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MSH2 promoter hypermethylation in circulating tumor DNA is a valuable predictor of disease-free survival for patients with esophageal squamous cell carcinoma

Z.Q. Ling [a,*], Q. Zhao [b], S.L. Zhou [a], W.M. Mao [a,b,*]

[a] *Zhejiang Cancer Research Institute, Zhejiang Province Cancer Hospital, No. 38 Guangji Rd., Banshanqiao District, Hangzhou 310022, PR China*
[b] *Department of Surgical Oncology, Zhejiang Province Cancer Hospital, No. 38 Guangji Rd., Banshanqiao District, Hangzhou 310022, PR China*

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Abstract

Background: Tumor-specific alterations of DNA methylation in circulating DNA have been associated with tumor burden and malignant progression. A wealth of information indicating the potential use of DNA methylation in circulating DNA for cancer screening, prognosis and monitoring of the efficacy of anticancer therapies has emerged. In this study, we examined prospectively whether the presence of plasma DNA with tumor characteristics before oesophagectomy is a predictive factor related to disease-free survival (DFS).
Methods: Promoter hypermethylation of *MSH2* was analyzed using real-time methylation-specific PCR (real-time MSP) in paired tumor and plasma samples of 209 patients with esophageal squamous cell carcinoma (ESCC).
Results: Aberrant *MSH2* methylation was found in 101 of 209 ESCC patients. Of these 101 patients, 77 cases exhibited the same alteration in their plasma DNA. No alterations were found in the plasma DNA of the remaining 108 patients. As a control, we screened for aberrant methylation in the plasma DNA of 60 health individuals. No methylation was found in plasma DNA of these control groups. Follow-up analysis indicated significantly lower DFS for patients with high *MSH2* methylation compared to those with *MSH2* unmethylation after surgery.
Conclusions: It was suggestted that *MSH2* methylation in the plasma would be a good predictor of DFS for these ESCC patients before oesophagectomy.

Keywords: MSH2; Methylation; Real-time methylation-specific PCR (real-time MSP); Disease-free survival (DFS); Esophageal squamous cell carcinoma (ESCC)

Background

Esophageal cancer (EC) is a major cause of cancer-related mortality worldwide.[1] ESCC is the predominant histological subtype of EC worldwide comprising almost 95% of cases, which is characterized by a high mortality rate and regional variation in incidence in China.[1–4] Despite advances in diagnostic methods and combined treatment modalities, the majority of patients with ESCC are diagnosed at advanced stages and the overall 5-year survival rate remains 40%.[5–7]

Although multiple genetic and epigenetic alterations have been detected in ESCC,[8] the precise molecular mechanisms of carcinogenesis and progression of ESCC remain unknown. Epigenetics is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence. Examples of such changes might be DNA methylation or histone deacetylation, both of which serve to suppress gene expression without altering the sequence of the silenced genes.[9] Aberrant DNA methylation is an important epigenetic alteration that is intimately involved in the silencing of DNA mismatch repair (MMR) genes.[10,11] Methylation of the promoters of the major MMR genes (*MLH1*, *MSH2*, and *MSH3*) and subsequent loss of gene expression occur frequently in primary gastric carcinoma, colorectal carcinoma, etc.[10–13] However, very little is known about the status of the mismatch repair genes and their impact on esophageal cancer. In this study focuses on evaluating the incidence of epigenetic aberrations in *MSH2* genes and its clinical impact on the development of ESCC in a middle incidence area of China.

* Corresponding authors. Zhejiang Cancer Research Institute, Zhejiang Province Cancer Hospital, No. 38 Guangji Rd., Banshanqiao District, Hangzhou 310022, PR China. Tel.: +86 571 88122423; fax: +86 571 88122587.
E-mail addresses: lingzq@hotmail.com (Z.Q. Ling), maowm1218@163.com (W.M. Mao).

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Materials and methods

Patients

The Institutional Review Board on Medical Ethics, ZhejiangProvince Cancer Hospital approved the method of tissue collection including informed consent. The study was based on 209 patients who underwent oesophagectomy for sporadic ESCC without preoperative radio- or chemotherapy. Two hundred nine ESCC specimens were collected prospectively from patients in Zhejiang Province Cancer Hospital between Dec 2004 and Jul 2010. The median age of the patients was 60 years (range, 40–85; 184 male and 25 female). All tumor samples were identified histopathologically as ESCC by pathologists. The disease stage of the ESCC cases was classified according to the World Health Organization (WHO) and the Tumor-Node-Metastasis (TNM) classification criteria of the International Union Against Cancer (UICC)[14]: 66 were stage I/II, and 143 were stage III/IV (Table 1). Representative tissue samples from tumors and matching morphologically normal esophageal epithelium tissues (non-tumor) at 6–10 cm away from the tumors were sampled during surgery in each patient. All samples were fixed in 10% formalin for histological assessment and for DNA extraction. Two hundred nine corresponding plasma samples were obtained prior to surgery. Sixty plasma samples from normal volunteers with the age and sex matched were also collected as control. The patients were followed up until April 30, 2011.

Table 1
Clinicopathological correlations of *MSH2* hypermethylation in primary ESCC tissues.

Variables	Number of cases	Methylation status			
		Absent (n = 108) U	Present (n = 101) M	X^2	p-value[a]
Age					
≤60	115	91	24	77.185	0.000
>60	94	17	77		
Sex					
Male	184	94	90	0.213	0.676
Female	25	14	11		
Localization					
Upper-Mid	123	67	56	0.936	0.399
Lower	86	41	45		
Grade					
High-Mid	160	82	78	0.049	0.871
Low	49	26	23		
Tumor status					
T1/T2	50	22	28	1.550	0.257
T3/T4	159	86	73		
Nodal status					
N0	72	34	38	0.872	0.384
N1	137	74	63		
Metastasis					
M0	171	105	66	35.648	0.000
M1	38	3	35		
Stage					
I/II	65	34	31	0.015	1.000
III/IV	144	74	70		

Correlation between clinical characteristics of ESCC patients with tumor *MSH2* methylation status.
[a] Fisher's exact test.

Cell preparation by needle for DNA extraction

We used 27-G needles to collect the target cells from 209 primary ESCC cases. After hematoxylin and eosin (HE) staining, tumor cells and the corresponding normal esophageal epithelia were taken from 2 to 3 consecutive 7-μm-thick paraffin-embedded sections fixed in 70% ethanol, using a 27-G needle, and treated with 40 μl of 200 μg/ml proteinase K (Sigma–Aldrich) at 42 °C for 72 h. We captured a total of approximately 3000–5000 tumor cells for DNA extraction. For each sample, DNA was extracted and purified by standard phenol/chloroform methods; after centrifugation the pellet was dissolved in 15 μl TE buffer (tris HCL [pH 8.0] and 0.2 ml EDTA [0.5 M], pH 8.0). DNA in plasma samples was extracted using QIAamp DNA Blood Mini Kit (Qiagen Co., Germany).

Bisulfite treatment and real-time MSP analysis

Genomic DNAs were treated with bisulfite to convert all unmethylated cytosines to uracils, while leaving methylated cytosines unaffected. Briefly, 1–2 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite, then purified with phenol/chloroform. The primers for the methylated (M) and unmethylated (U) promoter regions were as follows: M_(F) 5′-GGT TGT TGT GGT TGG ATG TTG TTT-3′, (R) 5′-CAA CTA CAA CAT CTC CTT CAA CTA CAC CA-3′; U_(F)5′-TCG TGG TC G GAC GTC GTT C-3′, (R) 5′-CAA CGT CTC CTT CGA CTA CAC CG -3′. Bisulfite- modified DNA was used for real-time MSP using an SYBR *Premix Ex Taq* kit (TaKaRa, Japan) on the ABI7500 PCR system (Applied Biosystems). All samples were analyzed with primer sets for both methylated and unmethylated DNA. The relative amount of methylation in each unknown sample was calculated as %M as reference.[13] Human genomic DNA (Clontech, Palo Alto, CA) treated in vitro with *Sss*I methylase (New England Biolabs, Inc., Beverly, MA) was used as a positive control. PCR products were further confirmed on 2% agarose gels with ethidium bromide and visualized under ultraviolet illumination.

Immunohistochemical analysis of MSH2 protein

Immunohistochemical analysis of *MSH2* (mouse anti-human monoclonal antibodies, SANTA CRUZ Inc.) was performed with the diaminobenzidine (DAB) chromogenic method. The results were evaluated by the proportion of positive cells and the intensity of the color. The scoring of the positive cell proportion was as follows: <5% was scored as 0 points; 5%–25%, 1 point; 51%–75%, 3 points; and >76%, 4 points. The intensity of the