

Correlation between expression of miR-155 in colon cancer and serum carcinoembryonic antigen level and its contribution to recurrence and metastasis forecast

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ABSTRACT

الأهداف: تحليل العلاقة بين تعبير miR-155 في أنسجة القولون السرطانية ومضاد مضغني سرطاني مصلي (CEA) وعرض دوره في تنبؤ النكسة والنمو الثانوي للورم.

الطريقة: أجريت هذه الدراسة خلال الفترة من مارس 2009م حتى ديسمبر 2011م في مستشفى شيانغيانغ الوسطى، شيانغيانغ، وهوبي، الصين. تم جمع وتحليل 84 زوج من عينات سرطان القولون والأنسجة المجاورة والمناظرة السليمة من الأورام. تم اختبار تعبير miR-155 في كلا الأنسجة باستخدام تفاعل البوليميريز المتسلسل وتم قياس مستويات مصلي CEA قبل العملية وبعد العملية أيضاً كل شهرين خلال فترة متابعة امتدت عامين.

النتائج: وجدنا أن تعبير miR-155 في أنسجة القولون السرطانية أعلى من الأنسجة الطبيعية بشكل إحصائي $p < 0.05$. وكانت هنالك علاقة إيجابية واضحة مع مستويات مصلي CEA قبل العملية $p < 0.01$ ، وعلاقة سلبية مع طول المدة من ارتفاع مستويات مصلي CEA بعد العملية مرة أخرى $p < 0.01$. كان تعبير miR-155 في مجموعة النكسة والنقيلة أعلى من مجموعة غير النكسة والنقيلة $p < 0.05$. كان ارتفاع مستويات مصلي CEA بعد العملية مرتبط بشكل إحصائي مع نقيلة الورم والرجعة بعد العملية.

الخلاصة: ينظم تعبير miR-155 أنسجة القولون السرطانية. المزيح من مقايسة مستوى miR-155 في أنسجة القولون السرطانية ومستوى CEA المصلي سواء قبل أو بعد العمل الجراحي يمكن أن يعطي معلومات أكثر دقة في التشخيص وخصوصاً في تنبؤ النكسة والنمو الثانوي للورم بعد العمل الجراحي.

Objectives: To analyze the correlation between expression of miR-155 in colon cancer tissue and serum carcinoembryonic antigen (CEA) levels, and then explore its contribution to forecasting recurrence and metastasis.

Methods: Eighty-four pairs of colon cancer specimens and their corresponding non-tumor adjacent tissues were collected and analyzed between March 2009

and December 2011 in Xiangyang Central Hospital, Xiangyang, Hubei, China. The expression of miR-155 in both tissues was tested using reverse transcription-polymerase chain reaction (RT-PCR), the preoperative serum CEA level was assessed, and the postoperative serum CEA level was also assessed bimonthly during the follow-up period of 2 years.

Results: The expression of miR-155 in colon cancer tissue was significantly higher than that in normal tissues ($p < 0.05$), it had an obvious positive correlation with the preoperative serum CEA levels ($p < 0.01$), and a negative correlation with time of duration since the serum CEA level increased again postoperatively ($p < 0.01$). The expression of miR-155 in the recurrence and metastasis group was significantly higher (6.06 ± 3.73 times) than that in the non-recurrence and non-metastasis group ($p < 0.05$). An increase in the postoperative serum CEA levels was significantly correlated with recurrence and metastasis of the tumor postoperatively.

Conclusion: The expression of miR-155 is up-regulated in colon cancer tissue. A combination of miR-155 level assay in colon cancer tissue and the serum CEA level both pre- and postoperatively can afford more accurate information for diagnosis and prognosis, especially for predicting recurrence and metastasis postoperatively.

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Colon cancer is a common malignant tumor found in the digestive tract. The incidence of colon cancer has shown a trend of increasing year by year.¹ Though there are big advances in treatment, the overall death rate is still high. Tumor morbidity and metastasis is one of the main causes of patient death. Data reveal that approximately 50% of the total number of patients with colorectal cancer eventually died from tumor and metastasis though effective treatment had been given.^{2,3} The carcinoembryonic antigen (CEA) is the most commonly used tumor marker for colon cancer prognosis, curative effect observation, and recurrence and metastasis monitoring. In pilot studies, our research team found that the increased expression of miR-155 in colon cancer tissue is possibly closely associated with the fact that miR-155, as a protooncogene, participated in the development and progression of colon cancer as well as the metastasis. However, could it be possible that miR-155 becomes a new tumor marker so that it can contribute more in clinical diagnosis? There are rare clinical and experimental reports (such as in PubMed and Embase) on such a possibility. For this reason, our study explored the contribution of miR-155 in prognosis, observation of curative effect, and monitoring of recurrence and metastasis of colon cancer through analysis of the correlation between miR-155 and CEA, so as to establish a foundation and experimental basis for clinical staff to find a new and perfect tumor marker for colon cancer.

Methods. This was a prospective study. The Ethics Committee of Xiangyang Central Hospital approved the project. Eighty-four pairs of primary colon cancer specimens resected by surgeons from the Department of General Surgery, Xiangyang Central Hospital, Xiangyang, Hubei, China from March 2009 to December 2010, were analyzed. Each specimen had a corresponding normal mucosa, which was used as control. These specimens were resected from 46 male patients and 38 female patients with a median patient age of 59 years. All of these patients' tumors were pathologically confirmed, and the patients had not received radiotherapy and chemotherapy preoperatively. The resected specimens were placed into liquid nitrogen within 30 minutes of resection and then were stored in a refrigerator with a temperature setting of -70°C. The laboratory period lasted from March 2011 to September 2011 in the Central Laboratory of Xiangyang Central Hospital.

The Trizol RNA extraction reagents and reverse transcription (RT) kit were purchased from the US-based Invitrogen Corporation (City, State, USA)

and the miR-155 and U6 snRNA quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay kit were purchased from Shanghai GenePharma Co., Ltd, Shanghai, China. The PCR thermal cycler used was a Roche LightCycler (Kaiseraugst, Rheinfelden, Switzerland). The Abbott i2000 chemiluminescence apparatus and Abbott reagent were used for serum CEA level assay (Abbott Laboratories, Abbott Park, Illinois, USA). An increased CEA level meant CEA >5ng/ml.

Total RNA extraction from colorectal cancer tissues and normal mucosal tissues was carried out through a one-step extraction using Trizol agents, and the experimental steps strictly follow the instructions provided on the label. The ultraviolet spectrophotometer was used to assay the absorbance of RNA solution optical density (OD)₂₆₀ and OD₂₈₀. The RNA concentration and purity were calculated. In addition, the RNA integrity was assayed using 1% agarose gel electrophoresis.

Since micro RNAs (miRNAs) contains only around 20 nucleotides, the assay could not be carried out using traditional qRT-PCR. This study used the miR-155 assay kit (Hairpin-it™miRNAs RT-PCR Quantification Kit) from Shanghai GenePharma Co., Ltd., Shanghai, China, which contains 1µl miR-155 specific stem-loop RT-PCR primers. The extracted total RNA underwent reverse transcription reaction using this stem-loop primer. We mixed 2µl stem-loop primers, 1µl total RNA, and 1µl 10mM dinitro thiophosphate (dNTP), then added 12µl sterilized distilled water to blend, then the solution was heated at 65°C for 5 minutes. The heated mixture was soon cooled using ice and was centrifuged immediately. Then 4µl 5× first strand synthesis buffer, 2µl 0.1M DL-Dithiothreitol (DTT), and 1µl RNaseOUT™ ribonuclease inhibitor were added and blended well. Then, the blended solution was incubated in water at 37°C for 2 minutes; 1µl moloney murine leukemia virus reverse transcriptase (M-MLV RT) was added into the blended solution. The solution was further blended with gentle blowing and jiggling. Following 50 minutes of incubation at 37°C, the solution was heated at 70°C for 15 minutes to terminate the reaction process. After the reaction, the products of reverse transcription were stored at -20°C for later use. As an internal reference, U6 snRNA underwent the same reverse transcription, and the products were stored at -20°C for later use.

The Taq DNA polymerase, PCR reaction buffer, and specific miR-155 upstream and downstream primers were included in that kit. The upstream primer was 5'ACGCTCAGTTAATGCTAATCGTGATA 3', and the downstream primer was

5'ATTCCATGTTGTCCACTGTCTCTG 3'. The fragment length was 84bp. In this experiment, 10 μ l 2 \times PCR Buffer, 0.36 μ l (5 μ M) miR-155 upstream and downstream primers, 2 μ l cDNA, and 0.2 μ l 5U/ μ l Taq polymerase were used, and the reaction system was 20 μ l in volume. The reaction conditions were as follows: 95 $^{\circ}$ C for 3 minutes, 95 $^{\circ}$ C for 20 seconds, 60 $^{\circ}$ C for 50 seconds, with 40 cycles in total. The cycle threshold (Ct) value is the number of cycles experienced when fluorescence signal in each reaction tube reached the set threshold. As an internal reference, U6 snRNA went through the above-said quantification PCR reaction in which the upstream primer was ATTGGAACGATACAGAGAAGATT and the downstream primer was GGAACGCTTCACGAATTTG. The fragment length was 70bp. The relative quantitative method used in real-time quantification PCR was adopted, and $N=2^{-\Delta\Delta Ct}$ was used to indicate the change fold of expression of miR-155 in tumor tissue against normal tissue, in which, $\Delta\Delta Ct=(Ct_{miR155}-Ct_{U6})_{tumor} - (Ct_{miR155}-Ct_{U6})_{normal}$.

Postoperatively, the patients' serum CEA levels were assessed once before being discharged from the hospital. The follow-up period was scheduled as 2 years. During that period, the serum CEA level was tested every other month. The time the serum CEA level started to increase after reaching the lowest value (for at least half year) was recorded as the time the serum CEA level began to increase again after operation. Enteroscopy, chest x-ray, and B-mode ultrasonic examination or CT scans of the abdomen were conducted every 6 months postoperatively to confirm if patients had experienced recurrence and metastasis. Follow-up was mainly by re-examination in the outpatient clinic and a phone call interview.

The Statistical Package for Social Sciences version 13.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis, and the results were denoted using $x\pm s$ (mean and standard deviation). The comparison of measured data between the 2 groups was verified using paired sample t , while the comparison of ratios was verified using the McNemar test. The correlation analysis for bivariate data was verified using Pearson's correlation. A p -value <0.05 was considered statistically significant.

Results. *Expression of miR-155 in colon cancer tissue.* The melting curves of miR-155 and U6 real-time quantification PCR products were shown as unimodal, which indicated that the stem-loop RT-PCR was able to augment the corresponding target gene. The

quantification PCR products showed a single strip in 2.5% agarose gel electrophoresis, which further proved the specificity of the reaction. The results indicated that of the 84 colon cancer tissues, 65 tissues were found with upregulated expression of miR-155, compared with normal mucosa, of which, 24 tissues were upregulated more than 4 times, 11 tissues were upregulated more than 8 times; compared with normal mucosa, the expression of miR-155 in colon cancer tissues ($N=2^{\Delta\Delta Ct}$) was 4.03 ± 2.95 times, which is significantly higher than that in normal mucosal tissue (Table 1) (Figure 1).

The correlation between the expression of miR-155 in colon cancer tissue and serum CEA level. Cases in which the expression of miR-155 in tumor tissue was more than one fold were selected to draw a scatter diagram (the serum CEA level was indicated by the X axis, while the fold of expression of miR-155 in tumor tissue was indicated by the Y axis). The serum CEA values >1500 ng/ml were drawn as 1500). Through Pearson correlation analysis, it was found that the fold of expression of miR-155 in tumor tissue was positively correlated with serum CEA level ($r=0.713$, $t=8.008$, and $p<0.01$) (Figure 2).

During the 2 years of follow-up, 24 patients had increased postoperative serum CEA levels. The values

Table 1 - The Ct values of miR-155 and U6 in colon cancer and normal mucosal tissue.

Cycle threshold values	Ct value of tumor tissue	Ct value of normal mucous membrane	Fold upregulated	P-value
miR-155 (n=84)	23.57 \pm 6.83	28.61 \pm 7.03	4.03 \pm 2.95	<0.001
U6 (n=84)	18.74 \pm 5.67	19.33 \pm 6.02		

Ct - Cycle threshold, it is a measure of the number of polymerase chain reaction cycles needed to get a fluorescent signal.

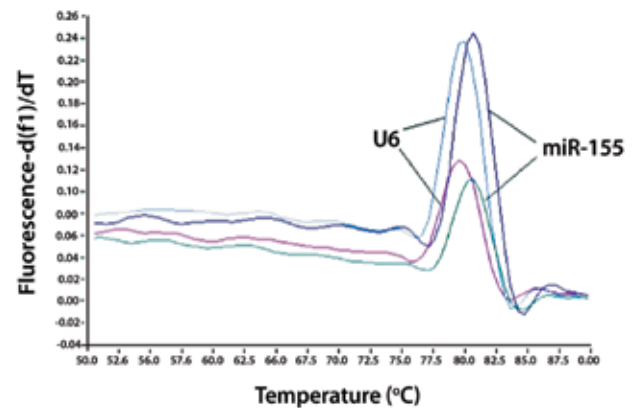


Figure 1 - The melting curve of miR-155 and the internal reference U6.

of CEA levels in these 24 patients were selected to draw a scatter diagram (the serum CEA level after operation was indicated by the X axis, while the fold of expression of miR-155 in tumor tissue was indicated by the Y axis). Through Pearson's correlation analysis, it was found that the fold of expression of miR-155 in tumor tissue had a significant negative correlation with postoperative serum CEA levels ($r=0.819$, $t=5.4920$, and $p<0.01$) (Figure 3).

Correlation between expression of miR-155 in colon cancer tissue and tumor recurrence or metastasis. During the 2 years of follow-up, 68 patients were diagnosed with recurrence or metastasis. Of these 68 patients, 19 patients reported recurrence or metastasis (not associated with patients' death), the recurrence and metastasis rate was 27.9%, of which, 7 patients reported hepatic metastasis (10.3%), 2 patients reported lung metastasis (2.9%), 5 patients reported lymph node metastasis (7.35%), 2 patients reported recurrence in anastomotic stoma (2.9%), and 3 patients reported intrapelvic or intra-abdominal metastasis (4.4%). The expression of miR-155 in tumor tissue in patients who had no recurrence or metastasis was 3.62 ± 2.55 times more than that in patients who had recurrence or metastasis. The difference between the 2 groups (group with recurrence or metastasis and group without

recurrence or metastasis) was statistically significant ($p=0.0144$) (Figure 4).

Correlation between length of duration since serum CEA level increased again after operation and tumor recurrence or metastasis after operation (this is not clear, please rephrase). During the 2 years of follow-up visits, 68 patients were with diagnosed recurrence or metastasis. Of these 68 patients, 19 patients reported recurrence or metastasis (not associated with patients' death), the recurrence and metastasis rate was 27.9%. Of the 24 patients who had an increased serum CEA level that lasted for less than 24 months postoperatively, 11 (45.8%) patients reported recurrence and metastasis.

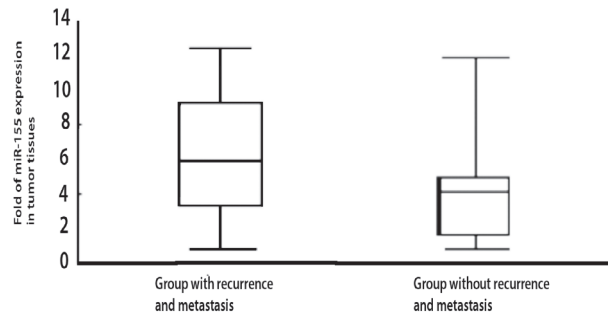


Figure 4 - Correlation between expression of miR-155 in colon cancer tissue and tumor recurrence or metastasis.

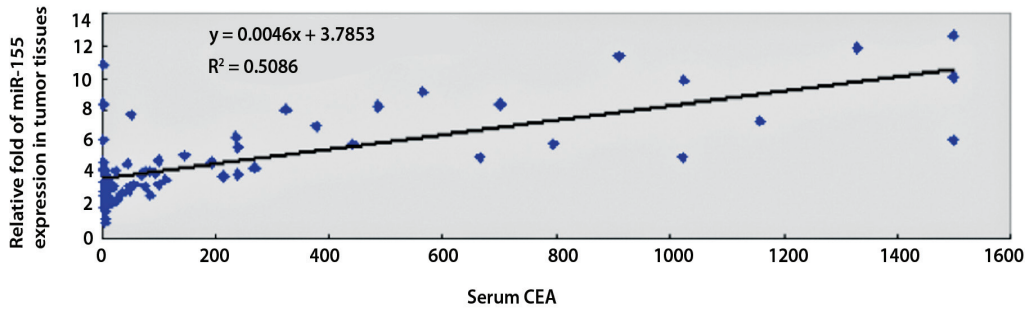


Figure 2 - The correlation between expression of miR-155 in colon cancer tissue and serum carcinoembryonic antigen (CEA) level.

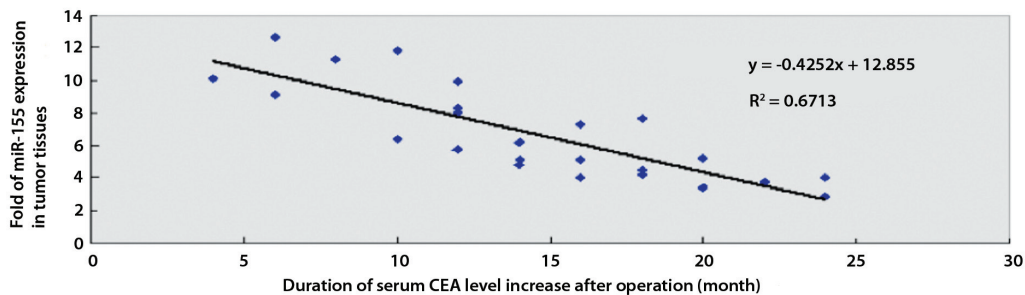


Figure 3 - Scatter distribution figure that shows the expression of miR-155 in colon cancer tissue and length of duration since serum carcinoembryonic antigen (CEA) levels increased after operation.

Table 2 - Correlation between length of duration since serum carcinoembryonic antigen (CEA) levels increased again after operation and tumor recurrence or metastasis after operation.

Duration of elevated postoperative CEA levels	Group with recurrence and metastasis	Group without recurrence and metastasis	Total	Recurrence and metastasis rate (%)
≤24 months	11	13	24	45.8
>24 months	8	36	44	18.2
Total	19	49	68	27.9

Of the 44 patients who reported that the increase in serum CEA levels lasted for more than 24 months after operation (the increase in serum CEA level was regarded as lasting more than 2 years if it was higher than its lowest value postoperatively at the end of the follow-up period), 8 (18.2%) patients reported recurrence and metastasis. Comparison showed that the difference between them was significant ($p=0.000$) (Table 2).

Discussion. The development and progression of colon cancer are a combined effect of oncogene activation and tumor suppressor gene deactivation, but the exact pathogenesis involved is still not clear. The discovery of the early diagnosis marker and therapeutic target is of great significance. In recent years, it has been found that miRNAs are closely associated with malignant tumors; the translation after transcription is suppressed primarily through specific binding to target miRNA molecule's 3' end non-coding area.⁴ Approximately 52% of regulated miRNAs' coding genes are located in tumor-related gene loci and chromosome areas.^{4,5} So, miRNAs may possibly become new molecule markers for tumor diagnosis, and molecular targets that serve as indicators for tumor treatment and prognosis.^{6,7} The miR-155 is one of the microRNA molecules that have been proved to have tumor-promoting effects, the expression of which is upregulated in many tumors, including breast cancer,⁸ lung cancer,⁹ colon cancer,¹⁰ pancreatic cancer,¹¹ and liver cancer.¹² In addition, the high expression of miR-155 is associated with the prognosis of lung cancer and pancreatic cancer.^{13,14}

The CEA is one of the most commonly used markers for colon cancer in clinical practice, which is mainly used for prognosis, observation of curative effect, and monitoring of recurrence and metastasis. Through pilot studies, our study team found that the expression of miR-155 in colon cancer tissue increased, but we did not study the correlation between expression of miR-155 in colon cancer tissue and serum CEA level in colon cancer and postoperative CEA levels, and there are few similar reports on this finding. For this reason, this study was conducted. This study used stem-loop qRT-PCR

technology to test the expression of miR-155 in colon cancer. The results indicated that of the 84 colon cancer tissues, compared with normal mucosa, the expression of miR-155 in colon cancer tissues was 4.03 ± 2.95 times, which is significantly higher than that in normal mucosal tissue. This indicated that the expression of miR-155 in colon cancer tissues was upregulated evidently, denoting that miR-155 is closely associated with the development and progression of colon cancer. In China, Wang et al¹⁵ used miRNA microarray analysis and qRT-PCR to test the miRNA expression in colon cancer, and reached the same conclusion. Bakirtzi et al,¹⁶ through an animal experiment, verified that silent miR-155 not only is able to prevent the formation of tumor cell aggregates, but also can slow down the growth of tumor cells, which further proves that miR-155 participates in development and progression of colon cancer.

Through data obtained from follow-up visits, this study found that expression of miR-155 in colon cancer is closely associated with tumor recurrence or metastasis postoperatively. That said, the higher the expression of miR155, the higher the possibility of tumor recurrence or metastasis after operation, and the poorer the prognosis of the patient. The study indicated that testing of miR-155 expression in colon cancer contributes greatly in predicting tumor recurrence or metastasis postoperatively. Currently, the exact pathogenesis involved in tumor invasion and metastasis has not been clarified completely, and plenty of research has been conducted. Kong et al¹⁷ verified that tumor transforming growth factor acts on RhoA protein via miR-155, and participates in metastasis of breast cancer. Gironella et al¹⁸ believed that unusual high expression of miR-155 in pancreatic cancer is the result of the following pathogenesis: the expression of TP53INP1 is decreased by suppressing its mRNA function thus, the tumor cell proliferation was boosted and the tumor cell apoptosis was suppressed. If the same pathogenesis is to be applied to colon cancer, it has yet to be proven by experiments. The miR-155 may guide a new research direction in the prevention

and treatment of colon cancer metastasis. The CEA is an acid glycoprotein that contains human embryonic antigen determinant, it is synthesized by juvenile cells and gastrointestinal tract tumor cells. A small amount of CEA is produced in normal digestive tracts of fetuses and adults, and by tumor cells that lose polarity that enter into the blood and lymph. It is commonly used in the auxiliary diagnosis of tumors in the digestive system and dynamic monitoring of the tumors and as indicators for curative effect and outcome.¹⁹ Currently, serum CEA is one of the most broadly used tumor markers in colon cancer, rectal cancer, lung cancer, pancreatic cancer, gastric cancer, breast cancer, liver cancer, and other malignant tumors. This increase is most evident in colon cancer and rectal cancer. Dynamic testing of postoperative serum CEA levels contributes greatly to the monitoring of colon and rectal cancer recurrence and metastasis after operation.²⁰ Experiment-based research found that the expression of miR-155 in colon cancer has significant positive correlation with serum CEA level, and it has significant negative correlation with the duration of elevated postoperative serum CEA levels. This indicates that during development and progression of colon cancer, the higher the expression of miR-155 in tumor tissue, the higher the serum CEA level, and the shorter the length of duration since serum CEA level increase again after operation. In addition, this experiment found that increase of postoperative serum CEA level implied the recurrence or metastasis.

Currently, clinical CEA testing is mainly used for prognosis, observation of curative effect, and monitoring of recurrence and metastasis of colon cancer. The above conclusions prove that miR-155 has certain contributions to prognosis, observation of curative effect, and monitoring of recurrence and metastasis of colon cancer. A combination of an assay of the expression of miR-155 in colon cancer tissue, and the serum CEA levels pre and postoperatively can afford more accurate information as indicators of clinical curative effect and prognosis, and can contribute to predicting tumor recurrence and metastasis postoperatively. In our study, there were still some limitations. The CEA level may be affected by many other factors such as smoking, diabetes and so on. During the follow-up period, 19.1% of the antcipants lost to follow-up. In further study, there are more factors to consider, and the sample size may be increased.

In conclusion, the expression of miR-155 in colon cancer tissue has a significant positive correlation with preoperative serum CEA levels, and higher expression of

miR-155 is related with ealier increase of postoperative serum CEA level. A combination with assay of expression of miR-155 in colon cancer tissue and the serum CEA levels before and after operation can afford more accurate information for clinical curative effect observation and prognosis judgment, especially contributing to tumor recurrence and metastasis forecast after operation.

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