

PROGNOSTIC SIGNIFICANCE OF MICRORNA-200B AND ERCC1 EXPRESSION IN TUMOR CELLS OF PATIENTS WITH ESOPHAGEAL CANCER

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Background: The development of malignant tumors, including esophageal cancer (EC), could be associated with impaired expression of oncosuppressive miRNA-200b and excision repair cross-complementing group-1 (ERCC1), a protein involved in DNA repair and alternative splicing. **Aim:** To investigate the features of expression of miRNA-200b and ERCC1 in tumor cells of patients with EC and to determine the possibility of their use for prediction of EC aggressiveness. **Materials and Methods:** 52 patients with EC of stages II–IV were enrolled. Expression of ERCC1 in tumor cells was assessed by immunohistochemical method, expression of miRNA-200b was evaluated by the real time reverse transcription-polymerase chain reaction. **Results:** The expression of miRNA-200b and ERCC1 in tumor cells of EC patients is associated with their life expectancy. The characteristic features of neoplasms in patients who died within 12 months are low expression of miRNA-200b (2.87 ± 1.65 a.u.) ($\rho = -0.42$; $p < 0.05$) and high expression of ERCC1 (191.0 ± 18.6 H-Score points) ($\rho = -0.48$; $p < 0.05$). The inverse correlations between the level of miRNA-200b expression and the tumor size were found both in the group of patients with survival < 12 months ($\rho = -0.42$; $p < 0.05$) and in the group of patients with survival > 12 months after surgery ($\rho = -0.53$; $p < 0.05$). **Conclusions:** The obtained data indicate the feasibility of using the expression of miRNA-200b and ERCC1 in EC cells to predict the aggressiveness of esophageal cancer. **Key Words:** esophageal cancer, miRNA-200b, ERCC1, aggressiveness of cancer.

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Esophageal cancer (EC) is an urgent problem of modern oncology both in Ukraine and in the world. It is one of the ten most common localizations of tumors and is one of the leading causes of cancer-related death [1, 2]. High aggressiveness of EC requires the use of a comprehensive approach to the treatment of patients with this pathology [3]. The most effective is the use of neoadjuvant chemoradiotherapy followed by the radical surgical treatment [4]. According to numerous studies, the use of neoadjuvant chemoradiotherapy reduces the bulk of the tumor in this group of patients, increases resectability, affects micrometastases, and reduces the risk of intraoperative contamination of the wound with tumor cells [5]. Despite the improved approaches to surgical treatment and chemotherapy of EC patients, long-term treatment outcomes vary widely and often remain unsatisfactory. The variability of EC regarding the course of the disease and the response to therapy is mediated by an increase in the number of patients with drug- and radiation-resistant tumors, which necessitates further studies of biological aspects of this cancer [6–8].

One of the current areas of fundamental oncology in recent years is the study of epigenetic disorders in the development and progression of malignant neoplasms. Particular attention is paid to the study

of miRNAs, as they are the main regulators of genes involved in carcinogenesis [9, 10]. MicroRNAs are small non-coding RNAs, approximately 22 nucleotides in length that regulate the expression level of mRNAs by interacting with their specific regions. MicroRNAs regulate more than 30% of human genes involved in many vital processes. That is why the impairment of microRNA regulation can affect all stages of carcinogenesis — from the initiation of malignant neoplasm to its progression [11, 12]. In recent years, there has been accumulated an evidence that the development of malignant tumors, including EC, may be associated with impaired expression of oncosuppressive miRNA-200b [13]. MicroRNA-200b is a member of the microRNA-200 family involved in the processes of proliferation, apoptosis and sensitivity to cytostatics by regulating transcription factors (Zeb1, Ets-1, Suz12). Changes in the expression of miRNA-200b promote epithelial-mesenchymal transition, correlate with the presence of cancer stem cells, high metastatic potential, resistance to chemoradiation therapy (including platinum derivatives), and are associated with an unfavorable course of different cancers [14–16].

Another promising prognostic marker that plays an important role in the development of many malignant tumors is excision repair cross-complementing group 1 (ERCC1) — a protein involved in DNA repair and alternative splicing [17]. ERCC1 is an indispensable component of the protein complex of ERCC1-XPF repair, the level of which in tumor tissue correlates with resistance to platinum-containing drugs and survival

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Abbreviations used: CDK2 — cyclin-dependent kinase 2; EC — esophageal cancer; ERCC1 — excision repair cross-complementing group 1.

rates of patients with gastric, ovarian, bladder cancer and other cancer types [18, 19]. Despite the significant interest in studying the role of microRNA-200 family and the marker of nucleotide excision repair ERCC1 in tumor growth, in the modern literature there are no data on the association of expression of these markers with EC aggressiveness. The expediency of their use as prognostic markers of EC has not been determined either.

In view of the above, the aim of this work was to investigate the features of miRNA-200b and ERCC1 expression in tumor cells of EC patients and to determine the possibility of their use for prediction of the EC aggressiveness.

MATERIALS AND METHODS

52 patients with stage II–IV EC were examined and treated at the Research Department of Thoracic Tumors of the National Cancer Institute of the Ministry of Health of Ukraine (Kyiv) from 2001 to 2016. All patients provided an informed consent on the use of their clinical data for scientific purposes. The general clinical and pathological characteristics of patients are presented in Table 1. The results of a comprehensive examination of patients showed that the majority of patients had tumors of category T3, the presence of metastases in regional lymph nodes was found in 26.92% of patients with EC. Morphological examination revealed that the vast majority of the studied tumors were squamous cell EC.

Examination of the patients was performed using esophagogastroduodenoscopy with tumor biopsy, computed tomography of the chest and abdomen with contrast enhancement, esophageal radiography, additional clinical and laboratory tests.

The stage of the tumor was determined according to the International TNM Classification (7th edition, 2009). Histological type of tumor was established according to the WHO classification (2006). Depending on the clinical indications, patients underwent organ-sparing operations according to the treatment standards adopted in Ukraine, as well as chemoradiation therapy.

Immunohistochemical study of ERCC1 expression in tumor cells was performed on 4–5 µm paraffin

sections using specific ERCC1 monoclonal antibodies (clone 4F9; Dako Cytomation, Denmark). To visualize the results of the reaction, a kit Mouse/Rabbit PolyVue Plus HRP/DAB Detection System, Diagnostic BioSystems (USA) was used in accordance with the manufacturer's recommendations, the sections were stained with Mayer's hematoxylin. Evaluation of the results was performed using optical microscopy (× 400) by the classic method of H-Score. The final result of the calculation was expressed in points: from 50 to 100 points — low expression level, from 101 to 200 points — medium, from 201 to 300 points — high.

To study the expression of miRNA-200b in tumor cells, the method of real time reverse transcription-polymerase chain reaction in was used. Total RNA was isolated from paraffin blocks with tumor tissue using the commercial RNeasy FFPE Kit (QIAGEN, Germany). The amount of isolated RNA was determined by spectrophotometry using NanoDrop 2000c Spectrophotometer (ThermoScientific, USA). The purity of the isolated RNA was assessed by the ratio of optical absorption values at wavelengths of 260 and 280 nm. RNA was dissolved in Tris-EDTA buffer and stored at –20 °C until use. Reverse transcription-polymerase chain reaction was performed on the Applied Biosystems 7900HT FastRealTime PCR System hardware detection system using a commercial TaqMan MicroRNA Assay RT-PCR kit (ThermoScientific, USA) according to the manufacturer's protocol. To determine miRNA-200b, we used a stem-loop primer for cDNA synthesis 5'-GTTGGCTCTGGTGCAGGGTCCGAGG-TATTCGCACCAGAGCCAACTCATCA-3' and a forward primer 5'-GTTTGGTAATACTGCCTGGTAA-3'. According to the stem-loop miRNA RT-PCR technique, a universal reverse primer 5'-GTGCAGGGTCCGAGGT-3' was used [20]. Primer sequences for the detection of miRNA-200b were obtained from the resource <http://genomics.dote.hu:8080/mirnadesigntool/> and synthesized by Metabion, Germany.

RNU48 microRNA was used as an endogenous control to objectify expression parameters. Primer sequences are taken from <https://www.ncbi.nlm.nih.gov> and synthesized by Metabion, Germany: RT-primer: 5'-CTCTGACC-3', forward 5'-AGTGATGATGACCCCAG-GTAACTC-3', reverse 5'-CTGCGGTGATGGCATCAG-3'. The relative expression of miRNA-200b was determined by a comparative dCT method (a.u.) [21]. The expression level of miRNA-200b was considered high if < 2 a.u., average — 2–4 a.u., low > 4 a.u.

Statistical analysis of the data was performed using the program Statistica 6.0. (Statistica Inc., USA), followed by graphical visualization of the results using Microsoft Excel 2010. Statistical processing was performed taking into account the nature of the distribution of the obtained data. The data are presented as $M \pm m$, where M is the arithmetic mean, m is the standard error of the mean or a percentage for a.u. In order to compare the two independent groups on a quantitative basis, we have used the Mann — Whitney U-test. The critical level of statistical significance

Table 1. General clinical and pathological characteristics of patients with EC

Characteristics	Number of patients, n	
	52	%
Sex		
M	45	86.54
F	7	13.46
Average age, years	62.7 ± 4.6	
Range	46–89	
Stage		
II	24	46.15
III	23	44.23
IV	5	9.62
T category by TNM		
T2	7	13.46
T3	45	86.54
N category by TNM		
Nx	21	40.39
N0	17	32.69
N1	14	26.92
Histological type		
Squamous cell carcinoma	51	98.00
Adenocarcinoma	1	2.00

was taken to be 0.05. Spearman's ratio was used to assess the relationship between the expression of the studied markers and the clinical and pathological characteristics of EC.

RESULTS AND DISCUSSION

Depending on the survival rates, the EC patients under study were divided into 2 groups. The first group consisted of 26 patients whose survival was less than 12 months, and the second group — 26 patients who lived more than 12 months after surgery. It was found that in patients of group 1 the level of ERCC1 was 1.5 times higher compared with patients of group 2 (Table 2). When analyzing the expression of miRNA-200b in EC cells, depending on the life expectancy of patients, it was found that in the group of patients who lived less than 1 year, the average level of miRNA-200b expression was twice lower in comparison with patients of group 1 (see Table 2).

A detailed analysis of ERCC1 and miRNA-200b expression in tumor tissue in EC patients with different survival showed that in both groups the tumors with a high level of ERCC1 expression were predominant (Fig. 1, 2). In particular, in group 1 and group 2, 60.9% and 56.3% of tumors, respectively, demonstrated ERCC1 expression level higher than 200 H-Score points (see Fig. 1). In contrast, a high level of miRNA-200b expression was detected in 15.4% of cases in group 1, which was 3.75 times lower compared to group 2 (57.4%). It was found that the percentage of cases with low ERCC1 levels in tumor cells was 5.75 times higher in group 2, while tumors with low levels of miRNA-200b in this group were not detected. It was shown that in patients who died within the first year after treatment, moderate ERCC1 expression in tumor cells was detected in 34.8% of tumors, which was 1.8 times higher than in patients who lived more than 1 year (18.8%), while the percentage of tumors with moderate miRNA-200b expression did not differ

Table 2. Relationship between ERCC1 and miRNA-200b expression and survival rates in EC patients

Index	Group 1	Group 2
ERCC1 expression, H-Score points	191.0 ± 18.6	124.4 ± 22.5*
miRNA-200b expression, a.u.	2.87 ± 1.65	6.40 ± 2.21*

Note: * $p < 0.05$ in comparison with group 1.

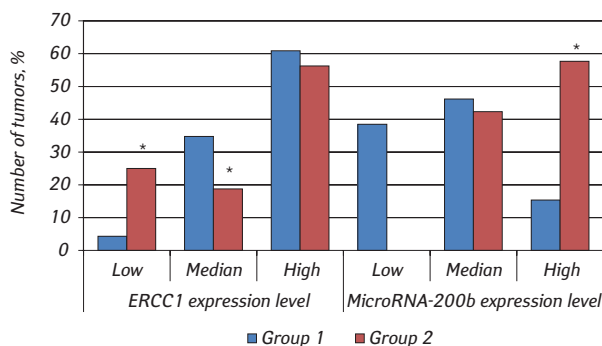


Fig. 1. Distribution of tumors by the levels of expression of ERCC1 and miRNA-200b depending on the survival rates of patients with MS. * $p < 0.05$ in comparison with group 1. Tumors with low levels of miRNA-200b in group 2 were not detected

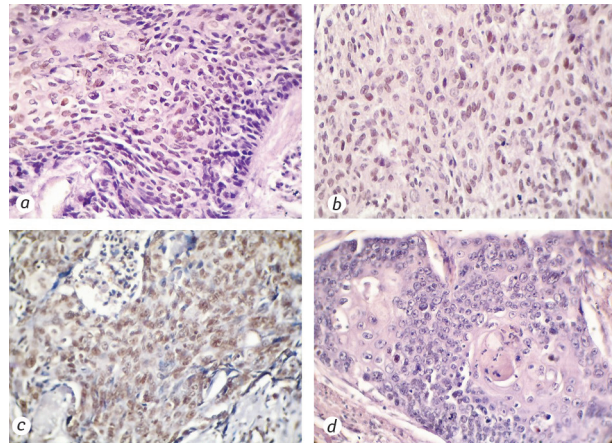


Fig. 2. Expression of ERCC1 in tumor tissue of patients with EC: a — low, b — medium, c — high, d — no expression. Immunohistochemistry. Chromogen DAB. Mayer's hematoxylin staining, × 400

significantly amounting to 46.2% and 42.3%, respectively.

When we have analyzed the dependence of ERCC1 and miRNA-200b expression levels on the clinical and pathological parameters of patients in both groups, the tendency for increasing expression of ERCC1 with more advanced clinical stage of EC in group 1 has been revealed (Table 3). No significant difference between the parameters of miRNA-200b in patients of group 1 with different clinical stage was found. In group 2, the expression of ERCC1 in tumor tissue of EC patients with stage II did not differ significantly from that in patients with stage III. The levels of miRNA-200b in the tumor tissue of patients with stage II and III did not differ statistically and were 1.9 and 1.6 times higher ($p < 0.05$) compared with stage IV.

There was also observed a tendency for increasing ERCC1 expression with the increased size of the tumor in group 1. In particular, the level of ERCC1 in patients with T2 tumors was 1.5 times lower than that in patients with T3 tumors (see Table 3). It was shown that in patients of groups 1 and 2 with neoplasms infiltrating the layers of the esophageal wall, including adventitia, the level of miRNA-200b was lower by 2.7 ($p < 0.05$) and 1.9 ($p < 0.05$) times, respectively, compared with tumors infiltrating the muscular membrane of the esophageal wall.

In the group of patients with a life expectancy of less than 12 months, a tendency for a decreasing level of ERCC1 and miRNA-200b expression in the presence of metastatic lesions has been observed. In particular, in patients of group 1 without metastases, the expression level of ERCC1 and miRNA-200b was by 1.2 and 1.5 times higher, respectively, than in the cases with lymph node involvement. In the group of patients with EC whose survival was more than 12 months, the presence of metastases was associated with higher levels of ERCC1. No significant difference in miRNA-200b expression in tumor tissue depending on the presence of metastases in the regional lymph nodes in this group of patients was found.

Table 3. Dependence of ERCC1 and miRNA-200b expression levels in tumor tissue of EC patients with different survival rates on the main clinical and pathological characteristics of the tumor process

Index	Group 1		Group 2	
	ERCC1 expression, H-Score points	MicroRNA-200b expression, a.u.	ERCC1 expression, H-Score points	MicroRNA-200b expression, a.u.
Average value	191.0 ± 18.6*	2.87 ± 1.65	124.4 ± 22.5	6.40 ± 2.21*
Individual range	0–283.0	0.77–5.27	0–285.0	2.26–11.63
Clinical stage				
II	156.3 ± 30.8	3.30 ± 2.88	110.7 ± 32.6	8.29 ± 0.83
III	207.4 ± 26.0	3.39 ± 1.19*	137.2 ± 36.5	6.93 ± 1.92
IV	222.5 ± 60.5	2.44 ± 0.89	-	4.29 ± 1.45 ·
T category				
T2	124.3 ± 73.1	4.18 ± 1.37	-	8.13 ± 2.33*
T3	192.6 ± 19.7*	1.54 ± 0.56*	124.2 ± 23.2	4.23 ± 1.26*
N category				
Nx	148.3 ± 30.1	3.21 ± 2.50	95.7 ± 38.4	5.02 ± 1.42
N0	238.4 ± 30.0*	3.53 ± 0.25	130.2 ± 34.1	7.73 ± 2.17
N1	193.6 ± 33.7	2.28 ± 0.64" · *	156.2 ± 51.2	5.81 ± 2.59

Note: *the difference in comparison with the group 2 index is significant ($p < 0.05$); · the difference in comparison with the index of patients with stage II is significant ($p < 0.05$); *the difference in comparison with the index of patients with T3 is significant ($p < 0.05$); "the difference in comparison with the rate of patients without metastases to regional lymph nodes is significant ($p < 0.05$).

Table 4. Correlation of ERCC1 and miRNA-200b expression levels with the main clinical and pathological parameters of EC patients with different survival

Pairs of correlations		Group 1		Group 2	
		ρ	p	ρ	p
Expression of ERCC1	Clinical stage	0.08	> 0.05	0.07	> 0.05
	Tumor size	0.25	> 0.05	0.11	> 0.05
	Metastases in regional lymph nodes	-0.07	> 0.05	0.004	> 0.05
Expression of microRNA-200b	Clinical stage	-0.18	> 0.05	0.07	> 0.05
	Tumor size	-0.42	< 0.05	-0.53	< 0.05
	Metastases in regional lymph nodes	-0.26	> 0.05	-0.21	> 0.05
Expression of ERCC1	Expression of microRNA-200b	-0.48	< 0.05	-0.45	< 0.05

Spearman's correlation analysis (Table 4) demonstrated the lack of correlations between the expression of ERCC1 in tumor tissue and the main clinical and pathological characteristics in both groups of patients with EC, what is consistent with previously published data by Bilen *et al.* [22]. It should be noted that the authors also did not find correlations between ERCC1 expression and albumin or hemoglobin levels, but revealed a correlation between ERCC1 expression and tumor size [22].

The discrepancies between obtained results can be explained by the relatively small size of the studied group of patients and significant heterogeneity in the expression values. In addition, it is necessary to take into account the various methodological approaches used to determine ERCC1 expression — the study of *ERCC1* gene polymorphisms, the levels of mRNA or ERCC1 protein, as well as the lack of large randomized studies. The inverse moderate correlation between the level of miRNA-200b expression and tumor size was found both in the group of patients whose survival was less than 12 months ($\rho = -0.42$; $p < 0.05$) and in the group of patients with a survival of more than 12 months after surgery ($\rho = -0.53$; $p < 0.05$), being in line with the results of other studies providing the evidence that miRNA-200b inhibits the expression of important proteins, cell cycle activators cyclin-dependent kinase 2 and PCNA-associated factor contributing to the activation of tumor growth [13, 18].

No relationship was found between the level of miRNA-200b expression in tumor tissue and such important clinical and pathological characteristics of patients with EC as the stage of the disease and the presence of metastases in regional lymph nodes in both study groups, although the tendencies to the decreasing miRNA-200b expression with tumor pro-

gression has been traced similarly to the data by other authors [13, 18]. For example, a direct correlation was found between the decreased levels of miRNA-200b expression in EC patients and increased risk of tumor invasion as well as metastasis to adjacent lymph nodes. We revealed an inverse correlation between the levels of ERCC1 and miRNA-200b in patients of both groups (see Table 4), which may indicate their role in the formation of the malignant phenotype of tumor cells. According to the literature and the results of bioinformatics analysis, such a relationship between the expression level of ERCC1 and miRNA-200b can be explained by the indirect effect of miRNA-200b on the expression level of ERCC1. It is known that ERCC1 is part of the ERCC1-XPF repair complex, the increased level of which in the tumor cell indicates a high probability of the resistance to anticancer therapy. Using the online tool TargetScan, we found that the gene encoding XPF (ERCC4) is a target for miR-200 family, which, in turn, can potentially lead to a decrease in the level of ERCC1-XPF complex in tumor tissue [13]. However, experimental data are insufficient to confirm this hypothesis, which once again emphasizes the importance of a deeper study of the role of a miR-200 family in tumors of different histogenesis, in particular EC. The relation between the expression levels of the studied markers and the survival of patients with EC has been confirmed by the literature data for tumors of other localizations [13, 23, 24].

To sum up, the unsatisfactory treatment outcomes of EC patients raise an issue for searching prognostic markers, which at the early stages, during therapy and after treatment allow identifying patients at high risk of recurrence. Our results indicate the possibility of using ERCC1 and miRNA-200b expression in tumor cells as markers of EC aggressiveness.

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