėžinių skrandžio būklių gleivinės audiniuose nustatytas toks pats metilinimo lygmuo visoje P. Correa kaskadoje (N *vs.* CG/AG *vs.* N-GC 64,48 ± 2,93 proc., 65,08 ± 3,37 proc., 65,75 ± 4,56 proc., P > 0,05). Netgi palyginus N, N-GC ir N-CRC mėginius, nustatytas toks pats LINE-1 metilinimo lygmuo skirtingose anatominėse virškinamojo trakto vietose (P = 0,2). Išskirsčius SV tiriamųjų grupę į pogrupius pagal pacientų klinikinius parametrus nebuvo nustatyta reikšmingų sąsajų tarp LINE-1 metilinimo lygmens ir SV anatominės vietos, TNM stadijos, vėžio diferenciacijos lygmens, Laurén histologinių subtipų, pacientų lyties ar *H. pylori* infekcijos (visais atvejais P > 0,05). Skrandžio vėžiu sergančių pacientų išgyvenamumo sąsajų su LINE-1 metilinimo lygmeniu analizė buvo atlikta išreiškus kiekybinius LINE-1 metilinimo duomenis kaip kokybinį kintamąjį (nustatyta 60 proc. metilinimo lygmens riba, suskirstanti tiriamąją grupę į „žemo LINE-1 metilinimo“ ir „aukšto LINE-1 metilinimo“ grupes). Statistinė analizė nenustatė reikšmingų sąsajų tarp SV pacientų grupių su žemu ar aukštu LINE-1 metilinimo lygmeniu ir išgyvenamumo prognozės (P = 0,59). Kategorizavus SV pacientų grupę pagal Laurén histologinius vėžio subtipus, taip pat nenustatyta reikšmingų pacientų išgyvenamumo sąsajų su LINE-1 metilinimo lygmeniu.

Studija III. Mūsų studijos rezultatai parodė, jog tirtų polimorfizmų genotipų pasiskirstymas kontrolinėje grupėje atitiko Hardy-Weinbergo

dėsnį. Buvo nustatytos kelios tendencijos apie galimas genetines tirtų polimorfizmų bei SV sąsajas: miR-196a-2 (rs11614913) heterozigotinis CT genotipas galėtų būti siejamas su padidėjusia SV rizika (ŠS: 1,46, 95 proc. PI: 1,03 – 2,07, P = 0,032), taip pat kaip ir miR-608 (rs4919510) GG genotipas (ŠS: 2,34, 95 proc. PI: 1,08 – 5,04, P = 0,029). Recesyvinis genotipų palyginimo modelis taip pat parodė tendenciją, jog miR-608 CC + CG *vs*. GG genotipai galėtų didinti SV riziką (ŠS: 2,44, 95 proc. PI: 1,14 – 5,22, P = 0,021). Vis dėl to, pritaikius daugybinio palyginimo Bonferonni korekciją, minėtos sąsajos nebuvo statistiškai reikšmingos (P > 0,01). Atskyrus tiriamąją SV grupę į atskirai žarninio ir difuzinio tipo grupes, statistiškai reikšmingų sąsajų taip pat nebuvo nustatyta.

Išvados

1. MiR-137 koduojančio geno promotoriaus metilinimas didėja palaipsniui priklausomai nuo skrandžio gleivinės pažeidimo lygmens pagal P. Correa klasifikaciją. Žarninio ir mišraus tipo skrandžio vėžio audinyje miR-137 koduojančio geno metilinimo lygis buvo didesnis palyginti su difuziniu tipu. Nustatyta atvirkštinė koreliacija tarp miR-137 koduojančio geno metilinimo ir raiškos lygmens.

2. Nustatytas sumažėjęs LINE-1 metilinimo lygmuo vėžio pažeistuose skrandžio gleivinės audiniuose palyginti su histologiškai sveikais šalia esančiais audiniais. LINE-1 metilinimo lygmuo nesiskyrė palyginus įvairaus ikivėžinių pakitimų lygio skrandžio gleivinės audinius – sveikus, lėtinio/atrofinio gastrito apimtus bei šalia vėžinio audinio esančius histologiškai sveikus audinius. Sąsaja tarp LINE-1 metilinimo lygmens ir skrandžio vėžiu sergančių pacientų išgyvenamumo nenustatyta.

3. Sąsaja tarp miR-27a, miR-146a, miR-196a-2, miR-492 ir miR-608 koduojančių genų vieno nukleotido polimorfizmų ir rizikos sirgti skrandžio vėžiu bei atrofiniu gastritu nebuvo nustatyta europiečių populiacijoje.

SUPPLEMENT

NUORAŠAS

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LEIDIMAS ATLIKTI BIOMEDICININĮ TYRIMĄ

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

Biomedicininio tyrimo pavadinimas: **„Virškinimo sistemos ligų tiriamosios medžiagos biobankas".**	
Protokolo Nr.:	**1**
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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/

CURRICULUM VITAE

Rūta Steponaitienė (Čedavičiūtė)

CONTACT INFORMATION

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EDUCATION

2013 09 – 2018 07	Lithuanian University of Health Sciences, Academy of Medicine, Kaunas, Lithuania **PhD student in biomedical sciences**
2011 09 – 2013 06	Vytautas Magnus University, Faculty of Natural Sciences, Kaunas, Lithuania **MSc, molecular biology and biotechnology (VD Nr.004342)**
2007 09 – 2011 06	Vytautas Magnus University, Faculty of Natural Sciences, Kaunas, Lithuania **BSc *cum laude*, biology (BD Nr. 004691)**
1994 09 – 2006 06	Steponas Darius and Stasys Girėnas Gymnasium, Kaunas, Lithuania **Maturity Certificate *cum laude* (P Nr. 001509)**

RESEARCH EXPERIENCE

2018 11 – present	Institute for Digestive Research, Laboratory of Clinical and Molecular Gastroenterology, Kaunas, Lithuania "Multi-layer omics approach to gastric cancer: circulating biomarker profiling in the blood on genetic, epigenetic and microbiome levels (MULTIOMICS)" Funded by the Research Council of Lithuania (Project no. 09.3.3-LMT-K-712-01-0130). **Project Junior Scientist**
2014 03 – 2015 03	Institute for Digestive Research, Laboratory of Clinical and Molecular Gastroenterology, Kaunas, Lithuania "Functional role of miR20b, miR451, miR29c, miR125b in pathogenesis of gastric and colorectal cancer" Funded by the Research Council of Lithuania (Project no. MIP-007/2014) **Project Junior Scientist**
2013 08 – present	Institute for Digestive Research, Laboratory of Clinical and Molecular Gastroenterology, Kaunas, Lithuania **Junior Scientist**

2008 09 – 2013 08 Institute for Digestive Research, Laboratory of Clinical and Molecular Gastroenterology, Kaunas, Lithuania. **Laboratory Technician**

SCIENTIFIC INTERNSHIPS

2014 04 – 2014 06 GI Research Laboratory, Department of Gastroenterology, Hepatology and Infectious Disease, Otto-von-Guericke University Hospital, Magdeburg, Germany
Scientific Internship

2013 02 – 2013 04 GI Research Laboratory, Department of Gastroenterology, Hepatology and Infectious Disease, Otto-von-Guericke University Hospital, Magdeburg, Germany
ERASMUS Traineeship

2012 04 13 – 15 Young Investigator meeting YIM 2012, Vienna, Austria
International Research Course

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