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Lack of association between *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* gene polymorphisms and colorectal cancer

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Colorectal cancer (CRC) is one of the most common cancers worldwide with high mortality rates. MicroRNAs (miRNAs) have an established role in the development of different cancers. Single nucleotide polymorphisms (SNPs) in miRNA related genes were linked with various gastrointestinal malignancies. However, the data on association between miRNA SNPs and CRC development are inconsistent. The aim of the present study was to evaluate the association between miRNA-related gene polymorphisms (*miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608*) and the presence of CRC in European population. Gene polymorphisms were analyzed in 621 subjects (controls: n = 428; CRC: n = 193). *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. Overall, all genotypes and alleles of miRNA SNPs were distributed equally between control and CRC groups. We observed a tendency for *miR-146a* C allele to be associated with lower risk of CRC when compared to G allele, however, the difference did not reach the adjusted *P*-value (odds ratio (OR) = 0.68, 95% confidence interval (CI) 0.49–0.95, *P* = 0.025). In conclusion, gene polymorphisms of *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492*, *miR-492*a and *miR-608* were not associated with the presence of CRC in European subjects.

Colorectal cancer (CRC) is the second most common cancer in females and the third in males with 1.2 million new annual cases and 608,700 deaths in 2008 worldwide[1]. The vast majority of cases (90%) occur in people over 50[2]. Apart from hereditary CRC syndromes, the development of this cancer type is still poorly understood[3,4]. Both germline and somatic genetic variations have been proposed as contributing factors in CRC development[5–7]. Recent large scale studies reported significant risk of different germline variations for CRC development[6,8]. Novel reports suggest a potential influence of single nucleotide polymorphisms (SNPs) of microRNA-related genes (miRNAs) for the risk of cancer development[9].

The first description of miRNA appeared in 1993[10,11]. The discovery of these small non-coding RNAs has opened a new investigation platform in molecular biology[12]. MiRNA are ~22 nucleotide sequences of RNA found in both prokaryotes and eukaryotes that are intimately involved in cell differentiation, cell cycle progression and apoptosis[10,11]. Recent studies have shown aberrant miRNA expression patterns in a range of human diseases including many cancers[13,14]. Deregulation of miRNAs can influence carcinogenesis through mRNA targets encoding tumor suppressor genes or oncogenes[5]. Either phenomena – over expression and silencing or switching off of specific miRNAs, have been described in the carcinogenesis of CRC[15]. The discovery of miRNAs has opened new opportunities for SNPs in cancer research[16,17]. MiRNAs have potential to regulate multiple genes; therefore, variations in genes encoding or related to miRNAs may produce pronounced regulatory effects that may modify the risk of different human diseases including cancer[18]. SNPs of the genes encoding miRNAs can alter miRNA expression and may influence cancer risks[19,20]. Growing number of case-control studies have shown associations between the polymorphisms of the genes encoding miRNAs and the risk of different malignancies. In this study,

we selected five different SNPs *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), which have been reported to influence cancer risks[9,21–23] and performed a case-control study among CRC patients. Above mentioned SNPs are located in mature or pre-mature regions of miRNAs with potential implications in gene expression regulation[9,16]. It is also worth pointing out, that all of these miRNAs have been shown to be deregulated in colorectal cancer or target carcinogenesis related genes[13,15].

Selected miRNAs and their polymorphisms have been studied in CRC as well as other cancer types[9,24,25]. The list of previously published case-control studies on rs895819, rs2910164, rs11614913, rs2289030 and rs4919510 SNPs in CRC is presented in Table 1. Several studies have linked *miR-196a-2* gene polymorphism (rs11614913) with increased risk of CRC[26–28], meanwhile other studies could not confirm this association[29–32]. A meta-analysis by Srivastava et al. found that *miR-196a-2* gene polymorphism was associated with colorectal cancers[9]. *MiR-146a* has been shown to play an important role in tumor genesis by promoting cell proliferation and colony formation in NIH 3T3 cells[33,34]. SNP of *miR-146a* (rs2910164) was shown to be associated with decreased risk of CRC in Asian populations[26,35,36], while no significant role of this polymorphism was observed in European population[31,32] as well as in one Japanese study[37]. Pathi *et al.* showed that novel anti-inflammatory drug ethyl 2-((2,3-bis(nitrooxy)propyl)disulfanyl)benzoate (GT-094) decreases *miR-27a* expression in colon cancers cells[38]. Hezova *et al.* study did not show significant associations between *miR-27a* rs895819 polymorphism and CRC susceptibility in European population[31], but studies on this SNP in other population are missing. *MiR-492* is deregulated in colorectal cancer tissues when compared to normal colon mucosa[39], but rs2289030 of this miRNA has not been previously studied with respect to CRC risk. Several reports showed that SNP of *miR-608* (rs4919510) was linked with prognosis and survival in patients with CRC[40–42], but not overall cancer risks[42].

Most of the currently available genotyping studies related to *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) in CRC patients come from Asian populations and report partially conflicting results (Table 1). Furthermore, to our best knowledge there are no previous reports on *miR-492* rs2289030 and CRC risk. Here in this study we evaluated the role of five above mentioned miRNA SNPs in a case-control study including 428 controls and 193 CRC cases of European descent.

Results

Characteristics of the subjects. Characteristics of the subject groups are presented in Table 2. Control group consisted of 428 individuals: 112 (26.2%) males and 316 (73.8%) females; mean age was 63.2 ± 10.6 years. There were 193 subjects within CRC group: 109 (56.5%) males and 84 (43.5%) females; mean age was 67.2 ± 10.3 years. In accordance with real-life age, subjects differed significantly according to age and gender distribution between the groups ($P < 0.001$). There were more males in CRC group and this groups was older than the controls; however, then performing logistic regression analysis odds ratios (OR) were adjusted for these covariates as explained in the methods section below. Individuals in control and CRC groups were recruited in Lithuania and Latvia (Table 2).

Associations of miRNA SNPs and risk of CRC. Genotype distributions for all five polymorphisms in the study control group were similar to those expected for Hardy-Weinberg equilibrium: rs895819 ($P = 0.780$), rs2910164 ($P = 0.583$), rs11614913 ($P = 0.128$), rs2289030 ($P = 0.546$), rs4919510 ($P = 0.075$). 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 control and CRC patient groups are presented in Table 3. No significant associations were observed for diseases under study following correction for multiple testing. We observed a tendency for *miR-146a* C allele to be associated with lower risk of CRC when compared to G allele (OR = 0.68, 95% CI 0.49–0.95, $P = 0.025$), but difference did not reach the adjusted significance threshold. *MiR-492* C allele was marginally associated with higher risk of CRC when compared to G allele (OR = 1.56, 95% CI 1.00–2.42, $P = 0.047$).

Table 1 | Previous case-control studies on *miR-27a* (rs895819), *miR-146a* (rs2910164), *miR-196a-2* (rs11614913), *miR-492* (rs2289030) and *miR-608* (rs4919510) SNPs and colorectal cancer risk

SNP	Study	Controls, n	CRC, n	Population	Effect of SNP
miR-27a T>C rs895819	Hezova R et al. 2012 [31]	212	197	Caucasian	No association
miR-146a G>C rs2910164	Lv M et al. 2013 [26]	540	353	Asian	CC genotype↓
	Hezova R et al. 2012 [31]	212	197	Caucasian	No association
	Min KT et al. 2012 [27]	502	446	Asian	No association
	Ma L et al. 2013 [35]	1203	1147	Asian	GC/CC genotypes↓
	Parlayan C et al. 2014 [37]	524	116	Asian	No association
	Hu X et al. 2014 [36]	373	276	Asian	CG genotype↓
	Vinci S et al. 2013 [32]	178	160	Caucasian	No association
miR-196a-2 C>T rs11614913	Lv M et al. 2013 [26]	540	353	Asian	T allele↑
	Zhan JF et al. 2011 [29]	543	252	Asian	C allele↑
	Chen H et al. 2012 [30]	407	126	Asian	No association
	Hezova R et al. 2012 [31]	212	197	Caucasian	No association
	Min KT et al. 2012 [27]	502	446	Asian	CC genotype↑
	Zhu L et al. 2012 [28]	588	573	Asian	CC/CT genotype↑
	Vinci S et al. 2013 [32]	178	160	Caucasian	No association
miR-492 G>C rs2289030	No studies				
miR-608 C>G rs4919510	Ryan et al. 2012 [42]	245	446	African-American/Caucasian	No association

SNP – single nucleotide polymorphism;
CRC – colorectal cancer;
↑- increased risk;
↓- decreased risk.

Table 2 | Characteristics of subjects within control and colorectal cancer groups

	Controls (n = 428)	Colorectal cancer (n = 193)	ANOVA (Age) Chi-squared test P value
Age			
Mean ± SD	63.2 ± 10.6	67.2 ± 10.3	<0.001
Gender			
Male	112 (26.2%)	109 (56.5%)	<0.001
Female	316 (73.8%)	84 (43.5%)	
Country of birth			
Latvia	201 (46.9%)	64 (33.2%)	<0.001
Lithuania	227 (53.1%)	129 (66.8%)	

All the other comparisons between control and CRC groups did not reveal significant associations or trends for five SNPs of miRNAs under different genetic models.

Discussion

In this paper we present a case-control study of five gene polymorphisms – *miR-27a* (rs895819), *miR-146a* (rs2910164), *miR-196a-2* (rs11614913), *miR-492* (rs2289030) and *miR-608* (rs4919510) including 428 controls and 193 CRC patients of European descent. MiRNA deregulation is a well-established event in colorectal carcinogenesis[5,15]; therefore, we expected that SNPs related to miRNAs could be associated with CRC. The polymorphisms mentioned above have been linked with the risk of different cancers; however, the data of these polymorphisms in CRC patients are scarce and reports show

Table 3 | Genotype frequencies of *miR-27a, miR-146a, miR-196a-2, miR-492, miR-608* SNPs in controls and colorectal cancer patients

		Controls (n = 428)		CRC (n = 193)				
SNP	Genotypes/Alleles	n	%	n	%	aOR	95% CI	P value
miR-27a T>C[a]	TT	203	47.4	87	45.5	1		
rs895819	TC	191	44.6	79	41.4	0.94	(0.65–1.36)	0.736
	CC	34	7.9	25	13.1	1.68	(0.93–3.02)	0.084
	TT vs. TC + CC					1.05	(0.74–1.48)	0.790
	TT + TC vs. CC					1.73	(0.99–3.03)	0.055
	Allele T	597	69.7	253	66.2	1		
	Allele C	259	30.3	129	33.8	1.18	(0.91–1.52)	0.219
miR-146a G>C[b]	GG	275	64.9	140	72.9	1		
rs2910164	GC	134	31.6	50	26.0	0.76	(0.51–1.12)	0.163
	CC	15	3.5	2	1.0	0.20	(0.06–1.23)	0.092
	GG vs. GC + CC					0.71	(0.48–1.04)	0.078
	GG + GC vs. CC					0.30	(0.07–1.33)	0.113
	Allele G	684	80.7	330	85.9	1		
	Allele C	164	19.3	54	14.1	0.68	(0.49–0.95)	0.025
miR-196a-2 C>T[c]	CC	199	46.6	79	40.9	1		
rs11614913	CT	174	40.8	87	45.1	1.28	(0.88–1.86)	0.193
	TT	54	12.6	27	14.0	1.25	(0.73–2.14)	0.420
	CC vs. CT + TT					1.27	(0.90–1.81)	0.177
	CC + CT vs. TT					1.10	(0.67–1.83)	0.700
	Allele C	572	67.0	245	63.5	1		
	Allele T	282	33.0	141	36.5	1.17	(0.91–1.50)	0.228
			428					
miR-492 G>C	GG	377	88.1	159	82.4	1		
rs2289030	GC	49	11.4	32	16.6	1.58	(0.97–2.59)	0.068
	CC	2	0.5	2	1.0	2.36	(0.32–17.38)	0.401
	GG vs. GC + CC					1.61	(1.00–2.61)	0.052
	GG + GC vs. CC					2.21	(0.30–16.28)	0.436
	Allele G	803	93.8	350	90.7	1		
	Allele C	53	6.2	36	9.3	1.56	(1.00–2.42)	0.047
miR-608 C>G[d]	CC	318	74.7	138	71.9	1		
rs4919510	CG	96	22.5	47	24.5	1.12	(0.74–1.68)	0.592
	GG	12	2.8	7	3.6	1.36	(0.52–3.59)	0.533
	CC vs. CG + GG					1.15	(0.78–1.69)	0.495
	CC + CG vs. GG					1.33	(0.50–3.48)	0.568
	Allele C	732	85.9	323	84.1	1		
	Allele G	120	14.1	61	15.9	1.15	(0.82–1.61)	0.407

CRC – colorectal cancer; aOR – adjusted odds ratio (age, sex, country); CI – confidence interval;
[a]two individuals failed to be genotyped for rs895819;
[b]five individuals failed to be genotyped for rs2910164;
[c]one individuals failed to be genotyped for rs11614913;
[d]three individuals failed to be genotyped for rs4919510.

contradictory results. Our data suggest that *miR-27a*, *miR-146a*, *miR-196a-2*, *miR-492* and *miR-608* gene polymorphisms are not associated with the development of CRC in European population. To our best knowledge, there are only two reports on association between rs2910164, rs11614913 and CRC in Caucasian population, while rs2289030 has not been examined in any of the previous studies.

MiR-196a-2 SNP (rs11614913) was investigated in several case-control studies with respect to CRC risk. A report by Lv *et al.* showed that CT, TT genotypes and T allele were associated with an increased risk of CRC compared with the CC genotype and C allele (CT vs. CC: OR = 7.34, 95% CI 3.76–14.34, P < 0.001; TT vs. CC: OR = 1.99, 95% CI 1.63–2.42, P < 0.001, respectively)[26]. Opposite results were shown by Zhan *et al.* who found that CC genotype and C allele were associated with a significantly increased risk of CRC compared with the TT genotype and T allele (CC vs. TT: OR = 1.74, 95% CI 1.11–2.73, P = 0.015, C vs. T: OR = 1.32, 95% CI 1.06–1.65, P = 0.014), however, no significant association between this polymorphism and CRC progression was observed[29]. CC genotyped was also named as a risk genotype for CRC by Min *et al.*[27] and Zhu L *et al.*[28] studies. The other studies by Chen *et al.* and Hezova *et al.* did not observe the link between *miR-196a-2* SNP and CRC risks[30,31]. Although a recent meta-analysis of seven studies suggested that rs11614913 might contribute to the reduced risk of CRC[43], our results do not support these findings.

MiR-146a SNP (rs2910164) has been extensively studied in different cancers, but reports on CRC patients, especially in Caucasian populations, still remain scarce. Lv *et al.* found that carriers of rs2910164 CC genotype had a significantly decreased risk of CRC (OR = 0.58, 95% CI 0.37–0.93, P = 0.02) and C allele was associated with reduced risk of CRC (OR = 0.80, 95% CI 0.66–0.97, P = 0.02)[26]. Similar findings for *miR-146a* SNP have been detected in a study by Ma *et al.* including 1203 controls and 1147 CRC patients, which showed that carriers of GC/CC genotypes had a reduced the risk of colon cancer[35]. Opposite results were revealed in European studies by Hezova *et al.*[31] and Vinci et al.[32] where *miR-146a* SNP was not linked with CRC; furthermore, negative results have also been previously reported in Asian populations[27,37]. The results of our study support the negative findings of the latter studies as no significant association between *miR-146a* polymorphism and the risk of CRC was determined. A similar conclusion on rs2910164 and CRC risks is drawn by a meta-analysis by Wan *et al.*[43].

Gene polymorphism of *miR-27a* has been poorly investigated in CRC patients in comparison to other types of cancer. Meta-analysis by Hu *et al.* concluded that *miR-27a* rs895819 polymorphism may contribute to the susceptibility of overall cancer risk[24]. To date, only one study carried out by Hezova *et al.* examined the role of this SNP in CRC development in Caucasians[31]. The results of the latter study did not show any associations between *miR-27a* rs895819 polymorphism and CRC susceptibility in 212 controls and 197 CRC patients. These results are in concordance with our study, but more extensive comparison of present results due to the lack of studies cannot be performed.

Polymorphism of *miR-492* (rs2289030) has been yet little explored in cancer related case-control studies. In a study with non-small cell lung cancer no risk was determined for carriers of different rs2289030 genotypes[44]. The results our own group showed no role of this SNP in the development of gastric cancer[45]. A study on colorectal cancer by Lee *et al.* 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[22], but the risk of CRC with respect to rs2289030 genotypes or alleles has not been analyzed in this study. Our present data do not show a significant role of *miR-492* SNP for CRC development, but further studies including other populations from different ethnical backgrounds are warranted.

A recent meta-analysis did not detect significant associations for *miR-608* rs4919510 in terms of overall cancer risks or specific types of cancer[24]. Previous studies that have analyzed the role of *miR-608* (rs4919510) SNP in relation to CRC risk are scarce. A case-control study including 245 colorectal cancer patients and 446 controls did not find an association with CRC 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[42]. Our results are in line with this study as rs4919510 genotypes or alleles of *miR-608* were not linked with the presence of CRC. Two other studies on rs4919510 were not aimed to determine overall risk of CRC development, but rather looked at looked prognostic role of this SNP. Their results showed that *miR-608* SNP was significantly linked with recurrence and survival in CRC patients[40,41].

Overall, our study did not show significant association between *miR-27a* (rs895819), *miR-146a* (rs2910164), *miR-196a-2* (rs11614913), *miR-492* (rs2289030) and *miR-608* (rs4919510) and the risk of CRC. These findings are in line with our previous research which did not reveal association between these SNPs and gastric cancer or premalignant gastric conditions[45]. Although some of the studies have observed significant differences for various cancer-types, the ultimate role of microRNA related SNPs in cancer development in still not clear. The differences among the conclusions drawn by separate studies may result from study design, subtypes of CRC or different ethical backgrounds[9,24]. The current meta-analysis suggest potential role of these SNPs in CRC development, but further research in this field is mandated[9,24,25,43,46]. To date there are 19 genome wide association studies (GWAS) in CRC patients according to National Human Genome Research Institute; but none of them have linked rs895819, rs2910164, rs11614913, rs2289030 and rs4919510 with the development of cancer[47]. Nevertheless, due to different limitations of GWAS studies, smaller association studies on potential candidate SNPs remain relevant.

Our study has certain limitations that have to be acknowledged. The number of individuals within CRC and control groups is not large for conclusive genotyping studies; however, we believe that our data will be valuable for future meta-analysis on miRNA related SNPs. Due to a small number of individuals we were not able to perform appropriate haplotype analysis in order to evaluate potential effects of combined genotypes of these miRNA SNPs. Unfortunately, we do not have a validation group; therefore, tendencies that were observed for *miR-146a* and *miR-492* SNPs should be explored in further larger scale studies. Our control group might be biased by the fact that control subjects were hospital based – outpatient dyspeptic patients without previous cancer history. Survival data as well as smoking and alcohol consumption data were available only for a small proportion of subjects; therefore, stratification analyses were not performed. Due to a small number of individuals we were not able to perform genotyping sub-analysis for CRC regarding disease stage, tumor location or molecular subtypes, which may have different genetic susceptibility.

Methods

Ethics statement. The study was approved by the Ethics Committees of the Lithuanian University of Health Sciences and Central Medical Ethics Committee of Latvia. All patients have signed an informed consent form to participate in the study. All the methods applied in the study were carried out in accordance with the approved guidelines.

Study population. Patients and controls were recruited during the years 2007–2013 at two gastroenterology centers in Lithuania (Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas) and Latvia (Riga East University Hospital and Digestive Diseases Centre GASTRO, Riga). Control group consisted of patients with dyspeptic symptoms, who had no alarm symptoms and no history of previous malignancy. CRC patients had histological verification of colorectal adenocarcinoma and were recruited from out-patient and stationary departments. In total 621 (428 controls and 193 CRC patients) individuals were included in the study. There were 265 subjects from Latvian group (201 controls and 64 CRC) and 356

subjects from Lithuanian group (227 controls and 129 CRC). All patients were of European ethnicity.

DNA extraction and genotyping. Genomic DNA from samples was extracted using salting out method from peripheral blood leukocytes. 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™ 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. After genotyping 5% of samples in each genotype group were selected for repetitive analysis with 100% concordance rate. Samples that failed to genotype were recorded as undetermined.

Statistical analysis. Age is shown as means and standard deviations, and was compared using unpaired Student's t-test. Statistical analysis of genotyping data was performed using PLINK software version 1.07[48]. Hardy-Weinberg equilibrium was assessed for each of SNPs. Association between control and CRC with gene polymorphisms was calculated using logistic regression analysis with adjustment for age, gender and country of birth with 95% confidence intervals (CI). The analysis was carried out using homozygous, heterozygous, allelic, recessive and dominant models. An adjusted significance threshold $\alpha = 0.01$ (0.05/5) was used to determine significant differences.

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Author contributions

J.K., J.S. and L.K. conceived and designed the experiments; I.B. and U.G. performed the experiments; J.K., J.S. and L.K. analyzed the data; L.J., S.J., G.K., M.L., H.P., A.T. and D.P. contributed reagents/materials/analysis tools; J.K. and I.B. wrote the paper.

Additional information

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