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ORIGINAL ARTICLE

Case Control Study

Methyl-methanesulfonate sensitivity 19 expression is associated with metastasis and chemoradiotherapy response in esophageal cancer

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Abstract

AIM: To investigate the clinical significance of methyl-methanesulfonate sensitivity 19 (MMS19) expression in esophageal squamous cell carcinoma (ESCC).

METHODS: Between June 2008 and May 2013, specimens from 103 patients who underwent endoscopic biopsy for the diagnosis of ESCC at the endoscopy center of Sun Yat-Sen University Cancer Center were collected; 52 matched-normal esophageal squamous epithelium samples were biopsied as controls. MMS19 protein expression was measured by immunohistochemistry. Of the 103 cases of ESCC, 49 received radical surgery following neoadjuvant chemoradiotherapy consisting of concurrent radiation in a total dose of 40 Gy and two cycles of chemotherapy with vinorelbine and cisplatin. Relationships between MMS19 expression, clinicopathologic characteristics and chemoradiotherapy response were analyzed.

RESULTS: The MMS19 protein could be detected in both the cytoplasm and nucleus of most specimens. High cytoplasmic expression of MMS19 was detected in 63.1% of ESCC samples, whereas high nuclear

expression of MMS19 was found in 35.0%. High cytoplasmic MMS19 expression was associated with regional lymph node metastases (OR = 11.3, 95%CI: 2.3-54.7; $P < 0.001$) and distant metastases (OR = 13.1, 95%CI: 1.7-103.0; $P = 0.002$). Furthermore, high cytoplasmic MMS19 expression was associated with a response of ESCC to chemoradiotherapy (OR = 11.5, 95%CI: 3.0-44.5; $P < 0.001$), with a high cytoplasmic MMS19 expression rates in 79.3% and 25.0% of patients from the good chemoradiotherapy response group and poor response group, respectively. Nuclear MMS19 expression did not show any significant association with clinicopathologic characteristics or chemoradiotherapy response in ESCC.

CONCLUSION: The results of our preliminary study suggest that MMS19 may be a potential new predictor of metastasis and chemoradiotherapy response in ESCC.

Key words: Chemoradiotherapy; Esophageal squamous cell carcinoma; Metastases; Methyl-methanesulfonate sensitivity 19; Surgery

Core tip: Methyl-methanesulfonate sensitivity 19 (MMS19) was first identified as a DNA repair protein, and recently as a part of cytoplasmic Fe-S assembly machinery that produce proteins involved in maintenance of genomic stability, such as DNA polymerase, DNA repair proteins, and DNA nuclease/helicase. However, the clinical significance of MMS19 protein expression in esophageal cancer has not been reported. This study shows that MMS19 is abnormally expressed in esophageal cancer, and the elevated cytoplasmic MMS19 expression is associated with lymph node and distant metastases, and response to chemoradiotherapy in esophageal squamous cell carcinoma.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive tumors, ranking fourth among the top ten cancer-related deaths in China[1,2]. In China, the histology of esophageal cancer is mainly ESCC, whereas esophageal adenocarcinoma is rare[3]. Because of the high recurrence and metastasis rates, the five-year survival rate of ESCC treated with surgery alone is poor, only approximately 25%, and in such circumstances, surgery plus radiotherapy and/or chemotherapy is increasingly adopted for locally advanced esophageal cancer[4]. The results from phase Ⅲ randomized trials of chemoradiotherapy (CRT) prior to surgery are encouraging; however, these studies reveal that only patients who are sensitive to CRT will ultimately benefit from the multimodality treatment[5-7]. Thus, the identification of patients who can benefit from CRT is crucial for the success of the combined treatment of CRT followed by surgery. However, there is currently no biomarker that can predict response of ESCC to CRT. Therefore, the discovery of biomarkers that can predict sensitivity of ESCC to CRT is an urgent need in clinical practice.

The methyl-methanesulfonate sensitivity 19 (*MMS19*) gene, also named as *MMS19L* or *hMMS19*, encodes a multifunctional protein involved in DNA metabolism and the maintenance of genomic stability[8]. Nucleotide excision repair (NER) plays a vital role in the development of carcinogen-induced cancers[9,10] and in tumor resistance to chemo- and radiotherapy[11,12]. By interacting with the core transcription factors of NER, MMS19 can affect NER functions[13,14]. In addition, Fe-S proteins are crucial for genomic instability[15], which is a hallmark of cancer[16]. As a part of the cytoplasmic Fe-S assembly machinery, MMS19 facilitates the transfer of the Fe-S cluster to target Fe-S proteins, which include DNA polymerase δ, xeroderma pigmentosum group D, Fanconi anemia pathway component J (also known as BACH1 or BRIP1)[17], DNA2 nuclease/helicase, RNase L inhibitor (also known as ABCE1), and endonuclease three-like glycosylase 2[18]. Thus, MMS19 is suggested to be involved in maintaining genomic stability[17,18].

At present, some studies have reported that single-nucleotide polymorphisms of the *MMS19* gene are associated with the risk of pancreatic cancer[19], chemotherapy toxicity of non-small-cell lung cancer[20], and chemotherapy response of osteosarcoma[21]. These polymorphisms could increase cancer susceptibility by altering conserved amino acids[22] and could affect cancer prognosis by modulating gene expression[23]. However, the cellular expression level of MMS19 protein in cancer and its clinical significance have not been reported. In this study, we investigated the cellular expression level and distribution of *MMS19* in ESCC and the relationships of *MMS19* expression with the clinicopathologic factors and CRT response of ESCC.

MATERIALS AND METHODS

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Medical Ethics Committee of Sun Yat-Sen University Cancer Center. All patients signed an informed consent form for this investigation.

Patients

Between June 2008 and May 2013, specimens from 103 ESCC patients who underwent endoscopic biopsy for diagnosis at the endoscopy center of Sun Yat-Sen University Cancer Center were collected. As controls, 52 samples of normal esophageal squamous epithelia (NESE) were biopsied from ≥ 5 cm from the primary lesion in the same patients. Patients who received any anticancer treatments before diagnosis were excluded. The biopsied specimens were diagnosed by two pathologists. Tumor staging was performed based on the combined results of physical examination, endoscopic ultrasonography, CT scan of the chest and abdomen, and color ultrasonography scan of the abdomen and neck. The tumors were staged according to AJCC (2002). The patients were aged from 42 to 83 years (median 59 years), including 84 men and 19 women. Two patients were classified as stage Ⅰ, 25 patients as stage Ⅱ, 58 patients as stage Ⅲ and 18 patients as stage Ⅳ. Among the 103 ESCC patients, a cohort of 49 patients with thoracic esophageal carcinoma staged Ⅱb and Ⅲ received neoadjuvant CRT followed by surgery.

Neoadjuvant chemoradiotherapy and surgery

Radiation treatment planning was designed according to CT simulation or three-dimensional conformal radiation therapy. The patients were treated with 6 or 8 MV photons delivered in a total dose of 40 Gy (20 fractions of 2 Gy per fraction in 4 wk) in anteroposterior fields including esophageal tumors and enlarged lymph nodes, with 3-cm proximal and distal margins, and an 0.8-cm radial margin. The patients received two cycles of vinorelbine and cisplatin. Vinorelbine (25 mg/m^2) was administered intravenously on days 1, 8, 22 and 29, and cisplatin (75 mg/m^2) was infused on day 1 and day 22 (or 25 mg/m^2 on days 1-4 and 22-25). Total thoracic esophagectomy through a right thoracotomy with radical mediastinal and abdominal lymph node dissection was performed ≥ 4-6 wk after the completion of CRT.

Evaluation of histopathologic response to preoperative CRT

For evaluation of response to CRT, surgical cancer samples from 49 patients who underwent CRT and surgery were obtained. The histopathologic response to CRT was evaluated by two experienced pathologists according to previously published criteria[24,25]. The percentage of residual viable tumor cells was estimated, and each patient was subsequently allocated to one of the following four groups: complete response group, no residual tumor cells; major response group, < 10% residual tumor cells; partial response group, 10%-50% of residual tumor cells; minor response group, > 50% of residual tumor cells. For the statistical analysis, the patients were divided into two groups according to CRT response: a good response group, consisting of patients with a complete or major response; and a poor response group, including patients with a partial or minor response.

Immunohistochemical staining

Immunohistochemical staining was performed on 4-μm-thick paraffin sections. The sections were deparaffinized in xylene and rehydrated through graded alcohol. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 min. For epitope retrieval, the tissue slides were immersed in EDTA buffer (pH 8.0) and heated for 2.5 min on high power in a microwave oven. After washing, the tissue slides were incubated with an anti-MMS19 antibody (16015-1-AP; Proteintech, Chicago, IL, United States) at a dilution of 1:50 for 50 min at 37 ℃ in a moist chamber. Subsequently, the secondary antibody (K5007, Real Envision/HRP; Dako of Agilent Technologies, Santa Clara, CA, United States) was applied to the tissue section for 30 min at 37 ℃. Finally, 3.3'-diaminobenzidine was used for color development and hematoxylin for counterstaining. Negative control slides in the absence of primary antibody were included for each batch of staining.

Cytoplasmic and nuclear MMS19 staining was evaluated separately. The immunochemistry staining for the MMS19 protein was evaluated under 400× high-power magnification. The positively stained cells in five separate areas of epithelial or intratumoral regions were counted. The quantification of MMS19 expression was performed according to a previous study[26]. The percentage of cells positively stained was scored as follows: 0 ≤ 5%, 1 = 6%-25%; 2 = 26%-50%; 3 = 51%-75%; 4 > 75%. The staining intensity was scored as follows: 0 = no staining, 1 = weak, 2 = moderate, 3 = strong. For each case, the final score for MMS19 immunostaining was calculated by multiplying the percentage score of positive cells with the staining intensity score. Immunostaining was independently evaluated by two experienced pathologists who had no knowledge of the patients' clinicopathologic information. If different scores for the same sample were made by the two pathologists, the sample was revaluated and, if needed, discussed to decide a final score. Then, a composite score scaled as 0, 1, 2, 3, 4, 6, 8, 9, and 12 was obtained. Based on the final score, each case was divided into a high expression group (≥ 6) or a low expression group (< 6).

Statistical analysis

The statistical analyses were performed using SPSS 20.0 software (IBM Corp., Armonk, NY, United States). Data are expressed as mean ± SE. The differences in *MMS19* expression levels between the different groups and the correlations between *MMS19* expression and clinicopathologic characteristics as well as response to CRT were analyzed by the χ^2 test. Spearman's rank correlation (r) was used to determine whether there

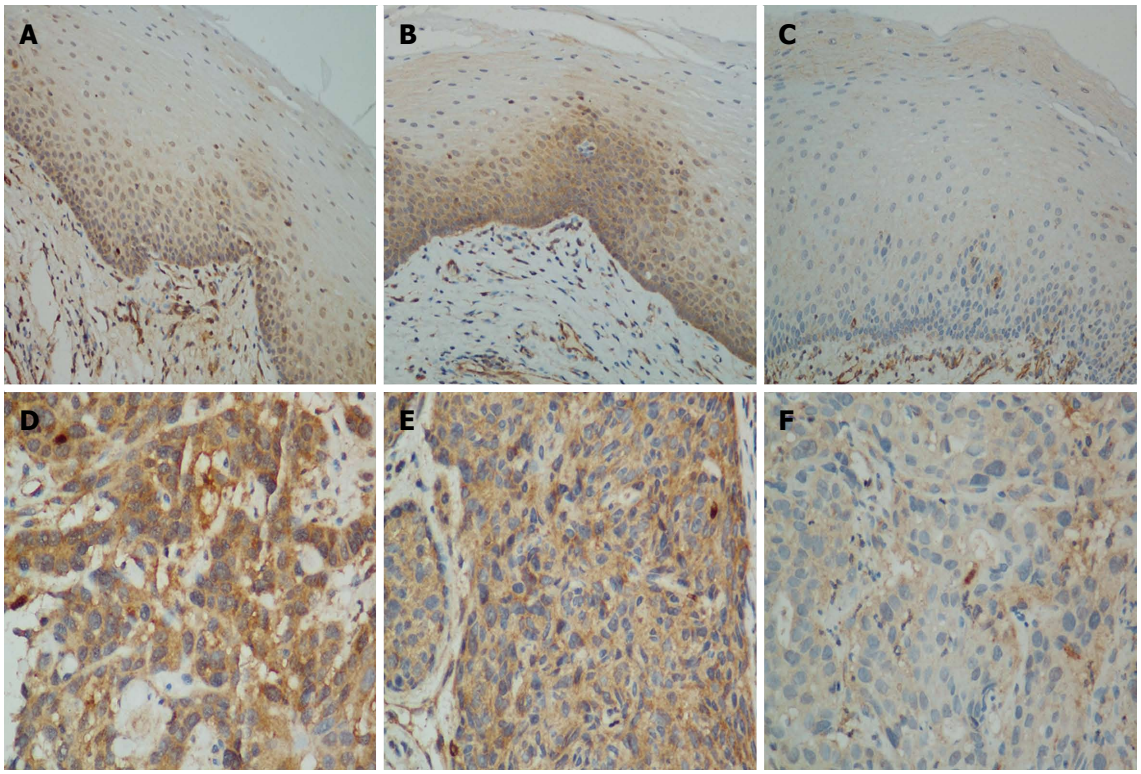

Figure 1 Methyl-methanesulfonate sensitivity 19 immunohistochemistry. Normal esophageal squamous epithelium with A: Strong nuclear but weak cytoplasmic staining; B: Strong cytoplasmic staining in the basal and suprabasal layers, with scattered strong nuclear staining in the normal epithelium area; C: Weak staining in both the cytoplasm and nucleus (Magnification × 200); Esophageal squamous cell carcinoma with D: Strong staining in the cytoplasm and nucleus; E: Strong staining in the cytoplasm and weak staining in the nucleus; F: Weak staining in both the cytoplasm and nucleus (Magnification × 400).

was a positive or negative correlation. Two-tailed $P < 0.05$ was considered statistically significant. The statistical methods of this study were reviewed by Qing Liu from Sun Yat-Sen University Cancer Center.

RESULTS

Different expression levels of MMS19 in biopsied NESE and ESCC tissues

Using immunohistochemistry, the MMS19 protein was detected in both the cytoplasm and nucleus of most endoscopic biopsied specimens (Figure 1), which is consistent with its cellular functions[13,18]. Cytoplasmic MMS19 staining in NESE was mainly found in the basal and suprabasal layers, with a gradually decreased staining from the basal layer to the superficial layer. In contrast, nuclear MMS19 staining in NESE was scattered throughout the entire layer (Figure 1A and B). The intensity of MMS19 staining was typically homogeneous within an ESCC specimen, but varied considerably among different tumors (Figure 1D and E). Figure 1C and F show weak staining in both the cytoplasm and nucleus of NESE and ESCC, respectively.

The mean scores for cytoplasmic MMS19 expression in the high expression group and low expression group were 7.78 ± 0.27 and 2.79 ± 0.21, respectively. Whereas, the mean scores of nuclear MMS19 expression in the high expression group and low expression group were 6.86 ± 0.32 and 2.68 ± 0.14, respectively. High cytoplasmic expression of MMS19 was detected in 63.1% of the ESCC samples, which was significantly higher than the 15.4% in NESE ($P < 0.001$, Table 1). High nuclear expression of MMS19 was found in 35.0% of the ESCC specimens, which was significantly lower than the 69.2% found in NESE ($P < 0.001$, Table 1).

Relationships of MMS19 expression in biopsied ESCC tissues with clinicopathologic features

First, associations of cytoplasmic MMS19 expression with clinicopathologic features were investigated. The results showed that high cytoplasmic MMS19 expression was significantly associated with regional lymph node metastases (LNM) (OR = 11.25, 95%CI: 2.31-54.73; $P < 0.001$) and distant metastases (DM) (OR = 13.10, 95%CI: 1.67-103.00; $P = 0.002$), suggesting that cytoplasmic MMS19 expression might be involved in cancer metastasis. The Spearman correlation coefficients of high cytoplasmic MMS19 expression with LNM and DM were 0.35 and 0.299, respectively, indicating that higher levels of MMS19 expression are positively correlated with ESCC metastasis. There was no significant association of cytoplasmic MMS19 expression with other clinicopathologic features, including histologic grade, invasion depth, patient age, tumor stage, or sex (Table 2). Nuclear MMS19 expression did not show any

Table 1 Methyl-methanesulfonate sensitivity 19 expression *n* (%)

Tissue	Cases (*n*)	Cytoplasmic expression		*P* value	Nuclear expression		*P* value
		High	Low		High	Low	
NESE	52	8 (15.4)	44 (84.6)	< 0.001	36 (69.2)	16 (30.8)	< 0.001
ESCC	103	65 (63.1)	38 (36.9)		36 (35.0)	67 (65.0)	

ESCC: Esophageal squamous cell carcinoma; High: Including composite score of 6, 8, 9 and 12; NESE: Normal esophageal squamous epithelium; Low: Including composite score of 0, 1, 2, 3 and 4.

Table 2 Associations of MMS19 expression with clinicopathologic features of esophageal squamous cell carcinoma

Characteristic	Cases (*n*)	Cytoplasmic MMS19			Nuclear MMS19		
		High	Low	*P* value	High	Low	*P* value
Total	103	65	38		36	67	
Sex							
Male	84	55	29	0.295	32	52	0.159
Female	19	10	9		4	15	
Age (yr)							
< 55	33	18	15	0.216	14	19	0.275
⩾ 55	70	47	23		22	48	
Site							
Upper thoracic	10	7	3	0.686	2	8	0.508
Middle thoracic	47	31	16		16	31	
Lower thoracic	46	27	19		18	28	
Differentiation							
Well	20	13	7	0.785	6	14	0.871
Moderate	52	34	18		19	33	
Poor	31	18	13		11	20	
TNM stage							
Ⅰ + Ⅱ	27	14	13	0.158	8	19	0.500
Ⅲ + Ⅳ	76	51	25		28	48	
Invasion depth							
T1 + T2	25	17	8	0.560	7	18	0.402
T3 + T4	78	48	30		29	49	
LNM							
No	12	2	10	< 0.001	4	8	0.900
Yes	91	63	28		32	59	
DM							
No	85	48	37	0.002	32	53	0.212
Yes	18	17	1		4	14	

DM: Distant metastases; ESCC: Esophageal squamous cell carcinoma; High: Including composite score of 6, 8, 9 and 12; LNM: Lymph node metastases; Low: Including composite score of 0, 1, 2, 3 and 4; MMS19: methyl-methanesulfonate sensitivity 19.

significant association with clinicopathologic parameters (Table 2).

Relationship of MMS19 expression in biopsied specimens with CRT response of resected ESCC

According to the histopathologic response to preoperative CRT, 24 cases showed complete response, 5 cases showed a major response, 9 cases showed a partial response, and 11 cases showed a minor response. Thus the good and poor response groups included 29 and 20 cases, respectively. Then, relationships of MMS19 expression with CRT response of ESCC were investigated. In the good response group, high cytoplasmic expression of MMS19 was observed in 23/29 (79.3%) patients. In contrast, high MMS19 expression was found in only 5/20 (25.0%) patients in the poor response group, and the difference in MMS19 expression between the two groups was statistically significant (OR = 11.5, 95%CI: 2.97-44.51; $P < 0.001$, Table 3). The Spearman correlation coefficient of high cytoplasmic MMS19 expression with a good response was 0.539, suggesting that high cytoplasmic expression of MMS19 is positively correlated with a good response to preoperative CRT. This result suggested that MMS19 might be a potential new biomarker to predict tumor response to preoperative CRT. However, nuclear MMS19 expression was not associated with CRT response, with a rate of high nuclear expression of 31.0% (9/29) in the good response group and 45.0% (9/20) in the poor response group (Table 3).

The relationships of CRT response with clinico-

Table 3 Clinical features of patients receiving neoadjuvant chemoradiotherapy followed by surgery, *n*

Characteristic	Good response (*n* = 29)	Poor response (*n* = 20)	*P* value
Age (yr)			
< 55	11	11	0.238
⩾ 55	18	9	
Sex			
Male	24	15	0.763
Female	5	5	
Tumor size (cm)			
< 5	12	7	0.652
⩾ 5	17	13	
Site			
Upper thoracic	3	2	0.405
Middle thoracic	14	6	
Lower thoracic	12	12	
Differentiation			
Well	5	4	0.936
Moderate	16	10	
Poor	8	6	
TNM stage			
Ⅱb	8	6	0.857
Ⅲ	21	14	
Cytoplasm			
High	23	5	< 0.001
Low	6	15	
Nucleus			
High	9	9	0.319
Low	20	11	

High: Including composite score of 6, 8, 9 and 12; Low: Including composite score of 0, 1, 2, 3 and 4; TNM: Tumor-node-metastasis.

pathologic features were also analyzed. However, there was no relationship between preoperative CRT response and clinicopathologic features, including tumor size, tumor site, differentiation, or Tumor-node-metastasis stage (Table 3). This result indicates that no clinicopathologic features should be used for predicting preoperative CRT response.

DISCUSSION

The results of the present study show, for the first time, that MMS19 expression in ESCC is upregulated in the cytoplasm and downregulated in the nucleus. The abnormal cellular distribution of MMS19 protein suggests that MMS19 is involved in the development and progression of ESCC. Furthermore, we found that MMS19 protein expression is associated with LNM and DM. More importantly, we found that cytoplasmic MMS19 protein expression is associated with the CRT response of ESCC. In clinical management, the therapeutic strategy for ESCC is primarily based on whether metastases exist, which is the most important determinant of patient outcome[27-30]. Furthermore, multimodality treatment only benefits patients who are sensitive to CRT[5-7]. Thus results of this study suggest that MMS19 has the potential to be a new biomarker for predicting metastasis and CRT response in ESCC.

In the present study, we found that the subcellular distribution of high MMS19 expression is changed from the nucleus in NESE to the cytoplasm in ESCC. Although the mechanism for this change in MMS19 expression in ESCC is not yet clear, a similar phenomenon has been reported for the DNA repair genes *Ape1/ref-1* and *JWA*[31,32]. The aberrant subcellular distribution of the MMS19 protein may implicate that the DNA repair function of MMS19 in the nucleus is attenuated. Conversely, as a cytoplasmic Fe-S assembly machinery component in the cytoplasm, MMS19 would promote the synthesis of many Fe-S proteins participating in DNA metabolism in the nucleus. Thus, we hypothesize that, as a consequence, DNA mutations will accumulate in cancer cells due to the impaired DNA repair function, with cell division and proliferation accelerating as a result of the increased DNA metabolism, exerting adaptive pressure on these cells[33-35]. Previous studies have reported that rapidly proliferating esophageal cancer cells are more sensitive to CRT[36,37] and that DNA damage in cancer cells is associated with the sensitivity of cancer to CRT[38,39]. Our finding that ESCC with higher cytoplasmic MMS19 expression is much more sensitive to preoperative CRT is in accord with these studies. Furthermore, ESCC with higher MMS19 expression will accumulate an array of mutations, facilitating cancer metastasis.

Previous studies reported that DNA repair genes are associated with chemo- or radiotherapy response. The low nuclear expressions of *ERCC1* and *XRCC1* are associated with a good chemotherapy response in non-small-cell lung cancer[40-42] and gastric cancer[32], respectively, whereas high nuclear expression is associated with the radio-resistance of laryngeal cancer[43]. Furthermore, high nuclear expression of *RRM1* is significantly associated with a lower disease control rate in non-small-cell lung cancer[44]. However, in the present study, we found that cytoplasmic MMS19 expression, but not nuclear MMS19 expression, is associated with CRT response. In addition to a role in DNA repair, MMS19 in the nucleus is also involved in mitotic segregation[45], histone modification[46], and interaction with regulator of telomere elongation helicase 1[17]. One reason that our study did not reveal an association between nuclear MMS19 and CRT response and metastasis may be that in the situation of abnormally expressed MMS19 in ESCC, the cytoplasmic function, but not the nuclear function of MMS19 plays the dominant role, underlying the development and progression of cancer cells.

In conclusion, the results demonstrate that MMS19 is abnormally expressed in esophageal squamous cell cancer. Elevated cytoplasmic MMS19 expression was associated with regional LNM, DM and a good preoperative chemoradiotherapy response of ESCC. These results provide novel preliminary evidence that MMS19 is involved in a mechanism of cancer development and progression, and has the potential to serve as a tumor biomarker that predicts metastasis

and chemoradiotherapy response in ESCC.

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COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive, malignant neoplasms. Surgery plus radiotherapy and/or chemotherapy is an effective treatment method for locally advanced esophageal cancer. However, there is no biomarker to predict the response of ESCC to chemoradiotherapy in clinical practice. Methyl-methanesulfonate sensitivity 19 (MMS19) is a multifunctional protein involved in DNA metabolism and genomic stability maintenance. Studies have demonstrated that single-nucleotide polymorphisms of the *MMS19* gene are associated with the risk of pancreatic cancer, chemotherapy toxicity of non-small-cell lung cancer, and chemotherapy response of osteosarcoma. So far, the clinical significance of the expression of MMS19 in ESCC is not clear.

Research frontiers

Fe-S proteins such as DNA glycosylases, primases, DNA helicases, and nuclease are crucial for genomic instability, which is a hallmark of cancer. As a part of the cytoplasmic Fe-S assembly machinery, MMS19 facilitates the transfer of the Fe-S cluster to target Fe-S proteins, such as DNA polymerase, Dna2 nuclease/helicase, RNase L inhibitor, and endonuclease three-like glycosylase 2. However, the roles of the components of this machinery in cancer have rarely been explored.

Innovations and breakthroughs

Previous studies investigated the relationships of single-nucleotide polymorphisms of the *MMS19* gene with cancer. We discovered, for the first time, that MMS19 is abnormally expressed in esophageal squamous cell cancer. Abnormally elevated cytoplasmic MMS19 expression is associated with regional lymph node metastases, distant metastases, and a good preoperative chemoradiotherapy response of ESCC. These results suggest that cytoplasmic Fe-S assembly machinery may play an important role in cancer development and progression, revealing new mechanisms of carcinogenesis and therapeutic targets.

Applications

The study results provide preliminary evidence that MMS19 has the potential to serve as a novel tumor biomarker for predicting metastasis and chemoradiotherapy response in ESCC.

Terminology

Methyl-methanesulfonate is an alkylating agent that can lead to DNA damage.

Peer-review

This is basically an interesting paper assessing a novel prognostic biomarker in ESCC with some potential to open up new lines of research.

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