A MiRNA-based Signature is Associated With Tumor Mutational Burden in Colon Adenocarcinoma

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Research

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Abstract

Background: Tumor mutation burden (TMB) has become an independent biomarker for predicting the response of Immune checkpoint inhibitors. MiRNA plays an important role in cancer-related immune regulation but the relationship between expression of miRNA and TMB is unclear in colon adenocarcinoma (COAD).

Method: The transcriptome profiling data, clinical data, mutation annotation data and miRNA expression profiles for cases with COAD were downloaded from TCGA database, and then COAD samples were randomly divided into training set and test set. The differential expression miRNAs of high and low TMB group in training set was obtained as a signature by the least absolute shrinkage and selection operator (LASSO) logistic regression, and it was verified in test set. Using LASSO method, principal component analysis (PCA) and ROC to verify the credibility of signature. In addition, the correlation between the miRNA-based signature and immune checkpoints was performed. In the end, enrichment analysis of the miRNAs in signature was performed to explore the biological function.

Results: 18 differential expression miRNAs were obtained according to LASSO method. According to LASSO method, principal component analysis (PCA) and ROC, we found that the credibility of signature, and the signature can discriminate the high and low TMB level. Furthermore, the results of correlation between the 18-miRNA-based signature and immune checkpoints showed that the miRNA-based model has a strong positive correlation with TMB, weak positive correlation with CTLA4 and CD274 (PD-L1). However, there is no correlation between the model and SNCA (PD-1). Finally, enrichment analysis of the 18 miRNAs demonstrated that the 18 miRNAs were involved in process of immunity and cancer pathways.

Conclusion: We established a novel miRNA-based signature integrating expression of miRNAs and TMB levels. The 18-miRNA-based signature can effectively predict and discriminate the TMB levels in COAD, and provides a potential guide of ICIs treatment.

Background

Colon adenocarcinoma (COAD) is a common malignant neoplasm of digestive system, has become the third most common cancer in the world and the second leading cause of cancer-related mortality [1]. Patients usually visits with diarrhea, abdominal pain and bloody stool, which has developed to the middle and late stages of the disease, and the quality of life of the patients is often low, and most of them have a poor prognosis. Although great progress has been made in recent years in the treatment of surgery, chemotherapy and targeted therapy, the five-year survival rate of patients with advanced COAD is still low. After nearly a hundred years of efforts, tumor immunotherapy has become one of the means to treat tumors [2]. Programmed death protein-1 (PD-1)/programmed death receptor ligand (PD-L1) immune checkpoint inhibitors (ICIs) have been approved by Food and Drug Administration (FDA) for the treatment of non-small cell lung cancer, melanoma, head and neck squamous cell carcinoma [3] etc. In recent years,

PD-1/PD-L1 ICIs have achieved encouraging results in the treatment of advanced COAD [4-6]. The expression of PD-L1 can be applied as a biomarker of PD-1/PD-L1 inhibitor therapy to help predict treatment response. However only less than 30% of patients perform long-term responses to immune checkpoint treatment and expression of PD-L1 probably not be the unique identify responder, therefore, it is necessary to find better responders.

Tumor mutational burden (TMB) has the prospect of becoming an effective biomarker for predicting the survival of cancer patients treated with ICIs and it is completely independent of PD-L1. TMB is defined as the total number of somatic gene coding errors, base substitution, gene insertion or deletion errors detected per million bases (MB), at present, the most commonly used method to detect TMB is the next generation sequencing (NGS). But there are some challenges in this method, for example, the high cost and requirement of vast tumor DNA and different platforms will lead to uneven results[7]. A high level of TMB may lead to the modification of a protein encoded by a mutant gene, which is recognized by the immune system as "non-self" and then activates a specific anti-tumor immune response as a new tumor-specific antigen, the probability of killing these tumor cells increases[8].

MicroRNAs (miRNAs) plays an important role in the post-transcriptional regulation of the translation of mutant genes into modified proteins. MiRNAs are classes of non-coding single-stranded RNA molecules encoded by endogenous genes with a length of about 22 nucleotides, they are involved in the regulation of post-transcriptional gene expression in animals and plants. The abnormal expression of miRNAs is often associated with many diseases, especially cancer[9, 10]. With the rise of miRNAs research in recent years, we realize that miRNA plays an irreplaceable role in cancer. Several studies have shown that miRNAs may be a prognostic marker for various cancers[10]. MiRNAs plays an important role in tumor microenvironment[11-14]. Studies have shown that the expression of miRNAs in some tumors is highly specific, and it plays a very important role in immune response, especially in early regulation. In the process of tumor development, the immune system plays a key role, and there is a close interaction between tumor cells and immune cells by releasing a variety of signals[15, 16]. It has been shown that miRNAs can be used as a medium of communication between tumor cells and immune cells. Therefore, the expression of miRNAs are supposed to correlate the TMB levels, and regarded as molecular markers for predicting TMB levels. In order to verify our above-mentioned point of view, the mutation annotation and miRNA expression datasets of COAD were downloaded from the cancer genome atlas (TCGA, <https://gdc.cancer.gov/>), and established a miRNA-based model to predict TMB levels.

Materials And Methods

Data collection and processing

The transcriptome profiling data, clinical data, mutation annotation data and miRNA expression profiles for cases with COAD were all obtained from TCGA database. Afterwards, processing aforementioned four groups of data according to Perl, including integration and normalization of transcriptome profiling data, extraction of clinical information, merging miRNA data and counting TMB levels of mutation annotation

data. When the quantity counted is greater than (or equal to) 10 means a high TMB level, otherwise it means a low TMB level. Merging TMB and miRNA expression data using “limma” package of R language [17]. As a result, a total of 323 tumor samples not only in mutation annotation data but also in miRNA data, and they were stochastically divided into training (60%) and test (40%) set through “caret” package in R [18], in the meantime, the two sets of clinicopathological data were statistically analyzed.

Extraction and analysis of differential expression miRNAs

The differential expression miRNAs of high and low TMB group in training set were analyzed by “limma” package of R language. Taking the fold change (FC) >1.5 and P value adjusted by false discovery rate <0.01 were statistically significant screening criteria. In addition, a heat map was drawn by “pheatmap” package of R to visualize the statistically significant differential expression miRNA in high and low TMB groups.

Least absolute shrinkage and selection operator (LASSO) and principal component analysis (PCA) of differential expression miRNAs

The differential expression miRNAs were obtained from training set. Least absolute shrinkage and selection operator (LASSO) is a powerful method to avoid error prediction. The LASSO logistic regression model was realized by the “glmnet” package of R language to predict the optimum differential expression miRNAs as a signature of discriminating TMB level [19]. Meanwhile, the differential expression miRNAs were applied to perform PCA using “ggplot” package in R, and then the optimum differential expression miRNAs were used for PCA to validate the reliability of the model [20].

The validation of miRNA-based model for predicting TMB level

The model was a miRNA-based signature discriminator for predicting TMB level was obtained from training set, we introduced the model into test set to verify the robustness and general applicability of the model. The discriminator index was created for each sample using the regression coefficients of the LASSO analysis to calculate the expression value of the optimum miRNAs with the following formula: $\text{index} = \beta_1 * \text{Exp}_{\text{miRNA1}} + \beta_2 * \text{Exp}_{\text{miRNA2}} + \beta_3 * \text{Exp}_{\text{miRNA3}} + \dots + \beta_n * \text{Exp}_{\text{miRNA}_n}$. The “ β ” is the regression coefficient of miRNA, and “Exp” means the expression of miRNAs. The efficiency of the model was evaluated by accuracy, sensitivity (SE), specificity (SP), positive predictive value (PPV), negative predictive value (NPV), and area under the receiver operating characteristic (ROC) curve. The ROC curves were created and analyzed by the “pROC” package of R [21].

Correlation analysis of the model in TMB and immune checkpoints

To assess the correlation between the model and TMB, the index of each sample in total set calculated was brought into the integrated data of TMB and miRNA using “limma”, “ggplot2” and “ggpubr” packages in R [22] and visualizing it. PD-1 (SNCA), PD-L1 (CD274) and CTLA-4 were three immune checkpoints. And

the index of each sample in total set calculated was brought into pre-processed transcriptome profiling data to analyze the correlation between the model and immune checkpoints.

Enrichment analysis for target genes prediction of miRNAs in model

Corresponding database files of TSV format were downloaded from TargetScan (http://www.targetscan.org/vert_72/), miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/php/index.php>) and miRDB (<http://mirdb.org/>). MiRNAs in model were introduced into three files to predict target genes by means of PERL. The standard for determining the target genes of miRNA was supported by all three databases. Through “clusterprofiler” package in R[23] the biological process and molecular function of miRNA were inferred by Gene Ontology (GO) analysis of target genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of target genes of miRNAs in the model was performed and visualized by “clusterprofiler” package in R.

Statistical analysis

Statistical analysis of clinical data was performed with the chi-square test. The expression of miRNAs in the high and low TMB group samples was performed with unpaired t test. All statistical analysis was completed in R language.

Results

Establishment and identification of differential expression miRNAs

The clinicopathological data of patients in the training set and the test set were analyzed and no statistical significance was found between the two sets, as shown in table 1. There were respectively 36 and 158 samples in high and low TMB group. $|\log_2 FC| > 0.263$ and P value adjusted by false discovery rate < 0.01 were used as screening criteria and it was determined that 48 miRNAs were differentially expressed in samples with high and low TMB levels. There were 22 upregulated and 26 downregulated differential expression miRNA in samples with high TMB level. Visualization of miRNA expression in high and low TMB groups was shown in Figure 1, the result of heatmap demonstrated that these differential expression miRNAs can basically identify samples with high and low levels of TMB.

Signature acquisition through LASSO and identification

The expression of 48 miRNAs in training set were analyzed by LASSO logistic regression to establish a model based on miRNA as a discriminator of TMB level. After the analysis of LASSO, 18 miRNAs with the largest AUC were identified as a signature of discriminating TMB level, as shown in Figure 2a. These 18 miRNAs are hsa-miR-296-5p, hsa-miR-155-5p, hsa-miR-6761-5p, hsa-miR-582-5p, hsa-miR-452-3p, hsa-miR-330-5p, hsa-miR-3127-5p, hsa-miR-146b-5p, hsa-miR-99a-5p, hsa-miR-874-3p, hsa-miR-132-3p, hsa-miR-625-3p, hsa-miR-552-5p, hsa-miR-195-3p, hsa-miR-452-5p, hsa-miR-224-5p, hsa-miR-582-3p and hsa-miR-592. Figure 2b and 2c demonstrate the PCA results of 49 differential expression miRNAs and 18

miRNAs in the model, respectively. It is confirmed that the 18 miRNAs identified by LASSO can clearly discriminate samples with high or low TMB level.

The validation of miRNA-based model

According to the LASSO regression analysis, the formula is as follows: $\text{index} = (-0.0218449096244075 * \text{EXP}_{\text{hsa-miR-296-5p}}) + (0.140980802896763 * \text{EXP}_{\text{hsa-miR-155-5p}}) + (-0.0618130806762854 * \text{EXP}_{\text{hsa-miR-6761-5p}}) + (0.792005574142893 * \text{EXP}_{\text{hsa-miR-582-5p}}) + (0.0400501457312491 * \text{EXP}_{\text{hsa-miR-452-3p}}) + (0.351535745471987 * \text{EXP}_{\text{hsa-miR-330-5p}}) + (0.321466850321636 * \text{EXP}_{\text{hsa-miR-3127-5p}}) + (0.306550028173435 * \text{EXP}_{\text{hsa-miR-146b-5p}}) + (-0.0129214249555584 * \text{EXP}_{\text{hsa-miR-99a-5p}}) + (-0.291557428450146 * \text{EXP}_{\text{hsa-miR-874-3p}}) + (0.312801874996257 * \text{EXP}_{\text{hsa-miR-132-3p}}) + (0.71353375237544 * \text{EXP}_{\text{hsa-miR-625-3p}}) + (-0.339018514344295 * \text{EXP}_{\text{hsa-miR-552-5p}}) + (-0.506312988185146 * \text{EXP}_{\text{hsa-miR-195-3p}}) + (-0.203390691480266 * \text{EXP}_{\text{hsa-miR-452-5p}}) + (-0.038165302905481 * \text{EXP}_{\text{hsa-miR-224-5p}}) + (0.0188966224605559 * \text{EXP}_{\text{hsa-miR-582-3p}}) + (-0.303904239862044 * \text{EXP}_{\text{hsa-miR-592}})$. Through the analysis of the model, the results are shown in the Table2, the accuracy in training set is 0.9753, 0.964 in test set and 0.9598 in total set. As it turns out, the model has a high credibility. Analysis of ROC curve shows that AUC in training set, test set and total set is 0.998, 0.958 and 0.982, it is implied that there is no significant difference between the training set and the test set, and verifies the accuracy of the model (Figure3a and 3b).

Correlation analysis between signature and TMB and immune checkpoints.

To analyze the correlation between the model and TMB, and the correlation between the model and immune checkpoint. The index of each sample in total set calculated was brought into the integrated data of TMB and miRNA, and simultaneously, the index was brought into the pre-processed transcriptome profiling data. As demonstrated in Figure3a-d, the miRNA-based model has a strong positive correlation with TMB (Pearson $R = 0.47$, $P < 2.2 \times 10^{-16}$, Figure 4a), weak positive correlation with CTLA4 (Pearson $R = 0.34$, $P = 4.2 \times 10^{-10}$, Figure 4b) and CD274 (Pearson $R = 0.39$, $P = 7.1 \times 10^{-13}$, Figure 4c). However, there is no correlation between the model and SNCA (Pearson $R = -0.034$, $P = 0.54$, Figure 4d).

Enrichment analysis for target genes prediction of miRNAs in model

The target genes corresponding to miRNAs were predicted from three databases. GO contains three aspects of functional information: the biological process in which genes are involved, the location of cells, and the function of molecules. And we found that through analysis the target genes were enriched in "DNA-binding transcription activator activity, RNA polymerase II-specific", "transforming growth factor beta receptor, cytoplasmic mediator activity" and "phosphatase binding" etc. KEGG is an understanding of advanced functions and biological systems at the molecular level, the result of KEGG analysis shows that the target genes of 18 miRNA were mainly enriched in pathways of cancers and cancer-related signaling pathways, for example, the "Colorectal cancer" and "MAPK signaling pathway".

Discussion

With the development of sequencing technology, the role of TMB as a biomarker in predicting the efficacy of ICIs has been confirmed in a number of clinical studies[24-26]. TMB provides a quantitative estimation of the total number of mutations in the coding region of the tumor genome, therefore, tumor cells with higher levels of TMB may be more likely to be recognized by the immune system, which in turn triggers a stronger immune response to ICIs. Previous studies have shown that blood-derived TMB detection does not need tissue samples, only plasma samples are needed to evaluate the TMB level[27]. Because many patients cannot provide biopsies for doctors to test for TMB, therefore, blood-derived TMB detection is a better substitute with low invasiveness and high flexibility[27, 28]. However, the evaluation of TMB by blood biopsy is faced with great challenges, the amount of DNA extracted from tumor cells in blood circulation is often very small which is a small part of the free DNA in the blood circulation. In recent years, a large number of literatures have reported that miRNA plays an important role in the differentiation and development of immune cells, the regulation of immune response and the occurrence and development of tumors in the immune system[29-31]. Given this, a blood-based miRNA signature was established for a large portion of patients with advanced COAD that were unable to undergo surgery. The relationship between miRNA expression and TMB has not previously been reported in COAD. For the current study, the differential expression miRNA in samples with high and low TMB level has been identified as a signature, which could basically discriminate the high and low expression of TMB in the sample. This signifies that changes in genomics may affect transcriptome to some extent. After selection of differential expression miRNA, a 18-miRNA-based model was obtained from training set and brought into test set to verify the robustness and general applicability. The accuracy in training set is 0.9753, 0.964 in test set and 0.9598 in total set. Analysis of ROC curves demonstrated that AUC in training set, test set and total set is 0.9984, 0.958 and 0.9817. As it turns out, the model has a high credibility. According to SP and NPV in training set, test set and total set, the model has high recognition for low TMB, as can be seen from PPV, the model also has high recognition ability for high TMB.

Previous studies suggest that TMB is a potential independent biomarker and has a certain predictive value of curative effect. A series of recent clinical studies around this problem have confirmed the positive correlation between TMB value and curative effect, and confirmed that TMB value is independently related to the efficacy of immunotherapy, but is not affected by PD-L1 level, while the immunotherapy effect of patients with high TMB and high PD-L1 level is better. Because the non-synonymous mutation of tumor cells can lead to the production of new antigens, and the existence of tumor-specific new antigens is related to the increase of tumor immunogenicity, the immune system can enhance the activity of T cells against tumors after recognizing a large number of new antigens, thus enhancing the efficacy of ICIs[32]. Some studies have mentioned and proved that if the tumor presents higher TMB and more new antigens, the tumor may have a better response to immunotherapy[33, 34]. In this study, there were multiple differentially expressed miRNAs between tumors with different TMB levels, which were associated with immunity. The target gene enrichment analysis of miRNA in the model showed that these miRNAs are related to the process of transcriptome and immunity. For example, the “DNA-binding transcription activator activity, RNA polymerase- α -specific” and “nuclear hormone receptor

binding” etc. are involved in the process of transcriptome. The “transforming growth factor beta receptor, cytoplasmic mediator activity” and “cell adhesion molecule binding” etc. are related to the immune process. The target genes of these 18 miRNAs are also enriched in pathways in cancer, including “colorectal cancer”. Thus it can be seen that the 18-miRNA-based model is robust and doable in TMB level predicting from this study but further experimental verification is of great necessity. According to previous research reports, miRNAs of plasma circulation were associated with immunotherapy of non-small cell lung cancer[35, 36].

In our study, we develop and verify a potential signature that predicts and discriminates the high and low TMB of COAD, but there are some deficiency of this signature. First, the threshold of TMB level discrimination may vary from method to method. Second, a larger cohort of COAD patients are required to improve the credibility of the signature. Third, the biological mechanism of these 18 miRNAs in tumor immunology needs further exploration.

Conclusion

For all these reasons, we find that miRNA expression of COAD patients is different at different TMB levels. A miRNA-based model was established as a signature to predict and discriminate the TMB levels in COAD.

Abbreviation

COAD: Colon adenocarcinoma; TMB: Tumor mutational burden; ICIs: Immune checkpoint inhibitors; FDA: Food and drug administration; MB: Million bases; NGS: Next generation sequencing; LASSO: Least absolute shrinkage and selection operator; PCA: Principal component analysis; ROC: Receiver operating characteristic; SE: Sensitivity; SP: specificity; PPV: Positive predictive value; NPV: Negative predictive value;

Declarations

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Authors' contributions

WX, YW and ZG designed this research. CY, YX, XC conducted analyses of the statistics. CG, CW carried out collection of data and processed the figures or tables. ZN and CZ drafted the manuscript. All of the authors reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

This manuscript is approved by all authors for publication.

Conflicts of Interest:

The authors have no conflict of interest to disclose.

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Tables

Table 1
Clinicopathology characteristics of patients wit COAD

Covariates		Training set	Test set	<i>P</i> -value
Age	<=65	81(41.75%)	57(44.19%)	0.7503
	>65	113(58.25%)	72(55.81%)	
Gender	FEMALE	95(48.97%)	61(47.29%)	0.8551
	MALE	99(51.03%)	68(52.71%)	
Stage	I-II	110(56.7%)	66(51.16%)	0.5004
	III-IV	79(40.72%)	57(44.19%)	
	unknow	5(2.58%)	6(4.65%)	
T	T1-2	34(17.53%)	30(23.26%)	0.2615
	T3-4	160(82.47%)	99(76.74%)	
M	M0	136(70.1%)	91(70.54%)	0.1279
	M1	37(19.07%)	14(10.85%)	
	unknow	21(10.82%)	24(18.6%)	
N	N0	116(59.79%)	72(55.81%)	0.5518
	N1-3	78(40.21%)	57(44.19%)	

Table 2
performance of 18-miRNA-based signature of TMB in COAD

ID	SE	SP	PPV	NPV	Accuracy	AUC
Train	0.8889	1	1	0.9753	0.9794	0.9984
Test	0.7647	0.9554	0.7222	0.964	0.9302	0.958
Total	0.8491	0.9815	0.9	0.9707	0.9598	0.9817

Figures

Figure 1

The 18 differential expression miRNAs can basically identify COAD samples with high and low levels of TMB.

Figure 2

LASSO logistic regression model and PCA. a: selection of arguments in the LASSO method. b: PCA of 49 differential expression miRNAs. c: PCA of 18 differential expression miRNAs selected by LASSO method.

Figure 3

ROC curves analysis of training set, test set and total set. a: ROC curves of training set and test set. b: ROC curve of total set.

Figure 4

Analysis of correlation between signature and TMB and immune checkpoints. a: the 18-miRNA-based signature has a strong positive correlation with TMB. b: the 18-miRNA-based signature has a weak positive correlation with CTLA4. c: the 18-miRNA-based signature has weak positive correlation with CD274 (PDL1). d: the 18-miRNA-based signature has no correlation with SNCA (PD1).

Figure 5

Enrichment analysis of these 18 miRNAs. a: GO analysis of the target genes of 18 miRNAs. b: KEGG analysis of the target genes of 18 miRNAs.