




IJCRR
Section: Healthcare
ISI Impact Factor
(2019-20): 1.628
IC Value (2019): 90.81
SJIF (2020) = 7.893

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Micro-RNA 155 - On the Crosspoint of Inflammation and Carcinogenesis

Antonia Atanassova^{1,3}, Avgustina Georgieva^{1,2}, Trifon Chervenkov⁴,
Assia Konsoulova^{5,6}

¹Gastroenterology Clinic, "St. Marina" University Hospital – Varna, Bulgaria; ²Second Internal Medicine Department, Medical University – Varna, Bulgaria; ³Department of Anatomy and Cell Biology, Medical University – Varna, Bulgaria; ⁴Department of Medical Genetics, Medical University – Varna, Bulgaria; ⁵Complex Oncological Center - Burgas, Bulgaria; ⁶Women for Oncology – Bulgaria.

ABSTRACT

Background: As miR-155 influences a wide spectrum of inflammatory mediators, the study of this miRNA may suggest a new insight over the cancer development mechanisms. This is why the investigation of the miR-155 expression may reveal a potential relation between inflammation and gastrointestinal cancer.

The aim of the current study is to assess the miR-155 expression levels in patients with Crohn's disease (CD) and ulcerative colitis (UC).

Materials and Methods: The expression of miR-155 was studied in 70 consecutive patients with a confirmed diagnosis of IBD: 35 with CD and 35 with UC and 30 healthy controls. Disease activity was evaluated by the clinical symptoms, biochemical inflammatory parameters (CRP, FCP) and validated indices for evaluating IBD activity (CDAI for CD, Montreal Classification, and partial Mayo score for UC). 25(OH)D serum concentrations were measured by a commercial paramagnetic particle chemiluminescent immunoassay for the quantitative determination of total 25 - hydroxyvitamin D [25(OH) vitamin D] levels use on Access 2 Immunoassay Systems. Serum expression of miR-155 by reverse transcriptase, a real-time quantitative polymerase chain reaction (RT-qPCR), was tested in all.

Results: The analysis of the results showed that the circulating miR-155 was increased in Crohn-colitis (3.51±5.22) and extensive UC (2.86±5.44). Levels of CDAI above 150 were a risk factor for detection of increased miR-155 expression levels (OR=10, 91 (1.194-99,688); p=0.017). An increased miR-155 expression was detected in patients, treated with corticosteroids (5.20±8.91 for UC and 3.39±3.10 for CD). There was an inverse proportional moderate correlation with the levels of FCP (r= -0.344 ;p<0.05). Vitamin D deficiency (25(OH)D) led to a 1.24 higher risk for increased serum level of miR-155.

Conclusions: The increased expression of miR-155 in patients with IBD is seen in disease localization in the colon, persistent inflammation, severe disease activity, vitamin D deficiency and treatment with corticosteroids.

Key Words: Fecal calprotectin, CRP, Crohn's disease, micro-RNA 155, Ulcerative colitis, Vitamin D

INTRODUCTION

Micro RNAs (miRNAs) are endogenous non-coding RNAs (ncRNAs), with length of about 22 nucleotides. The biogenesis of the miRNAs develops in several steps of post-transcriptional modifications in the nucleus and the cytoplasm.¹ Over 60% of all genes, that code proteins, are regulated by miRNAs^{2,3,4} and one miRNA has on average 200 targets.^{5,6,7}

miRNAs are important regulators of different cellular processes, including development, differentiation and signalization.^{8,9,10,11,12} The dysregulation of specific miRNAs may lead

to different diseases in humans: metabolic disturbances, cardiovascular or liver diseases as well as to immune dysfunction, including development of neoplasms.^{13,14,15}

The expression of miRNA-155 (miR-155) is related to different cardiovascular diseases, inflammation and cancers. Its multifunctional regulatory role defines the interest towards this particular miRNA, that is considered to be the most extensively studied miRNA.

Expression of miR-155 was first reported in human spleen and thymus and subsequently in liver, lungs and kidneys.^{10,16} Later on, it was established that this miRNA has

Corresponding Author:

Assist. Prof. Avgustina Chavdarova Georgieva MD, PhD, Varna, 1 Hristo Smimanski Bul., floor 11, Clinic of Gastroenterology, Multiprofile University Hospital for Active Treatment "Sveta Marina", city of Varna, Bulgaria; E-mail: avgustina_dobreva@yahoo.com

ISSN: 2231-2196 (Print)

ISSN: 0975-5241 (Online)

Received: 18.10.2020

Revised: 03.12.2020

Accepted: 27.01.2021

Published: 22.06.2021

an abnormally increased expression in different activated immune cells¹⁷, which defines the important role of miR-155 in immune response.¹⁸

miR-155 responds to many inflammatory stimuli as tumor necrosis factor alfa (TNF- α), interleukin (IL) -1b, interferons, pathogen-associated molecular patterns (PAMP), damage-associated molecular patterns, (DAMP)¹⁹, alarmins (e.g. IL-1a)²⁰, hypoxia²¹, as well as in Toll-like receptor ligands (TLR) in monocytes and macrophages B cells.²²

The expression of miR-155 is controlled by multiple signal pathways. The regulatory cytokines, including Transforming Growth Factor beta (TGF- β) may induce or inhibit the expression of miR-155.^{23,24}

In IBD, the negative feedback control over the inflammation is disturbed, leading to an excess activation of the inflammatory signal pathways.^{25,26}

The endogeneous and the synthetic glucocorticoids are highly effective to slow down the process of acute inflammation via suppression of the miR-155 expression in the glucocorticoid receptor and/or via nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B).^{27,28,29}

Chen Y, et.al, 2013³⁰ identified that the vitamin D receptor signal pathway blocks the activation of NF- κ B and thus leads to a decrease in miR-155 levels. As a result of this, the translation of SOCS1 is increased, allowing for an increase in the regulation of the negative feedback over the immune response.³¹

At least 15-20% of all types of human cancer are related to chronic inflammation: diseases as IBD, colorectal cancer, colitis associated cancer (in ulcerative colitis), chronic gastritis and *H. pylori* (gastric cancer, GC).³² As miR-155 influences a wide spectrum of inflammatory mediators, the study of this micro-RNA may suggest a new insight over the cancer development mechanisms. This is why the investigation of the miR-155 expression may reveal a potential relation between inflammation and gastrointestinal cancer.

The aim of the current study is to assess the miR-155 expression levels in patients with Crohn's disease (CD) and ulcerative colitis (UC).

MATERIALS AND METHODS

The expression of miR-155 was studied in 70 consecutive patients with a confirmed diagnosis of IBD: 35 with CD and 35 with UC. Patients were treated at the Gastroenterology clinic during a period of one year (April 2019 – April 2020). 30 healthy individuals were also tested in order to define a healthy control study group. All IBD patients were classified according to the Montreal classification. The clinical course and treatment regiments were assessed. Disease activity was

evaluated by the clinical symptoms, biochemical inflammatory parameters (C reactive protein - CRP, fecal calprotectin - FCP) and validated indices for evaluating IBD activity (CDAI for CD, Montreal Classification, and partial Mayo score for UC). Biochemical parameters (CRP and FCP) were assessed as either normal or abnormal: FCP was considered as normal if levels were < 50 mg/g; CRP was considered abnormal (elevated) if measured > 5 mg/l. 25(OH) D serum concentrations were measured by a commercial paramagnetic particle chemiluminescent immunoassay for the quantitative determination of total 25-hydroxyvitamin D [25(OH) vitamin D] levels use on Access 2 Immunoassay Systems. Vitamin D deficiency was defined as a serum level of 25OHD lower than 50 nmol/L; serum level above 50 nmol/L but lower than 75 nmol/L was classified as vitamin D insufficiency.

Levels of miR-155 were assessed in blood serum. 5 ml of blood was obtained via peripheral venous puncture with closed system BD Vacutainer™ SST™ II Advance (Becton Dickinson, USA). After withdrawal, the blood sample was held 30 minutes at room temperature for clotting. Subsequently, it was centrifuged at 500×g for 15 minutes at room temperature and the serum was separated and divided into aliquots of 500 μ l that were immediately stored at -80 °C until the moment of the analysis.

miRNAs were isolated from 200 μ l serum using a pre-existing commercial miRNeasy Serum/Plasma Kit, catalogue №217184 (QIAGEN, Germany) as per protocol of the manufacturer.

3,5 μ l control miRNA *C. elegans* miR-39: miRNeasy Serum/Plasma Spike-In Control, catalogue №219610 (QIAGEN, Germany), was added to each sample for normalization control; the samples were afterwards eluted in 14 μ l RNA-ase free water.

Each of the samples was subsequently submitted to reverse transcription via ready-to-use commercial kit miScript II RT Kit, catalogue №218161 (QIAGEN, Germany) as per manufacturer's protocol from 2,5 μ l eluted miRNA in a final volume of 10 μ l with HiFlex buffer and it was incubated at 37 °C for 60 minutes and the enzyme was inactivated at 95 °C for 5 minutes.

Each of the samples was then submitted to quantitative real time polymerase chain reaction (rt-PCR) via a ready-to-use commercial kit miScript SYBR Green PCR Kit, catalogue № 218073 (QIAGEN, Germany) and prepared primers miScript Primer Assay, catalogue №218300 (QIAGEN, Germany) as per manufacturer's protocol: 1 μ l complementary DNA (cDNA) in 10 μ l reactions in 3 - times repetitions for 3 target miRNA in plates. The used miScript Primer Assay primers, catalogue № 218300 (QIAGEN, Germany) were as follows (the reference number is in the brackets): Ce_

miR-39_1 (MS00019789), Hs_miR-155_2 (MS00031486), Hs_RNU6-2_11 (MS00033740). The used temperature parameters were as follows: maintenance for 15 minutes at 95 °C for enzyme activation; 40 cycles of 15 seconds at 94 °C; 30 seconds at 70 °C with fluorescent reading; analysis of the melting curve in order to prove the specificity of the amplification: primary denaturation for 15 seconds at 95°C and cooling to 55 °C for 60 seconds with an increase to 95 °C with velocity of +0,05 °C per second and fluorescent reading. The analysis was done by QuantStudio Dx instrument of Applied Biosystems (USA) company; a threshold cycle (Ct) was assessed for each sample. Receiver Operating Characteristic Curve (ROC) was calculated to detect diagnostic performance of the test, sensitivity, specificity, positive and negative predictive values. Significance of the obtained results was judged at the 5% level.

The results were calculated with SPSS, v. 20.0 for Windows. We used variation, correlation, regression analyses as well risk assessment and comparative analyses (χ^2 , t-test, ANOVA). $p < 0.05$ was used as a level of significance.

The clinical trial was initiated after approval and permission №82 / 28.03.2019 of the Ethics commission for scientific research at the Medical University – Varna, Bulgaria.

Results: Table 1 shows the characteristics of the patients with CD, UC and the healthy control subjects.

As there are no validated referent thresholds for the expression of any micro RNA, we calculated a cut-off threshold level in order to differentiate between healthy control subjects and patients with chronic inflammatory bowel diseases (IBD). This cut-off is specific for our cohort of studied healthy subjects and was used as a marker for assessment of the miR-155 expression in patients with UC and CD. In our study, the cut-off value for the miR-155 is calculated at 1.37 (AUC 0.620; 0.497-0.742) with a sensitivity of 61.4 % and specificity of 63.3 % (Fig. 1).

Table 2 shows the expression of miR-155 according to the characteristics of the patients with CD and UC.

The analysis of the results of the miR-155 according to the localization of the disease indicates that the circulating miR-155 was increased in Crohn-colitis (3.51 ± 5.22) and extensive UC (2.86 ± 5.44). In patients with CD, there was no significant difference in the miR-155 expression levels in inflammatory (B1) or stenotic (B2) form of the disease, whereas in patients with UC, not achieving a significant remission (chronic persistent form), the expression of miR-155 was significantly higher as compared to patients with chronic relapsing course of disease (5.53 ± 9.56 vs 1.54 ± 1.22 ; $P = 0.032$).

The correlation analysis showed a proportional moderate correlation between miR-155 expression CDAI ($r = 0.415$; $p = 0.015$). This indicates that the progression of the disease

activity led to an increase in the serum expression levels of miR-155. Patients with moderate CD activity had significantly increased expression of miR-155 as compared to patients in remission or with mild activity (6.08 ± 5.12 vs 2.11 ± 2.09 and 2.11 ± 1.07 ; $p = 0.009$). Levels of CDAI above 150 were a risk factor for detection of increased miR-155 expression levels (OR=10,91 (1.194-99,688); $p = 0.017$).

The analysis of the correlation between the severity of the UC and the expression of miR-155 showed that the severity index S (as per the Montreal classification) had a proportional moderate correlation with the increased expression of miR-155 ($r = 0.321$; $p = 0.048$). Patients with severe disease activity had a significantly higher expression levels of miR-155 ($p < 0.05$). On the other hand, the endoscopic activity, measured by the Partial Mayo score, did not show a correlation with the miR-155 expression – the miR-155 levels were increased in patients with moderate disease activity (4.82 ± 9.05) and decreased in patients with severe activity (2.23 ± 1.75). In patients with UC in remission or with mild disease activity, the miR-155 expression levels were close or just mildly below the threshold cut-off value, defined for the healthy control subjects.

Regarding the treatment, an increased miR-155 expression was detected in patients, treated with corticosteroids (5.20 ± 8.91 for UC and 3.39 ± 3.10 for CD). The expression of miR-155 was similar in patients, treated with immune modulators in both CD and UC. In patients with UC, receiving biological treatment, the levels of serum miR-155 expression are below the defined cut-off value for healthy controls.

Other widely used biomarkers, used to biochemically assess inflammation in patients with IBD, are CRP and FCP. The mean level of CRP levels in patients with IBD was $19.31 \text{ mg/l} \pm 32.69 \text{ mg/l}$ (0.09-160.0 mg/l), and for FCP it was $459.19 \text{ mg/g} \pm 490.74 \text{ mg/g}$ (2.00 – 1800 mg/g). The results of the analysis of the correlation between the miR-155 expression and CRP or FCP showed that the levels of the tested miR do not correlate with the levels of CRP, but there was an inverse proportional moderate correlation with the levels of FCP ($r = -0.344$; $p < 0.05$).

The mean 25(OH)D serum expression level in patients with IBD was $44.14 \text{ nmol/L} \pm 17.47 \text{ nmol/L}$ with a variation between 2.76 nmol/L and 90.65 nmol/L . These results showed that the expression of miR-155 was different according to the serum levels of 25(OH)D. In patients with 25(OH)D serum levels $< 50 \text{ nmol/L}$, the expression of miR-155 was significantly higher as compared to patients with 25(OH)D levels $\geq 50 \text{ nmol/L}$ (3.53 ± 6.35 vs 2.38 ± 2.23 ; $p = 0.041$). Vitamin D deficiency (25(OH)D) led to a 1.24 higher risk for increased serum level of miR-155.

DISCUSSION

Many studies show that the miR-155 expression is increased in patients with IBD, both in UC and CD.^{33,34,35} Our results also correspond with previous publications, showing a significantly higher miR-155 expression levels in patients with IBD as compared to healthy individuals.

The expression of miR-155 in patients with active UC is increased in comparison to healthy individuals. This is a result of the direct decrease in the expression of FOXO3a gene which may additionally activate the NF- κ B signaling pathway and increase the inflammatory cytokines and thus maintain inflammation.³⁶ These results are confirmed by our study as well.

In our trial, patients with persistent inflammation and no remission as well as a localization of their disease in the colon, have a significantly higher miR-155 expression. It is well known that chronic inflammation is the background of the IBD, UC-associated carcinoma as well of colorectal cancer.³²

Logically, the change in the expression of miR-155 levels during monitoring of inflammatory processes in IBD could be a potential biomarker for early discovery of dysplasia. Further larger prospective cohort trials in IBD patients are needed to explore the role of miR-155 in the tumorigenesis and to validate the change in its expression levels as a potential biomarker for occurrence of dysplasia and its transition into tumorigenesis.

There are publications that the localization and the disease course may be related to different miR-155 levels. Increased miR-155 levels in intestinal myofibroblasts and in mucosa of the colon are related to the control of the Th17 inflammatory pathway, targeting FOXO3a and IL-8, thus promoting fibrosis formation.^{37,38}

It has been proven that miR-155 plays a significant role in the control of the inflammation and the immune regulation and it is logical that its expression is significantly higher in patients with active disease, regardless of the method, assessing the activity of the disease.

Chen Y. et.al. describes a new mechanism, where vitamin D receptor signaling pathway controls the TLR4-mediated inflammation in IBD and the immune mediated diseases. This anti-inflammatory mechanism is mediated via miR-155 suppressing the regulation, decreasing SOCS1. This leads to enhancement of the regulation of the negative feedback. Vitamin D – receptor signaling pathway inhibits miR-155 via a transcription mechanism.³⁰ This process of suppression of the miR-155 expression is also confirmed in our study where we established an inverse proportional correlation between the vitamin D levels and the miR-155 expression. Vitamin D deficiency correlates with higher miR-155 expression levels.

Treatment with 5-ASA (5 – aminosalicylic acid) and biologicals is highly effective in patients with UC and leads to a decrease in the miR-155 expression to levels, close to or below the threshold cut-off values as in health individuals. On the other hand, in patients with CD, treated with 5-ASA, the levels of miR-155 do not decrease to such extent which shows the persistence of the inflammation and explains the difficult achievement of remission in patients with CD.

Treatment with corticosteroids is highly effective to suppress the acute inflammation via a change in the miR-155 expression.^{27,28} In our cohort of patients, treated with corticosteroids in order to induce remission, the levels of miR-155 are increased and this may be explained by the fact that the disease remission, induced by corticosteroids is short-term and unstable as there is a high risk of recurrence or complications of the disease.

CONCLUSION

The increased expression of miR-155 in patients with IBD is seen in disease localization in the colon, persistent inflammation, severe disease activity, vitamin D deficiency and treatment with corticosteroids. Decreased miR-155 expression with levels, close to the levels of healthy subjects is reported predominantly in patients with UC in remission, induced by biological therapy. As miR-155 has a significant role in the assessment of the inflammatory process and plays an important role in the tumorigenesis, a longer monitoring of the dynamics of its expression in patients with active IBD may reveal a potential crosspoint between inflammation and gastrointestinal cancer.

ACKNOWLEDGEMENT

Authors acknowledge the immense help received from the scholars whose articles are cited and included in references of this manuscript. We are also grateful to authors/editors/publishers of all those articles, journals and books from where the literature of this article has been reviewed and discussed.

Authors' Contribution:

Antonia Atanassova- conceived and designed the study, performed the statistical analysis, analyzed the data and drafted the manuscript

Trifon Chervenkov- performed the miRNA expression profiles in blood

Avgustina Georgieva- collected data and drafted the manuscript

Assia Konsoulova- critical revision of manuscript

All authors critically revised the manuscript, approved the final version to be published, and agree to be accountable for all aspects of the work.

Source of Funding: This studies as no fund from any authorities

Conflict of Interest: None to declare

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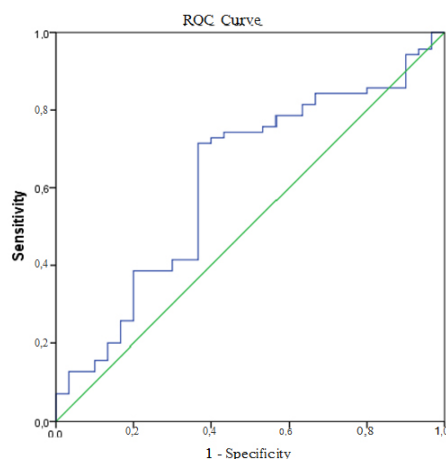
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Table 1: Characteristics of the patients with IBD according to the Montreal classification

Index		Active CD (n=20)	CD in remission (n=15)	Active UC (n=20)	UC in remission (n=15)	Healthy controls (n=30)
Age, years (mean±SD, range)	Current	41.1±12.9 (18-71)	42.1±14.8 (23-75)	37.9±13.8 (19-73)	46.4±16.1 (18-71)	26.2±6.2 (18-42)
	Diagnosis	35.5±11.6 (14-56)	36.8±15.1 (19-75)	30.8±11.5 (15-53)	35.3±14.3 (17-62)	-
Sex	Male	13/65.0%	5/33.3%	7/35.0%	8/53.3%	15/50.0%
	Female	7/35.0%	10/66.7%	13/65.0%	7/46.7%	15/50.0%
Localization of CD/UC	L1/E1	13/65.0%	6/40.0%	-	1/6.7%	-
	L2/E2	-	5/33.3%	8/40.0%	3/20.0%	-
	L3/E3	7/35.0%	4/26.7%	12/60.0%	11/73.3%	-
Behavior	B1	5/25.0%	12/80.0%	-	-	-
	B2	9/45.0%	2/13.3%	-	-	-
	B3	5/25.0%	1/6.7%	-	-	-
	B2-B3	1/5.0%	-	-	-	-
	Chronic relapsing	-	-	13/65.0%	15/100%	-
	Chronic persistent	-	-	7/35.0%	-	-
Duration of the IBD, months (mean±SD, range)		67.4±66.4 (3-204)	66.3±51.6 (3-156)	84.7±97.3 (1-336)	150.8±137.6 (12-492)	-
Treatment	5ASA	1/5.0%	1/6.7%	7/35.0%	-	-
	Corticosteroids	6/30.0%	1/6.7%	8/40.0%	5/33.3%	-
	Immune modulators	1/5.0%	1/6.7%	2/10.0%	9/60.0%	-
	Biological treatment	12/60.0%	12/80.0%	3/15.0%	1/6.7%	-
Vitamin D (25 OH D)	(mean±SD, range)	39.45±15.65 (2.76-71.14)	53.02±16.77 (26.37-73.17)	40.32±15.41 (25.66-77.0)	55.22±21.07 (26.41-90.65)	-
	< 50 nmol/L	15/75.0%	2/28.6%	11/73.3%	3/37.5%	-
	> 50 nmol/L	5/25.0%	5/71.4%	4/26.7%	5/62.5%	-
CRP (mg/l)	(mean±SD, range)	39.22±45.17 (0.17-160.0)	3.24±2.51 (0.09±8.34)	22.51±29.85 (0.47-91.9)	2.30±2.07 (0.13-6.90)	-
	< 5 mg/L	5/25.0%	11/73.3%	6/30.0%	10/76.9%	-
	> 5 mg/L	15/75.0%	4/26.7%	14/70.0%	3/23.1%	-
FCP (mg/g)	(mean±SD, range)	449.14±339.82 (110.0-910.0)	112.91±194.48 (2.0-648.0)	1054.89±413.67 (300.0-1800.0)	198.07±268.7 (12.5-759.0)	-
	< 50 mg/g	7/100%	6/60.0%	-	4/57.1%	-
	> 50 mg/g	-	4/40.0%	9/100.0%	3/42.9%	-

Table 2: Expression of micro-RNA 155 according to the main characteristics of the patients with CD and UC.

Baseline characteristics		miR-155 expression (mean±SD)	
		CD (n=35)	UC (n=35)
Location	L1/E1	2.51±2.42	2.19
	L2/E2	3.51±5.22	1.25±0.96
CD/UC	L3/E3	2.53±1.84	2.86±5.44
Behavior	B1	2.87±3.36	-
	B2	2.89±2.55	-
P=0.032	B3	1.86±1.06	-
	B2-B3	1.48	-
CDAI	chronic recurrent	-	1.54±1.22
	chronic persistent	-	5.53±9.56
P=0.009	Remission	2.11±2.09	-
	Mild	2.11±1.07	-
S (severity)	Moderate	6.08±5.12	-
	Remission	-	1.11±0.75
Mayo score	Mild	-	1.33±1.02
	Moderate	-	1.63±0.48
Treatment	Severe	-	4.92±7.93
	Remission	-	1.22±0.79
Vitamin D (25 OH D)	Mild	-	1.34±0.85
	Moderate	-	4.82±9.05
CRP (mg/l)	Severe	-	2.23±1.75
	5ASA	2.71±2.76	1.65±1.51
FCP (mg/g)	Corticosteroids	3.39±3.10	5.20±8.91
	Immune modulators	1.94±0.72	1.90±1.26
CRP (mg/l)	Biological treatment	2.51±2.85	1.23±0.86
	< 50 nmol/L	3.15±2.69	1.44±0.95
FCP (mg/g)	> 50 nmol/L	2.80±3.73	4.34±8.58
	< 5 mg/L	3.05±3.37	1.11±0.89
FCP (mg/g)	> 5 mg/L	2.34±2.16	3.63±6.18
	< 50 mg/g	4.04±3.98	1.63±0.70
FCP (mg/g)	> 50 mg/g	1.45±0.57	3.89±7.44

**Figure 1:** A receiver operating characteristic curve (ROC curve) for serum miR-155 expression to predict cases (from control).