ORIGINAL ARTICLE

# Elevated levels of serum metalloproteinase 9 in patients with esophageal squamous cell carcinoma

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**KEY WORDS**

esophageal squamous cell carcinoma, matrix metalloproteinase 9

## ABSTRACT

**INTRODUCTION** Matrix metalloproteinase 9 (MMP-9) is a proteolytic enzyme which is associated with tumor progression including invasion, migration, angiogenesis, and metastasis due to its ability to degrade type IV collagen.

**OBJECTIVES** The aim of the study was to assess clinical significance of MMP-9 measurement in the diagnostic evaluation of patients with esophageal squamous cell carcinoma (ESCC).

**PATIENTS AND METHODS** The study included 63 patients with ESCC and 30 healthy subjects. We assayed serum MMP-9 levels and squamous cell carcinoma antigen (SCC-Ag), a tumor marker. We defined diagnostic criteria for both markers.

**RESULTS** In ESCC patients serum levels of MMP-9 and SCC-Ag were found to be statistically higher compared with healthy subjects. Serum concentrations of MMP-9 and SCC-Ag tended to increase in patients with advanced cancer. The percentage of elevated MMP-9 concentrations (75%) was higher than that of SCC-Ag (68%) and increased for the combined use of both markers (97%).

**CONCLUSIONS** The results suggest the potential usefulness of MMP-9 in establishing the diagnosis of ESCC, especially when analyzed in combination with SCC-Ag.

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**INTRODUCTION** Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignant tumors associated with poor prognosis, rapid clinical progression, and a high rate of metastasis.[1,2] Degradation or breakdown of the extracellular matrix is the main structural change during the invasion and metastasis of ESCC.[3,4] The regulation of tissue remodeling is controlled by the expression or activity of matrix metalloproteinases (MMPs).[3] MMP-9 is capable of degrading type IV collagen, which is the major component of the basement membrane. Therefore, MMP-9 activity may be associated with disruption of the basement membrane and the potential for distant metastasis of cancer cells via vessel permeation.[5,6]

A number of studies have demonstrated enhanced tissue expression of MMP-9 in several malignant tumors, including colorectal[7], gastric[8], pancreatic[9] or esophageal squamous cell carcinoma[2,3,6,10]. It has been shown that expression of MMP-9 in ESCC tissue positively correlated with poorer differentiation of tumor, increased vessel permeation, deeper tumor invasion, and the number of metastatic lymph nodes.[2,10] The immunoreactivity of MMP-9 is also associated with a higher malignant potential of ESCC.[2] Moreover, Gu et al. have shown that MMP-9 expression might be a negative, independent predictor of disease-free survival of ESCC patients.[6] However, little is known about serum levels of MMP-9 in ESCC patients.

The aim of the present study was to determine serum MMP-9 concentrations in the ESCC patients, and to compare the results with healthy subjects (control group) and with a classic tumor marker – squamous cell carcinoma antigen (SCC-Ag). We also evaluated serum MMP-9 levels with regard to tumor stage. Moreover, the diagnostic sensitivity and area under the receiver operating characteristic (ROC) curves for both measurands were defined. To our knowledge, this is the first study which has assessed serum MMP-9 levels in patients with ESCC and their correlations with tumor stage as well as resectability of ESCC.

**PATIENTS AND METHODS** The study included 63 patients with ESCC (3 women and 60 men, aged 36–82 years) diagnosed by a group of oncologists and operated at the Department of Thoracic Surgery at the University Hospital of Białystok. The control group comprised 30 healthy subjects (17 women and 13 men, aged 20–69 years). A clinical diagnosis of ESCC was confirmed each time by microscopic examination of the material obtained during biopsy and/or surgery. Tumors were classified according to TNM (Primary Tumor, Regional Lymph Nodes, Distant Metastasis) classification (developed by the International Union Against Cancer [French: *Union Internationale Contre le Cancer*]).[11] Patients were divided into three groups: 13 patients in stage I + II, 35 in stage III and 15 patients in stage IV. Resectability of tumors was also determined. Out of 63 cancer patients, 30 underwent tumor resection and 33 had unresectable tumors (TABLE). The study was approved by the local Ethics Committee. All patients gave their informed consent.

Blood samples were collected from each patient before surgery using the S-Monovette blood collection system. Venous blood samples were centrifuged at 2000 × g to obtain serum samples and stored at –80°C until assayed. Serum levels of MMP-9 were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Abingdon, Great Britain). The sera were diluted prior to determination (100-fold). The manufacturer provided the intra-assay coefficient of variation (CV%) of 2% at a mean MMP-9 concentration of 0.833 ng/ml (standard deviation [SD], 0.017). In addition, chemiluminescent assay (CMIA) was used to determine SCC-Ag levels (Abbott Japan Co., Ltd., Tokyo, Japan). The intra-assay CV% for SCC-Ag determined by the manufacturer is 4.3% at a mean SCC-Ag concentration of 1.97 ng/ml (SD = 0.085). The cut-off levels of 480.0 ng/ml for MMP-9 and 2 ng/ml for SCC-Ag were defined using the Microsoft Office Excel software as values corresponding to the highest accuracy.

**TABLE** Characteristics of patients with esophageal squamous cell carcinoma

| Characteristics of patients | | Number of patients |
|---|---|---|
| | | 63 |
| gender | female | 3 |
| | male | 60 |
| age | <65 | 42 |
| | ≥65 | 21 |
| | median | 60 |
| | range | 36–82 |
| tumor stage | I + II | 13 |
| | III | 35 |
| | IV | 15 |
| resectability | resectable | 30 |
| | unresectable | 33 |

**Statistical analysis** The data we obtained did not follow a normal distribution, and therefore nonparametric statistical tests were used. Comparisons between two groups were performed by the Mann-Whitney U-test. Moreover, to assess the diagnostic value of MMP-9 and SCC-Ag measurements, diagnostic sensitivity and areas under the ROC curves were applied. The differences were considered statistically significant at $p < 0.05$.[12] Statistical analyses were performed using the STATISTICA 5.1 PL program (StatSoft Inc., Tulsa, OK, USA).

**RESULTS** **Serum levels of MMP-9 in patients with esophageal squamous cell carcinoma** In patients with ESCC serum levels of MMP-9 (FIGURE 1) and SCC-Ag (FIGURE 2) were found to be statistically higher compared with healthy subjects (control group). When the TNM staging was analyzed, serum MMP-9 levels showed a tendency to increase in advanced stage patients compared with those in the early stage of tumor growth. A similar correlation was observed for SCC-Ag. The highest MMP-9 level was observed in stage IV, and of SCC-Ag in stage III of ESCC. Moreover, concentrations of both analytes in each stage were significantly higher compared with healthy subjects (FIGURE 1, FIGURE 2). Moreover, serum MMP-9 and SCC-Ag were elevated in patients with unresectable tumors compared with those with resectable tumors. All these concentrations were significantly higher when compared with the control group (FIGURE 1, FIGURE 2).

**The diagnostic criteria for MMP-9 and SCC-Ag in patients with esophageal squamous cell carcinoma** FIGURE 3 shows the percentage of elevated concentrations of MMP-9 and SCC-Ag in ESCC. The percentage of elevated concentrations (diagnostic sensitivity) of MMP-9 (75%) was higher than SCC-Ag (68%) and increased for the combined use of MMP-9 and SCC-Ag up to 97%. The frequency of increased values for MMP-9 was higher in stage I + II (70%) and III (77%) when compared with SCC-Ag. The diagnostic sensitivity of MMP-9 (73%) in stage IV was lower than for SCC-Ag (80%); however, this value increased for the combined use of MMP-9

**FIGURE 1** The median concentrations of matrix metalloproteinase 9 (MMP-9) in patients with esophageal squamous cell carcinoma (ESCC)
**a** statistically significant at $p < 0.05$

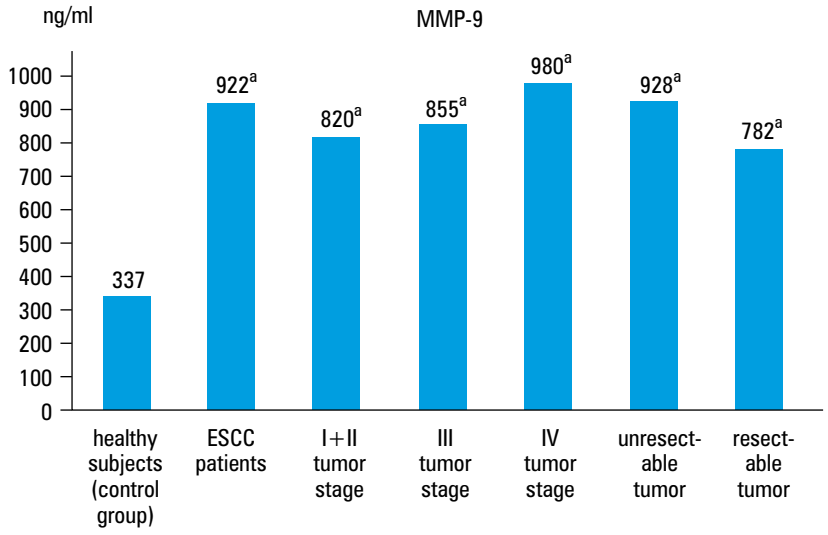



**FIGURE 2** The median concentrations of squamous cell cancer antigen (SCC-Ag) in patients with ESCC
**a** statistically significant at $p < 0.05$

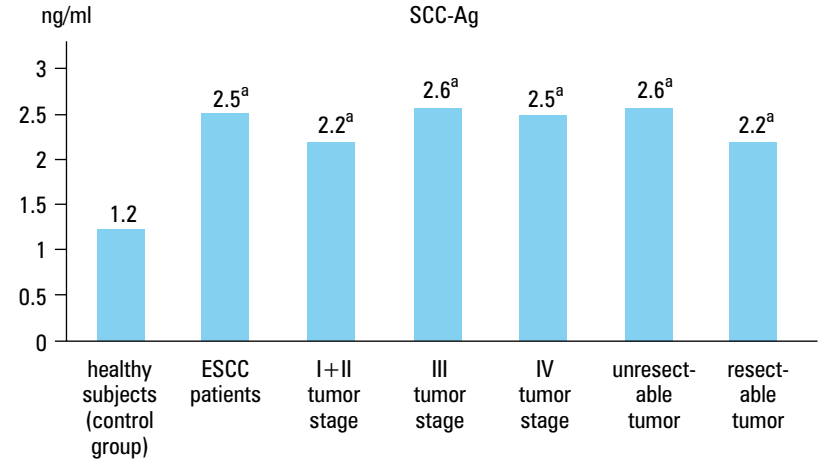
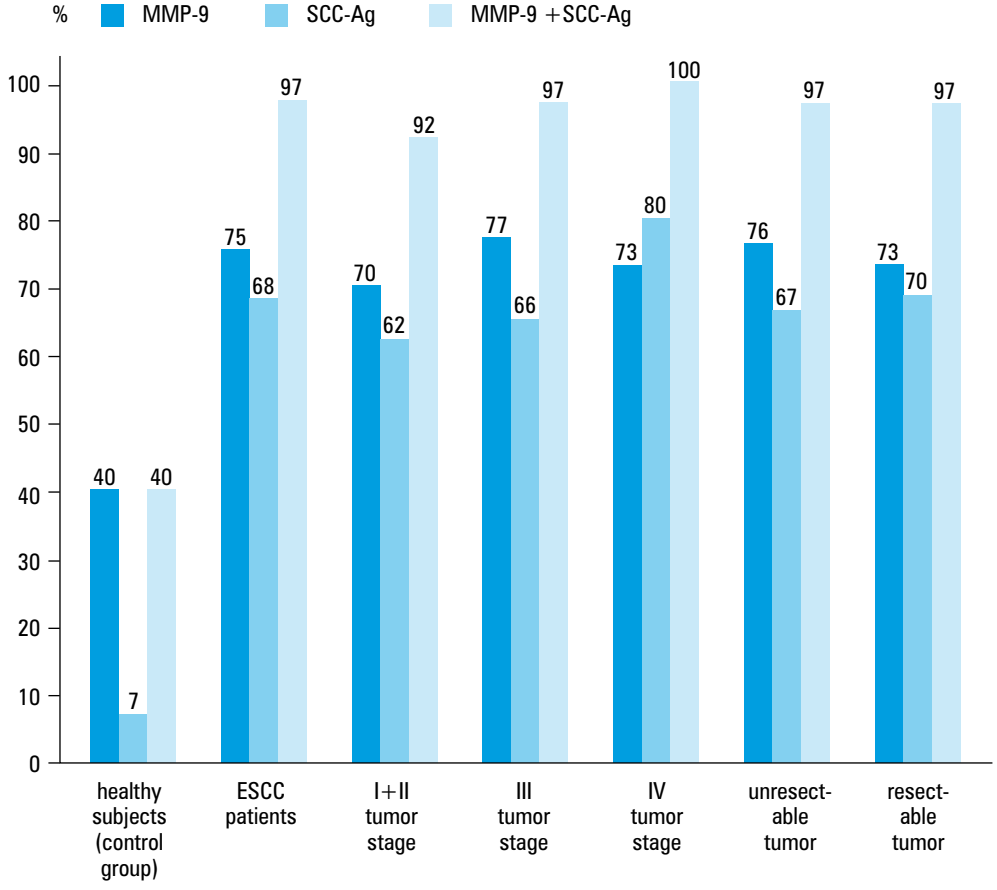



**FIGURE 3**
The percentages of elevated concentrations of MMP-9 and SCC-Ag in patients with ESCC



with SCC-Ag to 100%. Analysis of the percentage of elevated markers in each tumor stage, the highest diagnostic sensitivity was observed in stages III and IV (97% and 100%, respectively) (FIGURE 3). The area under the ROC curves for MMP-9 (0.78) was slightly lower than for SCC-Ag (0.82).

**DISCUSSION** Various factors influence the biology and prognosis of ESCC.[2] MMP-9 is a proteolytic enzyme which can degrade the components of basement membranes, especially type IV collagen. It plays a crucial role in the invasion and metastasis of malignant tumors, including ESCC.[2,3] The prognosis for patients with ESCC is poor and is worse than for other digestive tract cancers.[1,13]

Several studies have shown high MMP-9 expression in tissues of ESCC patients[3,6,10], while noncancerous esophageal tissues did not express detectable levels of the enzyme[3,10]. This may suggest that MMP-9 plays a role in the development of ESCC[10] and in esophageal tumorigenesis[3]. Moreover, other authors confirmed that the expression of MMP-9 correlated with clinical and pathological factors related to cancer, including the depth of tumor invasion, lymphatic vessel permeation, lymph node metastasis, and tumor differentiation.[2,6,10] The expression of MMP-9 is also associated with an increased malignant potential of ESCC.[2] Moreover, Gu et al. have shown that MMP-9 expression might be a negative, independent prognostic factor for ESCC patients' survival.[6] Therefore, the combined use of MMP-9 expression and TNM stage classification may provide a more accurate prediction of the post-operative outcome of patients with ESCC, improving individual treatment strategies.[6]

Several studies have indicated that MMP-9 expression is associated with ESCC.[2] However, the serum levels of this enzyme in ESCC have not been analyzed so far. To our knowledge, this is the first study to have assessed serum MMP-9 concentrations in ESCC patients in comparison to healthy subjects and a commonly used tumor marker, SCC-Ag. In the present analysis serum levels of MMP-9 and SCC-Ag were found to be statistically higher compared with the control group. High concentrations of MMP-9 from ESCC samples are consistent with the results obtained in patients with lung[14], gastric[15], colorectal[16], hepatocellular cancers[17], as well as with head and neck squamous cell carcinoma[18]. These findings are also in line with our previous study, in which we showed elevated MMP-9 concentrations in the sera of esophageal cancer patients, without the analysis within histopathological types of esophageal cancer, esophageal squamous cell carcinoma and adenocarcinoma.[19] Serum levels of MMP-9 and tumor markers (carcinoembryonic antigen [CEA] and SCC-Ag) were statistically higher in esophageal cancer patients compared with healthy subjects.[19] High concentrations of MMP-9 in the sera of patients might result from enzyme production by cancer cells.[20] It has been shown that MMPs are produced by physiological and neoplastic cells, and their synthesis in cancer may be induced by cytokines and growth factors.[21] Chen et al. have assessed the serum levels of MMP-9 in patients with squamous dysplasia, which is a precursor lesion for ESCC.[22] Serum MMP-9 concentration in patients with esophageal squamous dysplasia was significantly different from that of subjects without dysplasia and subjects with dysplasia/early cancer, which suggests that determination of serum MMP-9 concentration might possibly be used as a primary screening test for the ESCC, but it is insufficient on its own.[22] Moreover, Samantaray et al. have confirmed that alterations in MMP-9 levels may be early events in esophageal carcinogenesis.[3]

In the present study, serum MMP-9 and SCC-Ag concentrations showed a tendency to increase in patients with advanced stages in comparison with those in the early stage of tumor. The highest level of MMP-9 was found in stage IV, and SCC-Ag in stage III. In each stage concentrations of both markers were significantly higher in cancer patients than in healthy subjects. Similar correlation was observed in our previous study, where the highest level of MMP-9 was observed in stage IV of esophageal cancer.[19]

In the present study, the percentage of elevated concentrations of MMP-9 (75%) was higher than SCC-Ag (68%) and increased for the combined use of MMP-9 and SCC-Ag up to 97%. Moreover, the percentage of elevated concentrations of MMP-9 in the early stage of tumor development (70%) was higher than SCC-Ag (62%) and increased for the combined use of MMP-9 and SCC-Ag up to 92%. To our knowledge, this is the first study to have assessed the percentage of elevated concentrations of MMP-9 relative to tumor stages. Our results may substantially improve the early diagnosis of ESCC, which could ensure better treatment and prognosis for ESCC patients. Moreover, measurement of serum MMP-9 levels could be more helpful when combined with SCC-Ag because the percentage of elevated MMP-9 serum levels in ESCC patients increased significantly for the combined use of both markers up to 97%. The previous analysis similarly reported that the percentage of elevated MMP-9 serum levels in esophageal cancer patients was higher than the classic tumor markers (CEA and SCC-Ag), and the combined use of MMP-9 and classic tumor markers also increased their diagnostic sensitivity.[19]

In conclusion, this is the first study which has compared the serum levels of MMP-9 in ESCC patients with healthy subjects and with serum concentrations of the classic tumor marker (SCC-Ag). At the early stage of tumor growth the percentage of elevated concentrations of MMP-9 was higher than SCC-Ag (70 vs. 62%), and increased significantly for the combined use of MMP-9 and SCC-Ag up to 92%. These findings suggest that estimation of serum MMP-9 levels may help to increase diagnostic efficiency for early ESCC,

especially when combined with SCC-Ag. However, this issue requires further investigation.

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ARTYKUŁ ORYGINALNY

# Podwyższone stężenie metaloproteinazy 9 w surowicy chorych na płaskonabłonkowego raka przełyku

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**SŁOWA KLUCZOWE**

metaloproteinaza 9, płaskonabłonkowy rak przełyku

**STRESZCZENIE**

**WPROWADZENIE** Metaloproteinaza 9 (*matrix metalloproteinase 9* – MMP-9) należy do enzymów proteolitycznych, które mają zdolność degradacji kolagenu typu IV, ułatwiając progresję guza nowotworowego, w tym naciekanie, migrację komórek nowotworowych, angiogenezę oraz powstawanie przerzutów odległych.

**CELE** Celem pracy było określenie znaczenia klinicznego pomiaru MMP-9 w diagnostyce chorych na płaskonabłonkowego raka przełyku (*esophageal squamous cell carcinoma* – ESCC).

**PACJENCI I METODY** Badaniem objęto grupę 63 chorych na ESCC oraz 30 osób zdrowych. Oznaczono stężenia surowicze MMP-9 oraz markera nowotworowego – antygenu raka płaskonabłonkowego (*squamous cell carcinoma antigen* – SCC-Ag). Określono kryteria diagnostyczne dla obu wskaźników.

**WYNIKI** Wykazano, że stężenia MMP-9 i SCC-Ag w surowicy chorych na ESCC były statystycznie znamiennie wyższe w porównaniu z osobami zdrowymi. Stężenia surowicze MMP-9 i SCC-Ag wykazywały tendencję do wzrostu u pacjentów z zaawansowanym stadium nowotworu. Odsetek podwyższonych stężeń MMP-9 (75%) był wyższy niż SCC-Ag (68%) i wzrastał przy jednoczesnym oznaczaniu obu markerów (97%).

**WNIOSKI** Uzyskane wyniki sugerują potencjalną przydatność MMP-9 w diagnostyce chorych na ESCC, szczególnie przy łącznej analizie z SCC-Ag.

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