

• ESOPHAGEAL CANCER •

Loss of myeloid-related proteins 8 and myeloid-related proteins 14 expression in human esophageal squamous cell carcinoma correlates with poor differentiation

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Abstract

AIM: To study the expression of myeloid-related proteins (MRP)8 and myeloid-related proteins(MRP)14 in human esophageal squamous cell carcinoma and to investigate if there was any correlation between MRP8 and MRP14 expression level and histopathological grade in these tumors.

METHODS: In this study, 65 cases of advanced esophageal squamous cell carcinoma were assessed for MRP8 and MRP14 expression using immunohistochemistry. Statistical analysis was performed for the comparison of MRP8 and MRP14 expression in normal and tumor tissues, and their relationship with clinicopathological features.

RESULTS: Reduced or absent expression of MRP8 and MRP14 was observed in esophageal squamous cell carcinoma, with a significant difference between tumor tissues and normal tissues ($P<0.01$ and $P<0.01$ for MRP8 and MRP14, respectively). Poorly differentiated tumors presented a greater decrease than well and moderately differentiated tumors, with a correlation between their protein level and histopathological grading ($P<0.001$ and $P<0.001$, respectively). However, no significant association was found between MRP8 and MRP14 expression and age or gender ($P>0.05$).

CONCLUSION: These findings suggest that the decreased expression of MRP8 and MRP14 might play an important role in the pathogenesis of human esophageal squamous cell carcinoma, being particularly associated with poor differentiation of tumor cells.

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INTRODUCTION

Human esophageal squamous cell cancer (ESCC) is one of the most frequent cancers with a predominant distribution in North China, where the mortality rate ranks second^[1]. A wide variety of biological events and mechanisms appear to have roles in the development and progression of ESCC^[2-4]. Deregulation of differentiation is another hallmark of multi-step carcinogenesis^[5]. Recent studies have demonstrated that the disruption of normal squamous cell differentiation may be one of the mechanisms for esophageal cancer development^[6]. In consequence, the defect in the pathway of terminal differentiation is clearly one of the most important abnormalities in esophageal carcinogenesis. In the majority of ESCCs some differentiation-associated mechanisms must be involved to explain the early events leading to the induction of the neoplastic phenotype.

Human esophageal mucosa is lined by a stratified squamous epithelium and its differentiation is a multistep and highly heterogenous process requiring activation and deactivation of multiple and specific genes^[7,8]. Therefore it is worthwhile to investigate the tissue-specific molecules involved in the process of differentiation during esophagus tumorigenesis. Systematic approaches using microarray-based global transcriptome analysis might provide a powerful alternative with an unprecedented view scope in monitoring gene expression levels^[9]. By analyzing our cDNA microarray data, we have recently identified MRP8 and MRP14 as two down-regulated and differentiation-associated genes in a significant proportion of ESCCs^[10,11].

Myeloid-related protein 8 (MRP8; S100A8) and MRP14 (S100A9) are two calcium-binding proteins belonging to the S100 family^[12]. These proteins expressed during myeloid differentiation, are abundant in granulocytes and monocytes, and form a heterodimeric complex calprotectin in a Ca^{2+} -dependent manner^[13,14]. MRP8 and MRP14 also show a wide range of possible intracellular as well as extracellular functions. They have been shown to inhibit casein kinases I and II, to interact with cytoskeletal components to exert antimicrobial properties, especially against *Candida albicans*, to be involved in transcellular eicosanoid metabolism and to exhibit growth inhibitory activities against murine bone marrow cells, macrophages, and mitogen-stimulated lymphocytes^[15]. Typically, MRP8 and MRP14 are known to be differentially expressed at sites of acute and chronic inflammation^[16-19]. However, there is sparse information regarding the deregulation of MRP8 and/or MRP14 in several human common malignancies^[20-28]. And little is known about the possibility of abnormal expression of MRP8 and MRP14 in ESCC.

In this study, we investigated the expression of MRP8 and MRP14 immunohistochemically in a set of human esophageal squamous cell carcinoma tissues. We also evaluated the relationship between their expression level and clinicopathological features. Our data suggest that their down-regulation is an important event during ESCC progression, and may be involved in the dedifferentiation of neoplastic cells.

MATERIALS AND METHODS

Tissue samples

Sixty-five specimens of ESCC and adjacent normal mucosa were taken from patients who had not received radiotherapy or chemotherapy before surgery. Fresh samples were dissected manually to remove mixed connective tissues and stored in liquid nitrogen immediately after operation at the Cancer Hospital of Chinese Academy of Medical Sciences and Peking Union Medical College. The clinicopathological characteristics were evaluated by two senior pathologists according to the criteria of the WHO classification (1990).

Antibodies

Following antibodies were used in this study: anti-MRP8 and anti-MRP14 polyclonal antibodies (C-19, Santa Cruz Biotechnology Inc. Santa Cruz, CA). These antibodies were provided as goat polyclonal antibody against MRP8 and MRP14, respectively (Santa Cruz, CA) and were characterized extensively by Western blotting and enzyme-linked immunosorbent assays.

Immunohistochemical analysis

For immunohistochemical analysis, 5 μ m thick sections were cut from formalin-fixed paraffin-embedded tissue blocks, deparaffinized, rehydrated, dripped in 30 mL/L hydrogen peroxide solution for 15 min. Before staining, antigen retrieval was performed by heating the specimens in a microwave oven for 20 min in citrate buffer (pH 8.0). Then excess protein was blocked using normal rabbit serum for 30 min at room temperature. Goat anti-MRP8 polyclonal antibody and goat anti-MRP14 antibody were respectively applied to the sections at a 1:150 dilution, and sections were then incubated overnight at 4 °C. A streptavidinbiotin peroxidase detection system (Zymed Laboratories Inc. Francisco, USA) was used according to the manufacturer's instructions. 3,3'-diaminobenzidine was used as the chromogen and hematoxylin was used as the counterstain. Primary antibody was replaced by non-immune serum (Zymed Laboratories Inc. Francisco, USA) in the case of negative control^[29]. All slides were examined and scored independently

by two senior pathologists who were blinded to the pathological and clinical data, and a consensus was obtained between them.

Statistical analysis

The relationship of patients' demographic features, such as age and gender, to MRP8 and MRP14 expression was examined with the Fisher's exact test. The Chi-square test was used for the comparison of MRP8 and MRP14 expression levels between ESCC and the adjacent normal esophageal mucosa, and among different histopathological grades, including well, moderately and poorly differentiated ESCC. All data were analyzed using SigmaStat software (Jandel Scientific, San Rafael, CA, USA). Differences were considered statistically significant when *P*-values were less than 0.05.

RESULTS

Patients and tumor characteristics

In this study, we investigated 65 esophageal squamous cell carcinoma patients comprising 46 males and 19 females. The mean age of patients was 57.7 years and the median age was 57 years (between 38 and 75). Tumors were graded according to the World Health Organization classification: 30 tumors were well differentiated, 23 were moderately differentiated, and 12 were poorly differentiated.

MRP8 and MRP14 expression in ESCCs and matched normal esophageal mucosa

To verify the down-regulation of MRP8 and MRP14 in ESCC, we performed an immunohistochemical analysis on 65 advanced esophageal cancer specimens. In matched non-neoplastic esophageal epithelium, staining for both MRP8 and MRP14 started in the deepest suprabasal cells and increased in intensity toward the superficial layers. Staining was most pronounced in apical, more differentiated epithelial layers, but no staining was observed in columnar epithelial cells in the stratum basale (Figures 1A and 2A). Prominent MRP8 and MRP14 expression was also observed in monocytes and granulocytes. Of the 65 ESCC cases analyzed, MRP8 and MRP14 immunostaining was

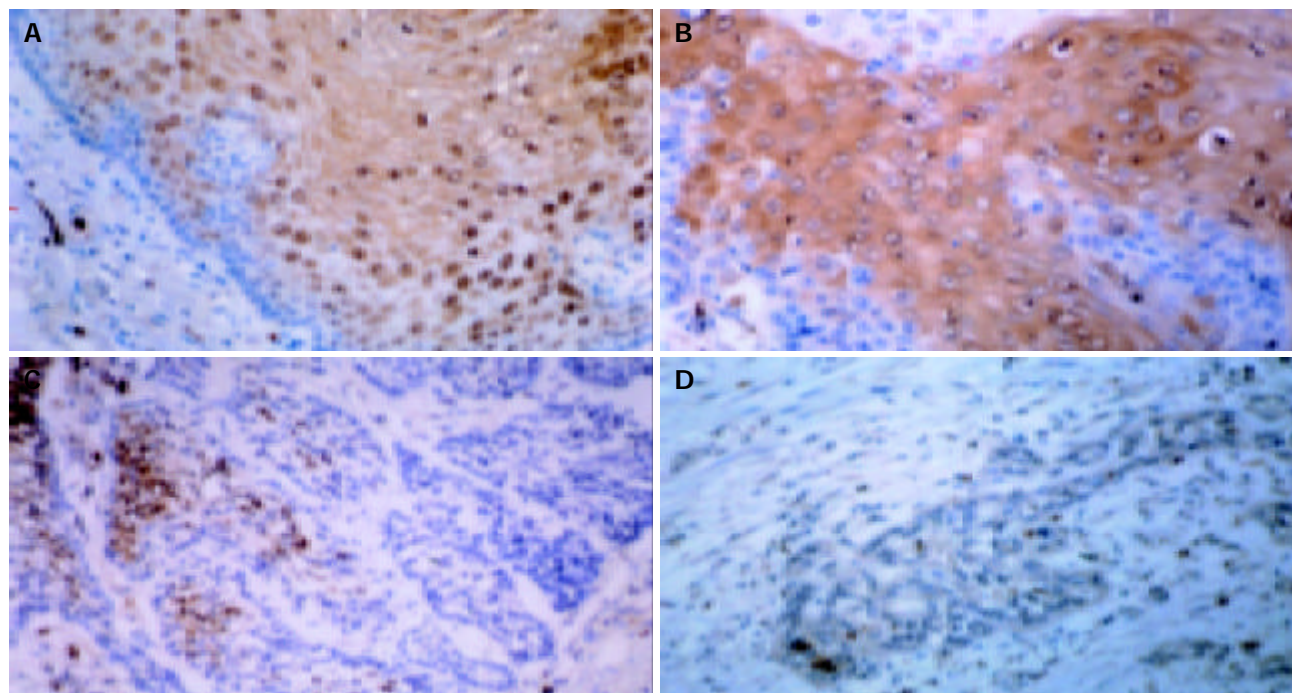


Figure 1 Immunohistochemistry for MRP8 in esophageal carcinoma tissues and normal epithelia (original magnification: $\times 400$). A: MRP8 expression in suprabasal layers of normal esophageal epithelium. B: MRP8 staining in well differentiated carcinoma, C: MRP8 staining in moderately differentiated carcinoma, D: MRP8 staining in poorly differentiated carcinoma.

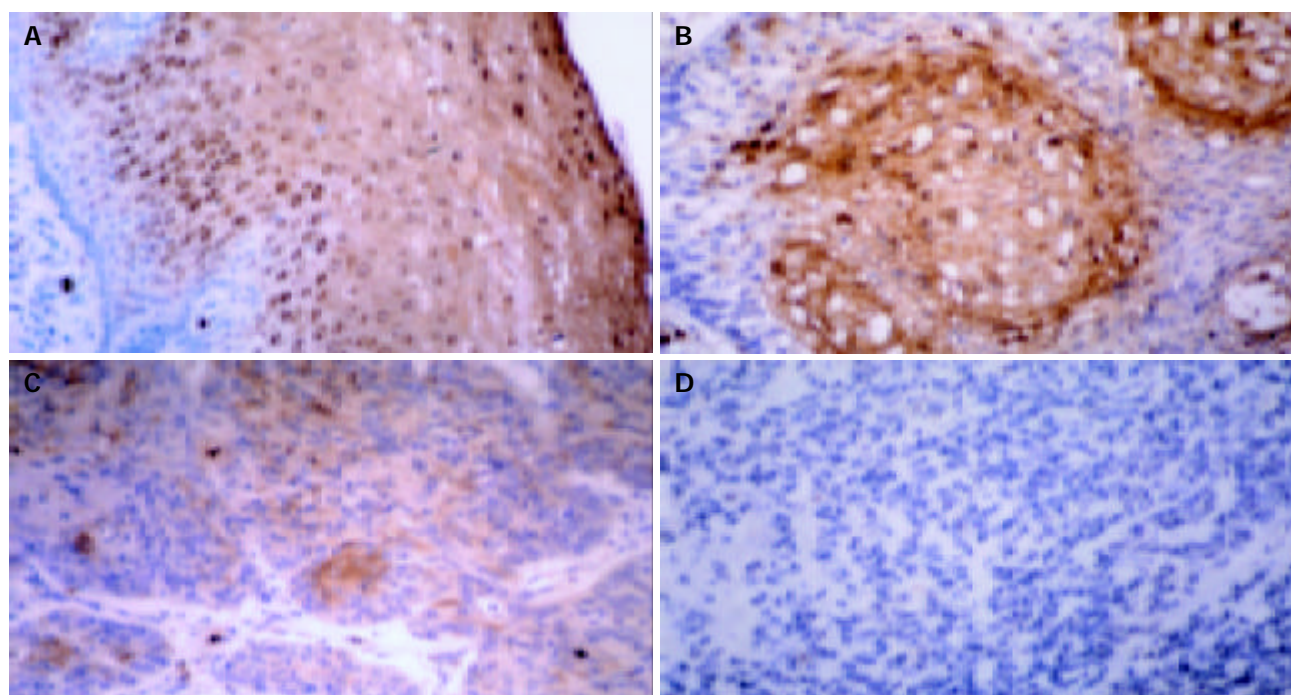


Figure 2 Immunohistochemistry of MRP14 in normal esophageal epithelium (A) and well (B), moderately (C) and poorly differentiated (D) carcinoma tissues. Normal esophageal epithelium showed strongly positive staining for MRP14. However, its staining in poorly differentiated carcinoma was significantly weaker than that in well and moderately differentiated carcinomas. Granulocytes and monocytes were also positively stained in all the sections. Original magnification: $\times 400$.

significantly reduced compared to the adjacent benign epithelia. The majority of tumors showed focal positive immunostaining in certain well differentiated areas, and undetectable in other less differentiated sections. In well differentiated carcinomas, the staining for MRP8 and MRP14 was positive in keratinized areas at the center of tumor foci, but decreased or undetectable in the marginal areas. However, in moderately and poorly differentiated carcinomas, the staining was sparsely weak or sporadic only in the well or moderately differentiated regions, whereas in other areas it was completely undetectable. The immunostaining was frequently heterogeneous even within one specimen, both in terms of percentage of positive cells and staining intensity (Figures 1B-D and 2B-D). The staining for MRP8 and MRP14 was predominant in the cytoplasm of benign cells and accumulated more in the nuclei of malignant cells. In surrounding stroma clear MRP8- and MRP14-positive monocytes and granulocytes could also be observed.

Relationship between MRP8 and MRP14 expression and clinicopathological characteristics in ESCCs

We further examined the relationship between MRP8 and MRP14 expression and clinicopathological findings in SCCs of the esophagus. Table 1 compares the expression of MRP8 and MRP14 in ESCC and matched non-neoplastic esophageal tissues. MRP8 was positive in 61 (93.8%) of 65 normal mucosae detected and in 49 (75.4%) of 65 ESCCs. MRP14 was found in all the normal mucosae detected and in 54 (83.1%) of 65 ESCCs. Among ESCC cases, grade 0 staining, which means lack of expression in tumor tissues, was recorded in 24.6% for MRP8 and 16.9% for MRP14 of ESCC. In total, 9.2% and 13.8% of ESCC cases had grade 1+ for MRP8 and MRP14, respectively. Percentage of moderate staining for MRP8 and MRP14 was 44.6% (MRP8) and 32.3% (MRP14). And 21.5% and 36.9% of cases showed strong staining for MRP8 and MRP14. In comparison with ESCC tissue, the matched non-neoplastic esophageal tissue showed 44.6% (29 of 65) and 92.3% (60 of 65) strong staining for MRP8 and MRP14, respectively. These results indicated a significant reduction of

MRP8 and MRP14 expression in malignant *versus* benign tissue (MRP8: $P < 0.01$, MRP14: $P < 0.01$).

Table 1 Summary of immunohistochemical analysis of MRP8 and MRP14 in esophageal epithelium and tumors. (Fisher's exact test)

Expression Level ¹	MRP8			MRP14		
	Normal (%)	Tumors (%)	P-value	Normal (%)	Tumors (%)	P-value
3+	29(44.6)	14(21.5)	$<0.01^b$	60(92.3)	24(36.9)	$<0.01^d$
2+	32(49.2)	29(44.6)		3(4.6)	21(32.3)	
1+	0(0)	6(9.2)		0(0)	9(13.8)	
0	4(6.2)	16(24.6)		2(3.1)	11(16.9)	

¹3+, $\geq 50\%$; 2+, $\geq 5\%$ and $<50\%$; 1+, $<5\%$; 0, negative; ^b $P < 0.01$ vs normal tissues; ^d $P < 0.01$ vs normal tissues.

Table 2 Relationship between MRP8 and MRP14 immunoreactivity and clinicopathologic factors. (Chi-square Fisher exact test)

	Case Number (n)	MRP8 (+)	P value	MRP14 (+)	P value
Age (yr)					
≤ 57	35	25	NS	27	NS
> 57	30	21		25	
Gender					
Male	46	34	NS	37	NS
Female	19	11		13	
Differentiation					
Well	30	29	$<0.001^b$	30	$<0.001^b$
Moderately	23	16		20	
Poorly	12	4		4	

^b $P < 0.001$ between MRP8 and MRP14 expression and histological grade, respectively.

In addition, we examined the correlation between MRP8 and MRP14 expression and clinicopathological features, and the

results are summarized in Table 2. According to clinicopathological grade, 65 primary ESCCs were classified into three groups: well moderately and poorly differentiated ESCC, respectively. To determine whether the altered expression of MRP8 and MRP14 had additional implications for poor differentiation, we performed Chi-square test in the three groups of ESCCs. There was a significant difference in the expression levels of MRP8 and MRP14 among different differentiation grades, being higher in the well differentiated tumors than in moderately and poorly differentiated tumors (MRP8: $P < 0.001$; MRP14: $P < 0.001$). However, their expression did not correlate with the other parameters such as age and gender ($P > 0.05$).

DISCUSSION

Squamous cell differentiation required the coordinated activation and repression of genes specific to the differentiation constitutes, and disruption of this program accompanied neoplasia^[30]. A better understanding of the factors and mechanisms regulating differentiation and of their disturbance in carcinogenesis would offer new possibilities to design novel tumor therapeutic strategies in the field of differentiation therapy^[5]. According to our cDNA microarray analysis, *MRP8* and *MRP14* were two genes in the down-regulated and differentiation-associated gene cluster, and their genetic alterations might contribute to esophageal tumorigenesis^[10,11]. However, information is limited regarding the possible biological significance of the altered expression of MRP8 and MRP14 during ESCC development. In this study, we revealed frequent loss of MRP8 and MRP14 expression in the majority of ESCCs and a significant correlation between MRP8 and MRP14 expression and clinicopathological parameters of ESCC.

MRP8 and MRP14 were preferentially expressed in the normal esophageal epithelia and lowly expressed in ESCCs. Previous reports have subsequently shown that the deregulation of MRP8 and/or MRP14 was associated with several common human malignancies, including carcinomas of the skin, stomach, colon, nasopharynx, lung, liver and anus, and follicular lymphoma as well^[20-28]. Therefore, our results further suggest that reduced expression of MRP8 and MRP14 in ESCC cells, but not in normal cells, plays an important role in esophageal carcinogenesis. It is interesting to note that MRP8 and MRP14 staining was decreased in poorly and moderately differentiated ESCCs when compared with well-differentiated ESCCs. These data suggest that loss of MRP8 and MRP14 expression in ESCC generally occurs along with worsening esophageal epithelial differentiation in histological grade. Identification of the grade of tumor malignancy would facilitate treatment strategies and provide important information for predicting the prognosis^[31]. To our knowledge, this is the first report on the relationship between MRP8 and MRP14 expression and histopathological grade of ESCC.

Although the fundamental role of MRP8 and MRP14 in myeloid differentiation and keratinocyte hyperproliferation is well established^[32,33], the detailed molecular processes that lead to down-regulation of MRP8 and MRP14 in esophageal epithelium during de-differentiation processes remain to be elucidated. Accumulation of MRP8 and MRP14 may play an important role in maintaining the differentiated status of well-differentiated ESCC, and preventing less differentiated ESCC. It is noteworthy that MRP14 expression level has a positive correlation with the differentiation degree of carcinomas derived from glandular cells, such as hepatocellular carcinoma, cholangiocellular carcinoma and pulmonary adenocarcinoma. In poorly differentiated adenocarcinoma (AC) cases, immunoreactivity of MRP8 and MRP14 was significantly higher than that in the moderately and well differentiated AC cases^[24,26]. These differences might reflect the different pathways or mechanisms that MRP8 and MRP14 were

involved in tumorigenesis of different cell origin.

The identification of MRP8 and MRP14 in esophageal epithelial cells may facilitate the elucidation of molecular mechanism of esophageal carcinogenesis. In normal human esophageal epithelia, MRP8 and MRP14 were expressed in regions containing differentiated cells but not in regions containing actively dividing cells, suggesting that MRP8 and MRP14 may be upregulated in the cellular differentiation status. Indeed, MRP8 and MRP14 were expressed in a tissue/cell-specific and differentiation-dependent manner, especially in the differentiation of monomyelocytes and keratinocytes^[34]. Warner-Bartnicki *et al* have reported that the expression of MRP8 and MRP14 was upregulated during mycophenolic acid and $1\alpha, 25$ -dihydroxyvitamin D₃-mediated differentiation of HL-60 leukemia cells^[35]. Similarly, terminal differentiation of various epithelial cells was also linked to their constitutive expression^[17]. Consistent with these notions, our observation of their spatial and temporal distribution in esophageal epithelium also supported that MRP8 and MRP14 might collaboratively act to maintain differentiated progeny, playing a growth-suppressive and differentiation-associated role^[36]. Based on these findings, we would propose that MRP8 and MRP14 may be closely associated with the early events in the terminal differentiation of human normal esophageal epithelial cells and the epithelial expression of these proteins is thought to require a certain state of proliferation and/or differentiation.

The *MRP8* and *MRP14* genes, together with other 11 *S100* genes and epidermal differentiation complex (EDC) genes, have been mapped to human chromosome 1q21, which is structurally conserved during evolution. This region has revealed a most remarkable density of genes that fulfill important functions in terminal differentiation of the human epidermis^[37]. Among those genes, the expression of *S100A2*, *S100A4*, *S100A12*, *SPRR3*, *TGM-3* and other genes has also been identified to be altered in ESCCs^[38-42]. Within this chromosomal region, a number of abnormalities such as deletions, rearrangements or translocations were considered to be associated with neoplasia^[43,44]. Thus, 1q21 may be a region of the structural and numerical aberration involved in esophageal carcinogenesis and progression. Furthermore, the clustered organization has posed the question whether each gene is regulated by its own elements or by possible superior locus control elements as suggested for the EDC genes^[45].

In summary, reduced expression of MRP8 and MRP14 was observed in a majority of ESCCs, suggesting that their down-regulation is a common event in esophageal carcinogenesis and progression. In addition, there was a significant correlation between MRP8 and MRP14 expression and clinicopathological parameters involved in differentiation of ESCC. Poorly differentiated carcinomas showed markedly reduced expression than well and moderately differentiated carcinomas. Thus we conclude that MRP8 and MRP14 may play a significant role in the dedifferentiation process of esophageal neoplastic cells. Whether MRP8 and MRP14 expression is an important predictor for histological grade needs to be confirmed by clinical follow-up studies.

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