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# Expression of MRP14 gene is frequently down-regulated in Chinese human esophageal cancer

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## ABSTRACT

Migration inhibitory factor-related protein 14 (MRP14) is one of calcium-binding proteins, referred as S100A9. The heterodimeric molecule formed by MRP14 with its partner MRP8 (S100A8) is the major fatty acid carrier in neutrophils. The MRP8/14 complex has been also implicated in the intracellular transport of arachidonic acid and its precursors in keratinocytes. We show here the involvement of MRP14 in human esophageal cancer. In an initial study, mRNA differential display - reverse transcription polymerase chain reaction (DD-PCR) was performed with two esophageal carcinomas, one esophageal adenocarcinoma and matched normal adjacent mucosa. DD-PCR with the arbitrary primer OPA3 showed that one cDNA band was highly expressed in normal tissues, but disappeared or substantially decreased in tumor counterparts. It was later identified to be the 3'-end of migration inhibitory factor-related protein 14 (MRP14). Northern blotting, RT-PCR and Western blotting corroborated the down-regulation of MRP14 in 58/64 squamous cell carcinomas and 2/2 adenocarcinomas as compared with adjacent normal epithelia of the esophagus. MRP14 was undetectable in 3/3 esophageal-carcinoma cell lines. Immunochemistry demonstrated that expression of MRP14 was restricted to normal esophageal epithelia. No mutation was found in the genomic DNA of the MRP14 gene by PCR and directed DNA sequencing. Our finding suggested that the reduction of MRP14 expression is a frequent event in Chinese human esophageal cancer.

***Keywords***: esophagus, carcinoma, MRP14, S100A9.

## INTRODUCTION

Esophageal cancer is a common malignant solid tumor of digestive system. It ranks the fourth cause of cancer death in China and among the 10 most frequent cancers in the world[1, 2]. Mortality rates of esophageal cancer are very close to incidence rates. Mutation, loss and abnormal expression of some candidate genes, such as *Cyclin D1*, *Rb*, *p53* and *p16*, were detected in esophageal cancer [3-6]. However, few genes specially related to the disease has been found until now. Further searching for esophageal cancer-associated genes remains to be done for revealing the molecular and genetic basis of cancer development and progression.

In order to isolate the differentially expressed genes between esophageal carcinomas and normal epithelia of esophagus, we performed messenger RNA differential display reverse transcription - polymerase chain reaction (DD-PCR). Herein we report our findings that expression of MRP14 significantly decreased in human esophageal carcinomas as compared with the matched normal adjacent mucosa.

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## MATERIALS AND METHODS

### Sample collection

Fresh tumor tissues and matched adjacent normal mucosa were obtained at surgery. 13 cases were collected by the Pathology Department of Anyang Cancer Hospital, Henan Province, China, and the others, from Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. Primary tumor regions and corresponding adjacent normal esophageal mucosa from the same patients were separately excised by experienced pathologists, and immediately placed in liquid nitrogen until use.

### Cell lines and cell culture

Esophageal carcinoma cell lines, EC109, EC8712 and EC9706, were established in our laboratory[7-9]. The cell lines were main

tained in M199 medium with 15% FCS and cultured at 37°C, 5% $CO_2$.

## RNA extraction and cDNA synthesis

Tumor samples were gross-dissected to trim away non-tumor tissues, and matched adjacent normal mucosa was separated from underlying muscle layers. Total RNA was prepared using Trizol reagent (Gibco) according to the manufacturer's instructions. RNA was treated with DNase I for 30-40 min at 37°C, extracted with phenol/isopropanol, precipitated in 3*M* NaAc (pH5.2) overnight at 4°C, and dissolved in DEPC-treated water. 5 μg of each RNA was reverse transcribed with 200 units Superscript II reverse transcriptase (Gibco) in the presence of 50 μ*M* 3'-anchored oligo (dT) primer in 20 μl RT buffer (1×PCR buffer, 2.5 m*M* $MgCl_2$, 10 m*M* DTT, 0.5m*M* dNTP), at 65°C for 5 min, followed by cooling to 37°C for 10 min. Reverse transcriptase was inactivated at 70°C for 15 min.

## mRNA differential display

mRNA differential display (DD-PCR) was performed with a modification of the procedure described by Liang and Pardee [10]. Briefly, PCR amplification was done using 1μl of the cDNA, primed with mixed anchored primers (GT15N, N=A, C and G, equal mole) and a 10-mer arbitrary primer (Operon). The cycle parameters were 94°C for 4 min, 39°C for 4 min, 72°C for 2 min, and then 35 cycles of 95°C for 20s, 39°C for 2 min, 72°C for 1 min. A final extension was carried out at 72°C for 5 min. The resultant products were run on an 8% polyacrylamide gel electrophoresis and showed by silver staining. Identified bands were excised from the gel, and the cDNA was recovered by incubation at 37°C in 0.1×TE overnight.

## Cloning and sequencing of differential display cDNA

PCR-amplified cDNA were cloned into the pGEM-T easy vector (Promega). Plasmid DNA was prepared by using Wizard Miniprep Purification System (Promega). The sequence reactions were performed by TaKaRa Corp (Dalian city, China).

## Northern blot analysis

Total RNA was denatured in the presence of 50% (vol/vol) and 2.2*M* formaldehyde, subjected to electrophoresis on 1.2% agarose/ formaldehyde gel. Following electrophoresis, the RNA was transferred to nylon membranes (Bio-Rad) by capillary transfer in 10×SSC. Northern blots were prehybridized for 1 h in 1*M* EDTA, 0.25 *M* $Na_2HPO_4$ (pH7.2) and 7% SDS at 65°C. The probe was prepared by Prime-a-Gene Labeling system in the presence of [$^{32}$P]-dATP and [$^{32}$P]-dCTP. Hybridization was carried out at 65°C for 18 h, and blots were washed first for 20 min in 1m*M* EDTA, 40 m*M* $Na_2HPO_4$, 5% SDS at 65 °C, followed by wash in 1m*M* EDTA, 40 m*M* $Na_2HPO_4$, 1% SDS. Then the membrane was autographed at -70°C for 16 h.

## RT-PCR detection

The primers were designed according to the sequences of *MRP14* and *MRP8* mRNAs (GenBank No.: NM_002965 and NM_002964). The sense primer for *MRP14* was 5'-CAGCTGG AACGCAAC-ATAGA-3' and the antisense primer, 5'-CCACAGCCAAGACA-GTTTGA-3'. The sense/antisense primers for *MRP8* were 5'-TCTTGTCAGC-TGTCTTTCAGAAG-3' and 5'CAGCCTCTG-GGCATAACTC-3' repectively. 5 μg of total RNA was used to synthesize the first strand of cDNA using superscript II (Gibco). The PCR amplification was carried out in 15 μl reaction volume containing 0.5 μl cDNA template, 1×PCR buffer, 1.5 m*M* $MgCl_2$, 200 m*M* dNTPs, 0.5 μ*M* primer, and 1 unit Taq polymerase. After a 4 min denaturation at 94°C, PCR was performed for 28 cycles. Each cycle consisted of 94°C for 30s, 60°C for 30s and 72°C for 1 min, followed by a 72°C elongation for 6 min. 5μl of each PCR product was electrophoresised on a 1.5% agarose gel. *β-actin* or *GAPDH* was meantime amplified as control with the same templates. The up-stream/downstream primers of *β-actin* was 5'-CGTG-GACATCCGTAAAGACC-3' and 5'-ACATCTGCTGGAAGG TGGAC-3', and those of GAPDH, 5'-GACCACAGTCCA-TGCCATC-3' and 5'-ACCAGG AAATGAGCTTGACA-3'. The expected PCR products of *MRP14*, *MRP8*, *β-actin* and *GAPDH* were 475bp, 353bp, 209bp and 416bp, respectively.

## Western blot analysis

Tissues were disrupted with a cocktail of 8M urea, 4% CHAPS, 40 m*M* Tris, 1m*M* PMSF, 20 m*M* spermin, 2 μg/ml aprotinin, 2 m*M* tetrabutylammonium phosphate, Carrier Ampholyte, DNase I and RNase. Lysate was kept on ice 1 h before centrifugation at 12,000g for 30 min. The supernatants were transferred to Eppendorf tubes, stocking at –70°C until use. Bradford method was used to assay the proteins concentration.

Total protein (40 μg) was run on 12% SDS-polyacrylamide gels and then transferred to nitro cellulose membranes. Blots were blocked overnight and probed with affinity purified polyclonal antibodies against MRP14. After washed, the blots were incubated with horse-radish peroxidase- conjugated secondary antibodies and visualized with the Amersham enhanced chemiluminescence ECL system.

## Immunohistochemistry

A protein produced by prokaryotic expression was used to immunize rabbits to obtain a polyclonal antiserum to MRP14. The resulting high titer antiserum was employed as a primary antibody to stain formalin-fixed and paraffin-embedded sections of esophageal carcinomas. The immunohistochemistry signals were visualized with a peroxidase-linked assay system (ZYMED). Hematoxylin was used as a counterstain.

## Mutation analysis—Direct DNA sequencing

The promotor region, the first exon and the upstream part of the first intron of the MRP14 gene were amplified by two pairs of primers: P1a. 5'-CCCCAAATCTCACCCTATGA-3' (position -489), P1b. 5'-CCACACAGAGTGTTTGCCAG-3' (position +15), and P2a. 5'-TATAAATGCCGAGCCTGCAC-3' (position -30), P2b. 5'-GCCCCAGCTTCACAG AGTATT-3' (position +512). Polymerase chain reactions were performed in a 50 μl volume containing 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 50μ*M* dNTPs, a 0.4 μ*M* concentration of each primer, 100 ng of genomic DNA, and 5 unit of pyrobest (Takara, Japan) DNA polymerase. After a 10 min denaturation at 95°C, PCR was performed for 35 cycles. Each cycle consisted of 94°C for 30s, 60°C for 30s and 72°C for 45s, followed by a 72°C elongation for 5 min. 2μl of each PCR product was electrophoresised on a 1% agarose gel. PCR fragments

were purified by Wizard PCR preps DNA purification system (Promega), then were sequenced with the ABI PRISM BigDye terminator cycle sequencing ready reaction reagent set according to the manufacturer's recommendations in conjunction with an ABI PRISM 377 DNA sequencer.

## RESULTS

### Differential expression of MRP14 mRNA between esophageal carcinomas and adjacent normal esophageal mucosa

Differential display was performed on RNAs isolated from esophageal carcinomas and adjacent normal esophageal mucosa. In order to minimize false positive of differential display, three cases of specimens were simultaneously analyzed. Only those bands consistently altered in all three cases were further investigated. With the arbitrary primer OPA3, one cDNA band of about 120 bp was found barely expressed in cancer tissues while highly expressed in matched adjacent normal mucosa (Fig 1). The identified band were recovered, reamplified, cloned and sequenced. Databases searching showed that this cDNA fragment was the 3'-end of migration inhibitory factor-related protein 14 gene (*MRP14*).

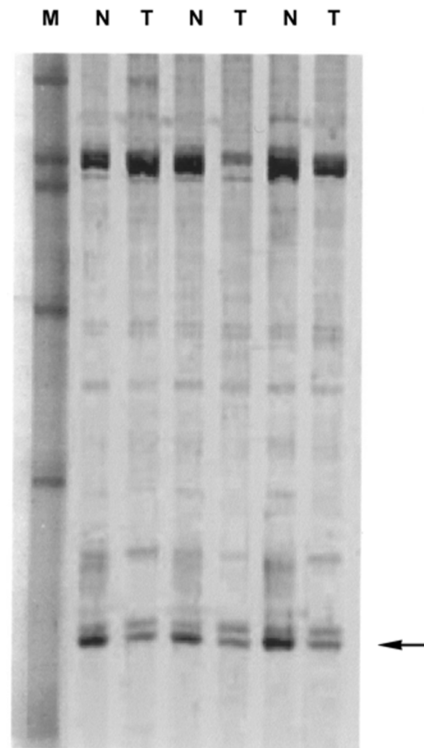


**Fig 1**. mRNA differential display of three esophageal carcinomas (T) and matched adjacent esophageal mucosa (N). Arrow indicates the recovered band. M-ϕX174 DNA/*Hae*III marker.

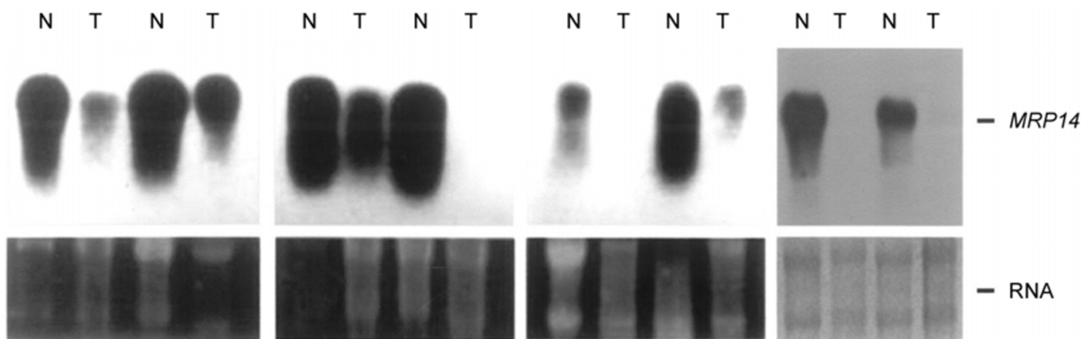


**Fig 2**. Northern blot analysis of *MRP14* in matched esophageal carcinomas (T) and adjacent histologically normal tissues (N).

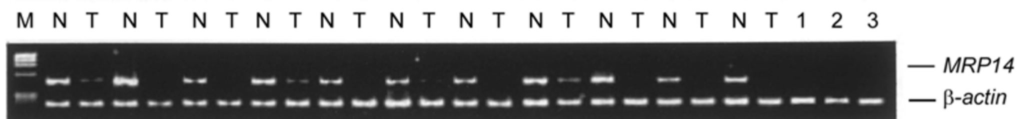


**Fig 3**. Representative RT-PCR analysis of *MRP14* in matched esophageal carcinomas (T) and adjacent histologically normal tissues (N). 1-3: Esophageal cancer cell lines EC109, EC8712 and EC9706; M-ϕX174 DNA/*Hae*III marker.

**Tab 1.** Basic clinical data and *MRP14* expression analysis of 66 esophageal tumors

| No. | Sample source[a] | Sex | Age | Tumor type[b] | TNM | Grade | MRP14 eduction | Analysis method[c] |
|---|---|---|---|---|---|---|---|---|
| 1 | H | M | 67 | AC | T3N1M0 III | G2 | Yes | DD, RT-PCR |
| 2 | H | M | 49 | SC | T3N0M0 IIa | G2 | Yes | RT-PCR |
| 3 | H | F | 65 | SC | T3N0M0 IIa | G3 | Yes | RT-PCR |
| 4 | H | F | 50 | SC | T3N0M0 IIa | G2 | Yes | RT-PCR |
| 5 | H | M | 70 | SC | T3N1M0 III | G3 | Yes | DD, RT-PCR |
| 6 | H | M | 58 | SC | T2N1M0 IIa | G3 | Yes | RT-PCR |
| 7 | H | F | 58 | SC | T2N1M0 IIb | G2 | Yes | RT-PCR |
| 8 | H | M | 58 | SC | T3N0M0 IIa | G2 | Yes | RT-PCR |
| 9 | H | M | 47 | SC | T3N1M0 III | G2 | Yes | RT-PCR |
| 10 | H | F | 50 | SC | T2N1M0 IIb | G2 | Yes | RT-PCR |
| 11 | H | M | 64 | SC | T3N1M0 III | G3 | Yes | RT-PCR |
| 12 | H | M | 49 | SC | T3N0M0 IIa | G2 | Yes | RT-PCR |
| 13 | H | F | 67 | SC | T2N0M0 IIa | G1 | Yes | DD, RT-PCR |
| 14 | L | F | 47 | SC | T3N0M0 IIa | G1 | No | RT-PCR |
| 15 | L | M | 60 | SC | T3N1M0 III | G1 | Yes | RT-PCR |
| 16 | L | F | 62 | SC | T3N0M0 IIa | G2 | Yes | N, RT-PCR |
| 17 | L | M | 63 | SC | T3N0M0 IIa | G2 | Yes | RT-PCR |
| 18 | L | F | 60 | SC | T3N0M0 IIa | G2 | Yes | RT-PCR |
| 19 | L | M | 45 | SC | T3N0M0 IIa | G1 | No | RT-PCR |
| 20 | L | M | 40 | SC | T3N1M0 III | G1 | Yes | RT-PCR |
| 21 | L | M | 75 | SC | T3N0M0 IIa | G1 | Yes | RT-PCR |
| 22 | L | F | 55 | AC | T3N1M0 III | G2 | Yes | RT-PCR |
| 23 | L | M | 66 | SC | T3N1M0 III | G1 | Yes | N, RT-PCR |
| 24 | L | M | 60 | SC | T3N1M0 III | G1 | Yes | RT-PCR |
| 25 | L | F | 53 | SC | T3N0M0 IIa | G3 | Yes | RT-PCR |
| 26 | L | M | 64 | SC | T3N1M0 III | G3 | Yes | N, RT-PCR |
| 27 | L | M | 56 | SC | T3N1M0 III | G2 | Yes | RT-PCR |
| 28 | L | M | 45 | SC | T3N1M0 III | G3 | Yes | RT-PCR |
| 29 | L | M | 50 | SC | T3N0M0 IIa | G1 | Yes | RT-PCR |
| 30 | L | M | 71 | SC | T3N0M0 IIa | G1 | Yes | N, RT-PCR |
| 31 | L | M | 38 | SC | T3N0M0 IIa | G2 | Yes | N, RT-PCR |
| 32 | L | M | 50 | SC | T3N1M0 III | G2 | Yes | RT-PCR |
| 33 | L | M | 58 | SC | T3N1M0 III | G2 | Yes | RT-PCR |
| 34 | L | M | 54 | SC | T3N1M0 III | G1 | No | RT-PCR |
| 35 | L | M | 54 | SC | T2N0M0 IIa | G1 | Yes | RT-PCR |
| 36 | L | F | 50 | SC | T2N1M0 IIb | G1 | Yes | RT-PCR |
| 37 | L | F | 59 | SC | T2N0M0 IIa | G1 | Yes | N, RT-PCR |
| 38 | L | M | 70 | SC | T3N1M0 III | G2 | Yes | RT-PCR |
| 39 | L | M | 49 | SC | T2N1M0 IIb | G3 | Yes | RT-PCR |
| 40 | L | M | 52 | SC | T3N0M0 IIa | G1 | No | RT-PCR |
| 41 | L | M | 51 | SC | T3N1M0 III | G2 | Yes | RT-PCR |
| 42 | L | M | 38 | SC | T4N1M0 III | G3 | Yes | RT-PCR |
| 43 | L | F | 50 | SC | T3N0M0 IIa | G1 | Yes | RT-PCR |
| 44 | L | M | 55 | SC | T4N1M0 III | G2 | Yes | RT-PCR |
| 45 | L | M | 70 | SC | T3N1M0 III | G3 | Yes | RT-PCR |
| 46 | L | M | 72 | SC | T3N0M0 IIa | G1 | Yes | RT-PCR |
| 47 | L | M | 48 | SC | T3N1M0 III | G2 | Yes | RT-PCR |
| 48 | L | M | 65 | SC | T4N1M0 III | G2 | Yes | RT-PCR |
| 49 | L | M | 63 | SC | T3N1M0 III | G2 | Yes | RT-PCR |
| 50 | L | M | 62 | SC | T3N0M0 IIa | G1 | Yes | RT-PCR |
| 51 | L | M | 56 | SC | T3N0M0 IIa | G3 | Yes | RT-PCR |
| 52 | L | M | 55 | SC | T3N0M0 IIa | G1 | Yes | RT-PCR |
| 53 | L | M | 68 | SC | T3N1M0 III | G1 | Yes | RT-PCR |
| 54 | L | M | 64 | SC | T3N0M0 IIa | G1 | No | RT-PCR |
| 55 | L | M | 60 | SC | T4N0M0 III | G2 | Yes | RT-PCR |
| 56 | L | F | 73 | SC | T3N0M0 IIa | G2 | Yes | RT-PCR |
| 57 | L | M | 47 | SC | T3N1M0 III | G1 | No | RT-PCR |
| 58 | L | F | 57 | SC | T3N0M0 IIa | G2 | Yes | RT-PCR |
| 59 | L | M | 67 | SC | T4N1M0 III | G1 | Yes | RT-PCR |
| 60 | L | M | 40 | SC | T2N1M0 IIb | G2 | Yes | RT-PCR |
| 61 | L | M | 78 | SC | T3N0M0 IIa | G2 | Yes | N |
| 62 | L | M | 64 | SC | T3N0M0 IIa | - | Yes | N |
| 63 | L | M | 55 | SC | T3N1M0 III | G2 | Yes | W |
| 64 | L | M | 64 | SC | T3N1M0 III | G1 | Yes | W |
| 65 | L | M | 77 | SC | T2N1M0 IIb | - | Yes | W |
| 66 | L | M | 56 | SC | T3N0M0 IIa | G1 | Yes | W |

Notes: [a]H-high incidence area of Anyang, Henan province, China, L-low incidence area of Beijing, China, respectively; [b]SC-squamous cell carcinomas, AC-adenocarcinomas; [c]DD-differential display, N-Northern blot, W-Western blot.

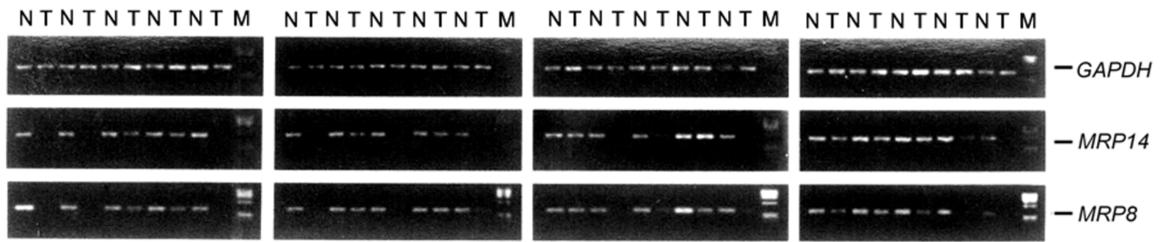


**Fig 4.** Simultaneous RT-PCR analysis of both *MRP14* and *MRP8* in matched esophageal carcinomas (T) and adjacent histologically normal tissues (N). M-ϕX174 DNA/*Hae*III marker.

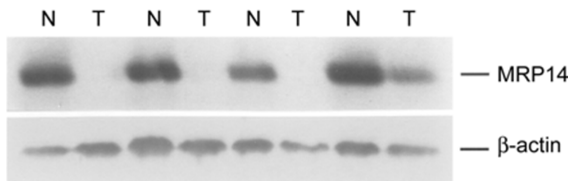


**Fig 5**. Decreased expression of MRP14 protein in esophageal cancer confirmed by Western blotting. Four cases of esophageal cancerous (T) and matched normal tissues (N) were analyzed using a polyclonal antibody against MRP14. Normalization of protein load was performed using an anti-β-actin antibody.

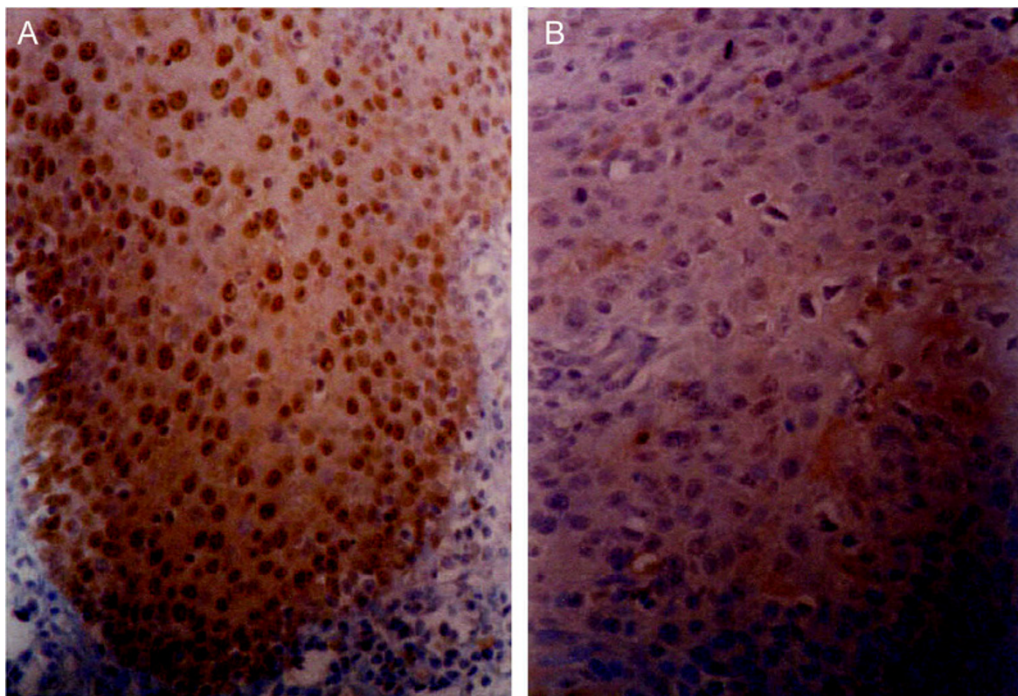


**Fig 6**. Immunohistochemistry staining of MRP14. Note that MRP14 (brown) highly expressed in histologically normal esophageal epithelium (**A**), but feebly stained in tumor tissues (**B**). 125×.

## Expression of *MRP14* were frequently decreased in esophageal cancer

For confirming the results from differential display and the repression of *MRP*14 in esophageal cancer, we carried out a pilot Northern-blot assay. The hybridization probe was prepared by PCR with the same primers as used in reverse transcription - polymerase chain reaction. Northern blotting showed that *MRP14* were preferentially expressed in 8/8 normal esophageal tissues and dramatically down-regulated in all the carcinoma counterparts (Fig 2).

The results of Northern blotting prompted us to further analyze the clinical significance of the MRP14 gene by reverse transcription - polymerase chain reaction (RT-PCR). Of the samples tested, 52/58 squamous cell carcinomas and 2/2 adenocarcinomas presented a decrease expression in comparison with the adjacent normal mucosa (Tab 1, Fig 3). MRP14 was undetectable in 3/3 esophageal-carcinoma cell lines. There was no statistically significant

correlation between MRP14 expression and gender, age, tumor grade or stage, and lymph node metastases. In addition, 20 pairs of cancer/normal tissues were analyzed by RT-PCR of both *MRP14* and *MRP8*. Simultaneous down-regulation of these two genes was observed in 16 cases of tumors (Fig 4).

Western blot analysis was performed in four esophageal tumors and matched normal epithelia. Complete or substantial loss of MRP14 was observed in all four tumors examined, whereas in the adjacent normal epithelium, MRP14 presented high expression (Fig 5). Putting together the results from differential display, RT-PCR, Northern blot and Western blot analysis, MRP14 expression was lost in 3/3 esophageal-carcinoma cell lines and decreased dramatically in 60/66 esophageal carcinomas compared to normal tissues.

Immunohistochemistry showed that MRP14 expressed in normal esophageal epithelia adjacent to tumor tissue, especially with strong staining in the nuclei (Fig 6). No signal was detected in connective cells and muscle cells. Esophageal tumor tissues presented no or very feeble staining, thus confirmed that expression of MRP14 was reduced in esophageal cancer as identified by Northern blot and RT-PCR analysis.

### No genomic mutation was detected for *MRP14* in tumor tissues and carcinoma cell line of the esophagus

In order to investigate possible mutations of the promotor region, the first exon and the enhancer (a part of the first intron) of the MRP14 gene[11], 18 esophageal tumors/matched normal epithelia, the esophageal carcinoma cell line EC9706 and human placenta (as control) were analyzed by PCR and directed DNA sequencing. No mutation was found in the -489~512 bp range of the MRP14 gene of all the tumors and in the whole *MRP14* genomic sequence of the EC9706.

## DISCUSSION

MRP14 is a member of the S100 family of calcium-binding proteins. It is referred variously as S100A9, P14, calgranulin B (GAGB), calprotectin, cystic fibrosis antigen or L1 heavy chain. MRP14 is frequently companied with another calcium-binding protein, MRP8 (S100A8, calgranulin A, GAGA or L1 light chain), forming specific MRP8/P14 heterodimeric complex by chemical cross-linking. This complex has been shown to inhibit the activity of casein kinase I and II[12]. Recent studies suggest that MRP8/P14 complex is the major fatty acid (FA) carrier in human neutrophils[13].

The expression of MRP8 and MRP14 is involved in monocyte/macrophage differentiation. In peripheral blood, the proteins MRP8 and MRP14 are only observed in granulocytes and monocytes[14]. Elevated plasma levels of MRP8 and MRP14 have been found in patients suffering from a number of inflammatory disorders including cystic fibrosis, rheumatoid arthritis, and chronic bronchitis. In all (acute and chronic) inflammation models tested, the cells arriving first at the lesion were MRP8/MRP14-positive[15].

Little information is available concerning the implication of MRP14 in human cancer. Down-regulation of MRP8 and MRP14 was observed in human lung carcinoma cell lines[16]. Endress et al. investigated 35 lung carcinomas and 5 healthy lungs with the antibodies recognizing different macrophage subtypes. Compared to healthy lungs, the infiltration of MRP8/MRP14-positive macrophages was reduced in lung carcinomas while the number of MRP8/MRP14—positive cells was enhanced [17]. Stulik et al. analyzed the expression pattern of MRP8 and MRP14 in 23 matched sets of colorectal carcinomas and normal colon mucosa using two-dimensional gel electrophoresis. They found that the level of these two proteins, as compared with matched normal colon mucosa, was significantly increased in malignant tissues of 16 patients (70%). The immunohistological analysis revealed the accumulation of MRP14 positive cells, macrophages and polymorphonuclear leukocytes along the invasive margi of co-lorectal carcinoma[18]. Quite the contrary, esophageal cancer presented a decreased expression of MRP14 compared to normal mucosa.

In the present study, MRP14 expression was undetectable in 3/3 esophageal-carcinoma cell lines. 58 out of 64 primary squamous cell carcinomas of the esophagus presented markedly lower expression of MRP14 than the corresponding normal tissues. These data indicated that down-regulation of MRP14 expression is a frequent event in esophageal squamous cell carcinomas. No statistically significant correlations were found between MRP14 expression and gender, age, tumor grade or stage, and lymph node metastases. Nevertheless, all the six tumors without alteration of MRP14 expression presented cytologically grade I (G1). On the other hand, the samples examined were from two different regions of China: esophageal cancer high-incidence area, Anyang, Henan province, and low-incidence area, Beijing. Compared to the down-regulation rates of MRP14 between the two different regions, no significant difference was found. Dietary factors, nitrosamine exposure and history of injury to the esophagus have been reported to somehow associated with the high incidence of this malignancy in North China[19, 20]. However, consistent high down-regulation rates of MRP14 in the two different areas indicated that abnormality of MRP14 gene might not be a downstream event contrib-

uted by the environmental and chemical risk factors found in the high-incidence area. Although the significance of MRP14 in esophageal cancer remains unclear, the reverse alteration of MRP14 expression in the disease, as compared to colorectal carcinomas, suggested that different molecular mechanisms might be involved regarding to the implication of MRP14 in the development or progression of these two kinds of tumors. Interestingly**,** we found that MRP8, like MRP14, was simultaneously down-regulated in esophageal cancer. It will be important to further investigate the precise role of these two genes in the development and progression of esophageal squamous cell carcinomas. It is noted that MRP14 was down-expressed in 2/2 esophageal adenocarcinomas, which leaves open the question of whether the repression of MRP14 was also involved in esophageal adenocarcinomas.

MRP8/MRP14 proteins present a variant subcellular localization in different types of cells. In monocytes, MRP8/MRP14 associate with the type III intermediate filament vimentin of cytoskeletal structures in a $Ca^{2+}$-dependent manner[21]. Buccal squamous carcinoma cell line TR146 displays a localization of MRP8/MRP14 in keratin intermediate filaments[22]. In the pancreatic cell lines, MRP8/MRP14 remains in the cytoplasm or in the plasma membrane, depending on the functional status of the cells [23]. We found that in normal esophageal mucosa, MRP14 expressed predominantly in the nuclei of the epithelial cells. This subcellular localization different from previous reports suggests MRP14 probably play a particular role in normal epithelial cells of the esophagus.

To investigate the mechanism of MRP14 expression reduction in esophageal carcinomas, we sequenced the full-length genomic DNA of the *MRP14* gene in esophageal cancer cell line EC9706 and human placenta (as control). Neither of mutation, deletion or rearrangement was found in the cell line EC9706. Then we analyzed the promotor region, the first exon and the enhancer (a part of the first intron) of the MRP14 gene in 18 esophageal tumors and matched normal epithelial tissues. No mutation was detected in the -489~512 bp range of the MRP14 gene of all the tumors. The results suggested possible involvement of epigenetic abnormalities in esophageal cancer, to which further studies should be addressed.

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