# Significance of microRNA-330/TYMS expression axis through colorectal tumorigenesis

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## Abstract

# Background

Colorectal cancer is one of the most common types of cancer worldwide. MiR-330 has been reported as a cell proliferation inhibitor by suppressing thymidylate synthase (TYMS) in FOLFOX, one of the major chemotherapy regimens used to treat colorectal cancers. A number of dysregulated miRNAs have been linked to CRC progression and treatment response and are thought to be promising prognostic biomarkers for this cancer. In the current study, miR-330, TYMS, and their interactions have been investigated in the absence of this chemotherapy to evaluate their therapeutic and diagnostic value for other treatment methods.

# Material and Methods

The expression levels of miR-330 and TYMS were evaluated in-silico using TCGA datasets for colorectal cancer. Data validation was performed on a set of internal samples (100 pairs of CRC tumor specimens and adjacent non-cancerous samples) were determined utilizing RT-qPCR assay. The linkage between clinicopathological parameters and expression levels was also investigated.

# Results

TCGA results illustrated that miR-330 and TYMS are significantly upregulated and downregulated through colorectal tumorigenesis, respectively. QRT-PCR results confirmed that the expression level of miR-330 was significantly higher in tumor tissues relative to margin tissues (p value = 0/0005) whereas TYMS was significantly down-regulated (p value = 0.0001). However, there was no significant association regarding TYMS and patient pathological features while miR-330 expression was associated with tumor stage and lymph node metastases.

# Conclusion

The microRNA-330 inhibited cell proliferation by suppressing thymidylate synthase (TYMS) in colorectal cancer. Therefore, suggesting that they are valuable factors for further studies of alternative treatment and diagnostic methods.

## Introduction

Colorectal cancer (CRC) between all types of cancer is ranked as third with regard to prevalence, and second in mortality rates [1, 2], contributing to over 700,000 fatalities per year worldwide [3, 4]. CRC incidence rates are relatively similar in both men and women [5, 6]. The notable difference in the worldwide annually reported frequency of CRC, illustrates the major effect of lifestyle influences on the

presence of cancer [3]. For instance, colorectal cancer prevalence rates are approximately 3 times higher in transitioned against developing countries [1]. To achieve a treatment for CRC, early diagnoses, personalized medication, and a clear view on the molecular mechanisms of its incidence and development, are essential [7]. But unfortunately, most CRC cases are found at an advanced phase in patients often at the beginning of metastasis, which contributes to the weak prognosis and the lower patient recovery possibilities [4, 8].

Given the advances achieved in understanding the molecular mechanisms involved in the CRC, several aspects are still vague. Despite that, molecular science developments have contributed to the identification of several prospective biomarkers that are important to colorectal cancer (CRC). Given the fact that in CRCs, a noticeable number of dysregulated miRNAs are identified to have been linked with disease progression and treatment response, microRNAs are considered to be hopeful prognostic biomarkers for this cancer in the latest researches [2, 4, 9, 10]. Compared to normal controls, miRNAs are therefore categorized as oncomiR and miRNA tumor suppressor, and some of them could be utilized as CRC diagnostic, prognostic and predictive biomarkers [11].

Micro-RNAs (miRNAs) are vast subgroups of small single-strand noncoding RNAs containing 19–22 nucleotides that by binding to the 3'UTR end of the mRNA could inhibit translation or degrade the mRNA before translation. MicroRNA expression irregularities are found to be involved in the dysregulation of cell apoptosis, angiogenesis, metastasis, and tumor development of different malignancies. Moreover, to regulate the expression of their target genes which are associated with CRC proliferation and metastasis, miRNAs can target long non-coding RNA (lncRNA), as well [4, 12–16]. The roles of these dysregulated miRNAs tend to be contextual, showing a double role as oncogenes and the tumor-suppressors regarding their cellular environment. The distinctive expressing characteristics of miRNAs, therefore, contribute to CRC diagnosis, prognosis, and therapeutic results [10, 14, 17]. The biogenesis of miRNAs is done by many enzymes and various cellular compartments and is a complicated multi-phase process with various steps [18].

The miR-330 gene is found on a fragile genome region of chromosome number 19 [19]. miR-330 as a key regulator for gene expression in some malignancies, such as colorectal cancer [20], prostate cancer [21], and melanoma [16], have been documented to be down-regulated. On the other hand, miR-330 is identified as an oncogenic factor due to overexpression in glioblastoma cells [22].

In CRC tissues, miR-330 expression was reported to be significantly lower than in adjacent non-tumorous tissues [23, 24]. As the decreased expression of miR-330 in CRC promotes proliferation and metastasis and decreases apoptosis, it can be considered as a therapeutic target and a molecular biomarker for CRC [20, 25]. MiR-330 has been observed in many studies as a regulatory factor, in CRC. For instance, induction of miR-330 inhibits cell proliferation through the suppression of post-transcriptional BACH1 expression [20]. Other results demonstrated that Cdc42 as an oncogene agent was negatively regulated by miR-330 via the specific target motif of Cdc42 3'UTR acting as a CRC tumor suppressor [26]. It has also been found that the downregulation of miR-330 expression may impact the development of CRC by

inhibiting the expression of ITGA5 through binding directly to the 3'UTR of ITGA5 mRNA [23]. We have also previously indicated that miR-330 functions as a miRNA tumor suppressor in CRC by suppressing HMGA2 expression and reducing cell viability, proliferation, and migration. Consequently, for patients with CRC, miR-330 could be proposed as a potential candidate for miRNA replacement therapy [27].

An analysis carried out on CRC cell lines indicated that miR-330 inhibited proliferation of colorectal cells and increased the chemo-sensitivity of CRC cells to 5-fluorouracil (5-FU) via the cell apoptosis pathway [24]. 5-FU is an antimetabolite medication that has a cytotoxic impact on inhibition of thymidylate synthase (TYMS) resulting in dTMP depletion [28, 29]. Hence, TYMS was recognized as a direct target gene of miR-330. Therefore, TYMS can represent a predictive cellular response biomarker for 5-FU and a therapeutic target for 5-FU-based chemotherapy [24].

Thymidylate synthase (TYMS) gene located on 18p11.32. TYMS catalyzes deoxyuridylate (dUMP) methylation to deoxythymidylate (dTMP) by employing 10-methylenetetrahydrofolate (methylene-THF) as a cofactor. This role protects the dTMP (thymidine-5-prime monophosphate) pool essential to DNA replication and repair. This enzyme has been studied as the target for cancer chemotherapies [19, 30, 31]. In several studies, dysregulation of TYMS in CRC has been recognized. In a study on ERCC1 and TYMS in CRC patients, both were reported to be over-expressed [32]. Although most studies have shown that Tumors expressing high levels of TYMS have a poorer overall survival (OS) contrasted with tumors expressing low levels, others have noted that increased TYMS protein and mRNA expression have been linked with higher relapse-free survival and OS [33–35].

Given these findings, maybe there is a diagnostic and predictive benefit to investigate the miR-330 and TYMS expression and the contribution of these genes in the pathogenesis of CRC, and this research may help to determine their value as a clinical biomarker for disease prediction.

# Materials And Methods

### Bioinformatic analysis

To initially evaluate the significance of miR-330 and TYMS dysregulation through colorectal tumorigenesis, their expression levels were investigated bioinformatically using the Cancer Genome Atlas (TCGA) datasets, as a public-funded project surveying genomic profiles of large cohorts of over 30 human cancers. For this aim, the expression data of miR-330 and TYMS were retrieved from TCGA Colon Cancer (COAD) and TCGA Rectal Cancer (READ) datasets using Xena Functional Genomics Explorer (https://xena.ucsc.edu/) and then analyzed.

### Research Population and Method for Clinical Sampling

Tumor tissues and associated non-tumor margin tissues were collected from 100 CRC patients (41 females, 59 males) during surgery which was the routine part of their treatment approach. All CRC cases have been diagnosed by surgical oncologists and referred to a pathologist for confirmation. These

operations were all performed at Imam Reza Hospital in Tabriz, Iran, between 2019 and 2020. The competence criteria for these patients were concentrated on the absence of any prior or simultaneous malignancies, chemotherapy background, radiotherapy, or medications that could affect the expression of the target genes. The research studied both patients with familial backgrounds and those described as their family's first probe of CRC. The categorized clinical characteristics of patients are shown in Table 1. These tissue samples were promptly placed in the solution of an RNase inhibitor (Qiagen, Hilden, Germany) and transported to the laboratory. Before sampling, signed written informed consent was gained from all participants. The research has also been confirmed by the Ethical Committee of Tabriz University of Medical Sciences.

### RNA Extraction Procedure

The total RNA of tissue samples was extracted using RiboEx reagent (Gene All biotechnology, Seoul, Korea) in accordance with the protocols of the manufacturer. By using the NanoDrop (Thermo Scientific™, USA) spectrophotometer at 260/280, the quality and quantity of total RNA was been verified. After that, the RNA samples were stored in -80 until the cDNA synthesis.

### Complementary DNA Syntheses and Quantitative Real-Time PCR

The cDNA synthesis process was performed separately for microRNA study (using stem-loop primers) and mRNA study refers to the kit standards using 2x RT-PCR Pre-Mix (Taq) (Universal cDNA synthesis kit (BioFACT TM, Seoul, South Korea) U6 and GAPDH were used as internal controls of this study. The qRT-PCR was performed using RealQ plus Master Mix Green (Amplicon, Denmark) and Step OneTM qRT- PCR System (Applied Biosystem, Foster City California, USA). The sequences of primer sets and stem-loops used for this study are shown in Table. 2.

### Statistical analyses

GraphPad Prism 6 (San Diego, CA, USA) was used to statistically analyze data. The distribution of normality of variables was evaluated with the Kolmogorov-Smirnov test. The statistical value of the differences between variables was evaluated by the Un-Paired Student T-test. Spearman correlation coefficient was used to investigate the expression relationship between the two genes and also the expression level of genes and clinicopathological characteristics of patients. P values smaller than 0.05 ($p<0.05$) were considered to be statistically significant.

## Results

### MiR-330 and TYMS dysregulation in TCGA colorectal cancer samples

For the pre-evaluation of miR-330 and TYMS dysregulation significance in colorectal cancer, we first analyzed TCGA datasets for CRC. As shown in Fig.1, the obtained results illustrated that miR-330 exhibits higher expression levels in CRC samples compared to colorectal normal tissue samples. Conversely,

TYMS was shown to be significantly upregulated through colorectal tumorigenesis. These results suggested that the miR-330/TYMS axis may be involved in the pathogenesis of CRC.

### MiR-330 and TYMS expression status in internal samples

To further confirm the obtained results from TCGA, expression alterations of the miR-330 and TYMS genes were examined in 100 colorectal cancer tissue samples in comparison to tumor margin samples and afterward statistically analyzed. In addition to the qRT-PCR review, clinicopathological characteristics of patients with CRC, such as the sex and age, along with tumor-related characteristics such as tumor stage (with AJCC staging system classification [36]), lymph-node metastasis, and distance metastases, were analyzed in the research groups. The findings of this study revealed a significant decrease in the expression level of miR-330 in tumor samples relative to the margin samples (p value =0/0005). Moreover, for TYMS the results showed a significant up-regulation in tumor samples in comparison with the margin samples (p value = 0.0001) (Fig. 2). The expression level of miR-330 was significantly associated with Stage (p value =0.016) and Lymph-node metastasis (p value =0.0024) in the study of clinicopathological characteristics of patients. There was no significant association between clinicopathological features and the TYMS expression level in our study (Table 3). Our results also revealed a significant inverse correlation between the expression level of miR-330 and the TYMS gene. In other words, the expression level of TYMS significantly decreased in samples with a higher level of miR-330 (Pearson r= -0/607 p value= 0.0001) (Fig. 3)

## Discussion

Colorectal cancer (CRC), which ranked third in terms of incidence and second in mortality rates, is one of the world's concerns. This issue has propelled the direction of researches towards finding new approaches for prevention, diagnosis, and treatment. [1, 2]. Despite the available diagnosis and therapies of this disease, the survival rate of patients is largely associated with the tumor stage and 40–50 percent of patients die due to distant metastasis [11]. For patients suffering metastatic colorectal cancer, the FOLFOX regimen, based on the association of 5-fluorouracil and oxaliplatin, is the most commonly suggested chemotherapy regimen [29, 37]. Epithelial-mesenchymal transition (EMT) has been documented to be involved in microRNA-driven modulation of the response of tumor cells to 5-fluorouracil and oxaliplatin [29]. TYMS as an EMT-associated factor, which along with other factors, is recognized to be a biomarker for the efficacy of 5-FU, is an antimetabolite drug that exerts its cytotoxic effect mainly through inhibition of thymidylate synthase (TYMS) leading to dTMP depletion. Analysis of CRC cell lines indicated that miR-330 inhibited colorectal cell proliferation and increased the chemo-sensitivity of CRC cells to 5-fluorouracil (5-FU) via the cell apoptosis pathway [24].

The diagnostic and therapeutic benefit of miR-330 has been investigated in numerous studies in all types of malignancies including CRC. In general, miR-330 expression was significantly down-regulated in CRC [23]. Guo et.al proposed mir-330 as a biomarker for CRC as part of the SH3PXD2A-AS1/miR-330-5p/UBA2 network that regulates the progression of CRC through the Wnt/β-catenin pathway [38]. In one

of our previous surveys, miR-330 has been studied alongside other micro-RNAs and has been reported to be down-regulated with a significant correlation among metastatic progression and expression levels and proposed as a valuable therapeutic and diagnostic factor in CRC [39].

The roles of miR-330 and TYMS, univariate and multivariate, have been investigated in the presence of 5-FU medication. In this research, the aim was to investigate the coloration between miR-330 and TYMS in absence of (5-FU) in order to evaluate the therapeutic and diagnostic value of miR-330 and its impact on TYMS in the progression of colorectal cancer. Based on the results of our study, the expression of miR-330 in tumor tissue decreased significantly compared to healthy non-cancerous tissue. Due to the combination of miR-330 with certain clinicopathological features of patients, this could be a possible diagnostic marker for colon cancer. On the other hand, TYMS, which is over-expressed in CRC tissues relative to healthy non-cancerous tissues, can also be used as a diagnostic and therapeutic marker for CRC. Our research suggests that the dysregulation of mir-330 and TYMS, and also miR-330 down-regulation which affects the TYMS expression level and leads to CRC progression, has the capacity to detect molecular mechanisms of CRC and developments in novel diagnosis and therapy approaches.

# Conclussion

Regarding the substantial risk of the CRC and the important involvement of the miRNAs, further studies should be conducted to have a better approach in understanding the molecular mechanisms of the pathogenesis of the CRC. It is anticipated that forthcoming multicenter studies and further investigation with a larger sample size regarding miR-330 and TYMS dysregulation would contribute to clearer insight into their role in the diagnosis and treatment of CRC cases. Apart from the therapeutic procedure about FOLFOX, miR-330, and TYMS dysregulation investigations are required in relation to other therapeutic methods.

# Declarations

### Ethics approval and consent to participate

This study was confirmed by Ethical Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1399.206) and written informed consent was obtained from all patients.

### Consent for publication

Written informed consent was obtained from all patients.

### Availability of data and material

All data in this article have been sent to the journal.

### Conflict of interests

The authors declare that they have no competing interests.

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### Authors' contributions

(I) Conception and design: Leila Karimi and Touraj Asvadi Kermani

(II) Administrative support: Milad jaberi

(III) Provision of study materials or patients: Milad Asadi

(IV) Collection and assembly of data: Habib Zarredar and Venus Zafari

(V) Data analysis and interpretation: Milad Asadi and Soghra Bornehdeli

(VI) Manuscript writing: Leila Karimi

(VII) Final approval of manuscript: All Author

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# Tables

**Table 1; Clinicopathological features of patients with CRC.**

| Clinical feature | classification | |
|---|---|---|
| Age | 55 | 57 |
| | 55 | 43 |
| Sex | female | 41 |
| | male | 59 |
| Lymph node metastasis | Present | 59 |
| | Absent | 41 |
| Stage (AJCC) | II, III | 64 |
| | IV | 36 |
| Distant metastasis | Present | 21 |
| | Absent | 79 |

**Table 2. Primer and stem-loop sequences used for cDNA synthesis and qPCR.**

| Name | Target sequence | TM (°C) |
|---|---|---|
| TYMS Forward | AGTACCTGGGGCAGATCCAAC | 59 |
| TYMS Reverse | ACGTTTGGTTGTCAGCAGAGG | |
| miR-330-5p Stem-loop | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAGCCTAA | |
| miR-330-5p Forward | CGAGCTGGTCTCTGGGCCTG | 59 |
| miR-330-5p Universal Reverse | CCAGTGCAGGGTCCGAGGTA | |
| U6 Stem-loop | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATAT | |
| U6 Forward | GCTTCGGCAGCACATATACTAAAAT | 59 |
| U6 Reverse | CGCTTCACGAATTTGCGTGTCAT | |
| GAPDH Forward | CAAGATCATCAGCAATGCCTCC | 59 |
| GAPDH Reverse | GCCATCACGCCAGTTTCC | |

**Table 3. Relationships between *miR-330* and *TYMS* expression levels in CRC tissue samples and clinicopathological features of CRC patients (N.S: non-significant differences)**

| Gene | miR-330 | TYMS |
|---|---|---|
| **Age** | N.S | N.S |
| **Sex** | N.S | N.S |
| **Stage** | 0.016 | N.S |
| **Lymph node metastasis** | 0.0024 | N.S |
| **Family story** | N.S | N.S |
| **Distant metastasis** | N.S | N.S |

# Figures

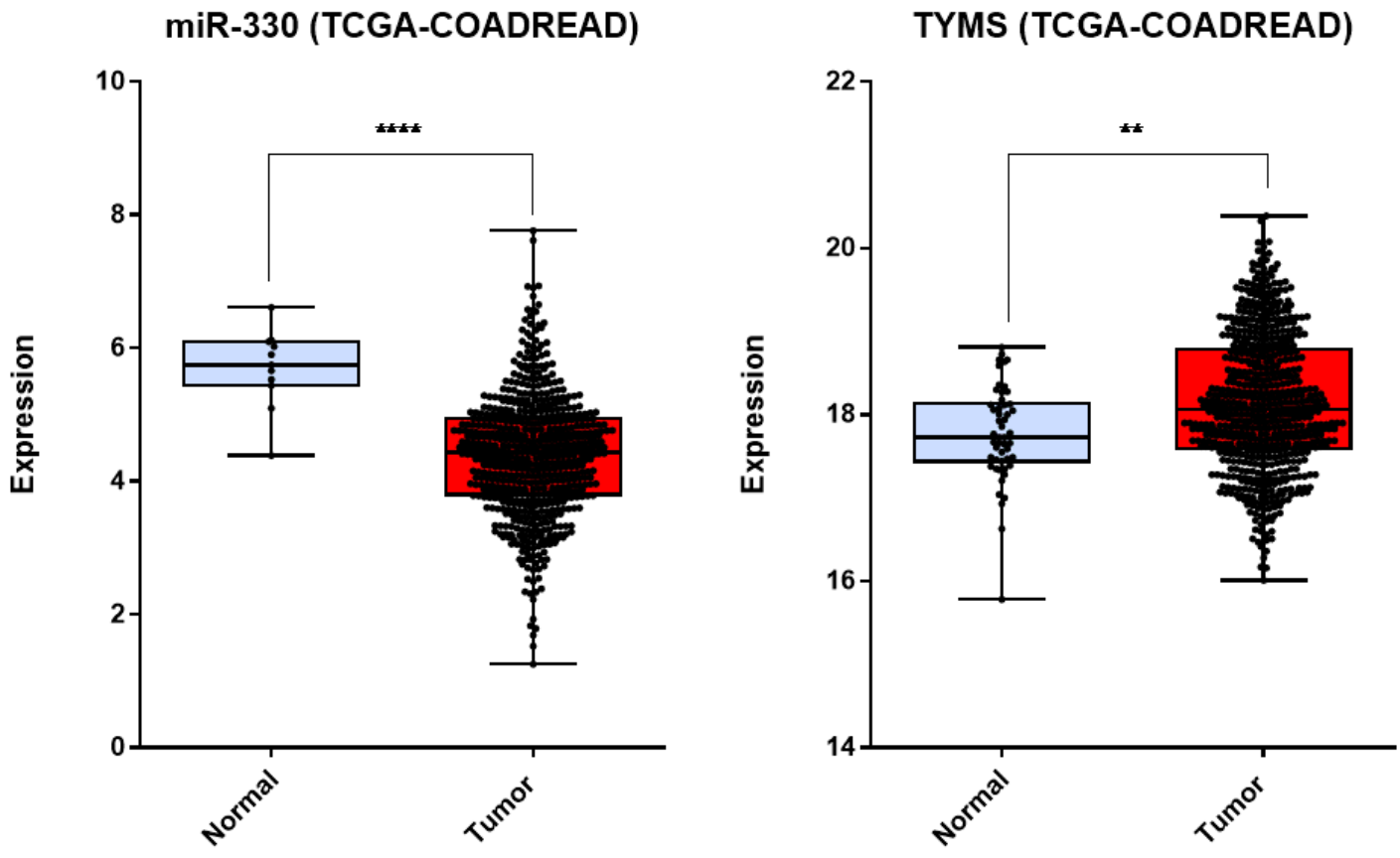


Figure 1

The expression levels of TYMS and miR-330 in TCGA datasets for CRC; **p<0.01 and ****p<0.00001.

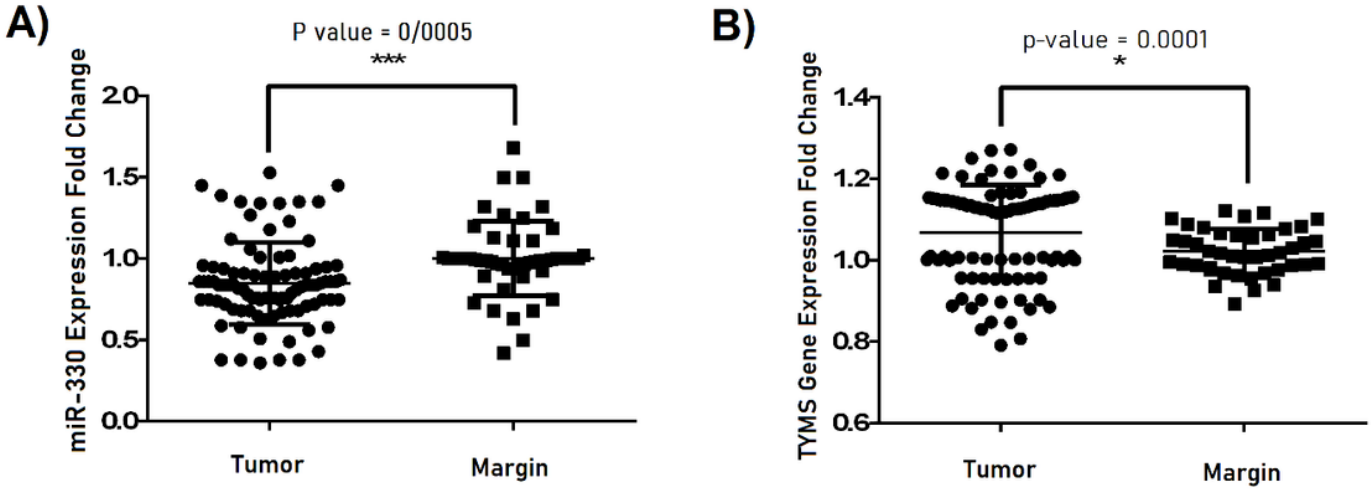


Figure 2

There was a significant decrease in the level of miR-330 expression (A) in tumor samples relative to the margin samples. Regarding TYMS (B), the results showed a significant up-regulation in tumor samples

compared to margin samples. Considering the significant changes both seen in miR-330 and TYMS in CRC, it is proposed that subsequent studies could contribute to the development of new therapeutic approaches for patients with CRC.

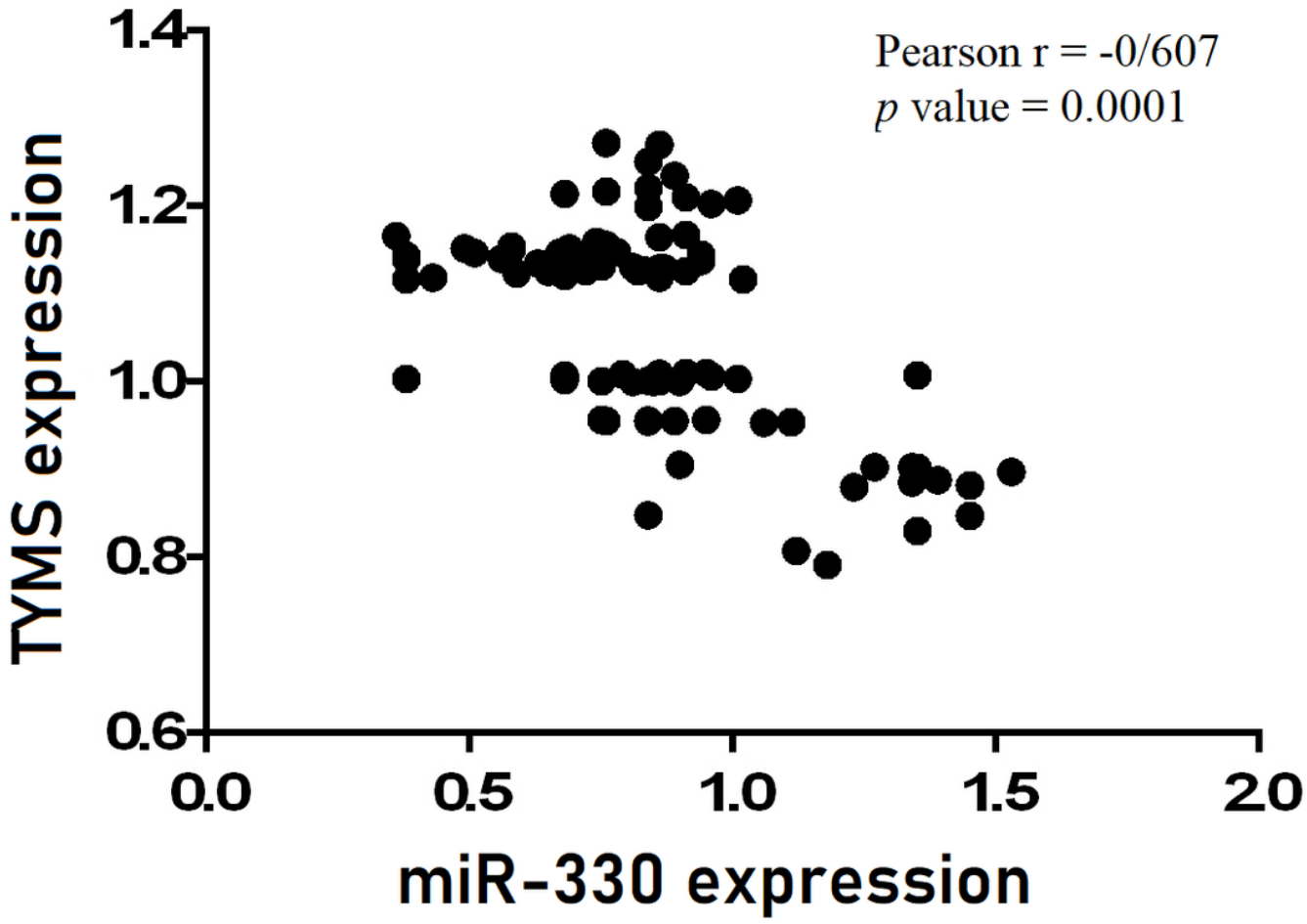


**Figure 3**

In samples with a higher level of miR-330, the expression level of TYMS decreased significantly.