ANTICANCER RESEARCH *24*: 3245-3250 (2004)

Serum Markers as Predictors of Esophageal Squamous Dysplasia and Early Cancer

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Abstract. *Background: Squamous dysplasia is the precursor lesion for esophageal squamous cell carcinoma (ESCC). A primary screening test for ESCC which identified this lesion could lead to a reduction in disease-specific mortality. Materials and Methods: We conducted a population-based screening study in Linzhou, China. All subjects provided blood samples and underwent endoscopy with Lugol's iodine staining and biopsy. We selected a subset of 84 subjects stratified on worst squamous histologic diagnosis in six categories and measured the serum concentrations of potential markers using commercially available ELISA tests for matrix metalloprotease-9, tissue inhibitor of matrix metalloprotease-1, copper/zinc superoxide dismutase, anti-p53 auto-antibodies, and soluble serum interleukin-2 receptor. Results: Serum matrix metalloprotease-9 concentration was significantly different by esophageal squamous dysplasia status with a median (interquartile range) for subjects without dysplasia of 150 ng/ml (80-225) and subjects with dysplasia/early cancer of 97 ng/ml (58-155), p=0.033), but the maximum sensitivity and specificity were low. The serum concentrations of the other markers tested showed no significant differences by category of worst histologic diagnosis. Conclusion: Serum matrix metalloprotease-9 concentration could contribute to a primary screening test for an ESCC, but is insufficient alone.*

Esophageal squamous cell carcinoma (ESCC) is the 8th most common incident cancer worldwide, but is the 6th most common cause of cancer death, causing nearly as many deaths as breast cancer (1). The anatomy of the esophagus allows tumors to grow and spread before inducing dysphagia, the typical first symptom in ESCC patients. Therefore, most subjects present at the doctor too late for therapy with curative intent. Esophageal squamous dysplasia is the precursor lesion for ESCC (2) and the natural target for a primary screening program. To reduce the burden of ESCC it will be necessary to develop non-invasive, patient-acceptable screening tests that can be used in high-risk populations and sub-populations.

Linzhou (formerly Linxian), China has one of the highest rates of ESCC and gastric cardia adenocarcinoma anywhere in the world. Incidence rates for both sexes exceed 100/100,000/year (3) making Linzhou an ideal place to study early detection methods for ESCC.

We conducted a population-based screening study that included 725 adult volunteers who underwent a battery of tests and provided biological samples for the testing of novel screening methods. Here we report the testing of five different serum protein concentrations as early detection markers for ESCC in a subset of 84 subjects who represent a spectrum of worst histologic diagnoses from normal through invasive cancer.

Materials and Methods

Subjects and biologic sampling. A screening study was conducted in Yaocun commune, Linzhou, Henan Province, People's Republic of China, in the spring of 2002. This study was conducted under the auspices of the Institutional Review Boards of CICAMS and the NCI. Subjects were unselected volunteers aged 40-65 from three villages. Blood was collected using Vacutainer tubes, separated by centrifugation and transported to Beijing on dry ice, where the serum was frozen at -80°C until used.

Endoscopy with Lugol's iodine and biopsy was performed as previously described (4). During endoscopy, the entire esophagus and stomach were visually examined, and one or more 2.8-mm biopsies were taken from all grossly abnormal appearing lesions. The entire esophagus was sprayed with Lugol's iodine solution and unstained areas were biopsied. If no focal lesions or unstained lesions were

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Key Words: Esophagus, dysplasia, MMP-9.

Table I. *Subject characteristics by worst squamous diagnosis.*

	Normal	Esophagitis Dysplasia	Mild Dysplasia	Moderate Dysplasia	Severe	CIS/ESCC
Number	17	15	14	16	15	7
Age (IQR), y	53 (51-56)	56 (51-59)	53 (51-60)	54 (51-61)	57 (55-62)	56 (54-61)
Sex, N (%) male	9 (53%)	7 (47%)	7 (50%)	8 (50%)	8 (53%)	5 (71%)
Smoke, N (%) ever	5 (29%)	5 (33%)	5 (36%)	7 (44%)	6 (40%)	2 (29%)
Family history, N (%) yes	9 (53%)	4(27%)	7 (50%)	10 (63%)	10 (67%)	4 (57%)

Table II. *Distribution of markers comparing no dysplasia and dysplasia.*

	No Dysplasia	Dysplasia or Cancer	*P**
MMP 9 (median, IQR), ng/ml	150 (80-225)	97 (58-155)	0.033
TIMP-1 (median, IQR), ng/ml	112 (103-118)	109 (87-122)	0.42
Cu/Zn SOD (median, IQR), ng/ml	17 (13-24)	15 (11-26)	0.35
anti-p53 (median, IQR), U/ml	0 (0-1.03)	0 (0-0.68)	0.59
sIL-2 r (median, IQR), pg/ml	611 (451-793)	607 (362-750)	0.60

* *P*-values come from Wilcoxon Rank Sum tests with the normal approximation

found, a standard site in the mid-esophagus was sampled. The biopsies were fixed in 95% ethanol, embedded in paraffin, cut in 5-μm sections and stained with hematoxylin and eosin.

From the 724 patients with adequate biopsies for histologic diagnosis, we randomly selected a subgroup of 84 subjects within strata of worst squamous diagnosis to test the utility of serum protein concentrations as a screening test.

Histologic categories. The biopsy slides were read independently by two pathologists (NL, SMD), without knowledge of the patient's history or the visual endoscopic findings. The histologic criteria were based on previous descriptions (5) and are described below:

Normal: A stratified squamous epithelium was present which showed no features diagnostic of the other histologic categories listed below. Mature squamous cells with abundant clear cytoplasm, scattered lymphocytes and compressed nuclear fragments ("squiggle cells") were occasionally seen in the epithelium. The lamina propria, if present, commonly contained a few scattered mononuclear inflammatory cells.

Esophagitis: One or more of the following three criteria were present: elongation of lamina propria papillae into the upper third of the epithelium together with basal cell hyperplasia >15% of total epithelial thickness; epithelial infiltration by neutrophils or eosinophils; or a dense non-follicular infiltrate of mononuclear inflammatory cells or neutrophils in the lamina propria.

Squamous dysplasia: Nuclear atypia (enlargement, pleomorphism and hyperchromasia), loss of normal cell polarity and abnormal tissue maturation were present in the lower third (mild), in the lower two-thirds (moderate), or in all thirds (severe) of the epithelium, without full-thickness involvement or invasion. Dysplastic biopsies, which could not be graded because of biopsy size or orientation, were categorized as squamous dysplasia, not otherwise specified (NOS).

Squamous cell carcinoma: Neoplastic squamous cells were present throughout the full thickness of the epithelium (CIS) or had invaded through the basement membrane (ESCC).

Only a small number of subjects were diagnosed with acanthosis, basal cell hyperplasia, or dysplasia not otherwise specified, as their worst squamous diagnosis so these subjects were excluded from this study.

Laboratory procedures. All laboratory measurements were carried out at the Diet and Cancer Epidemiology Laboratory, CICAMS. The concentration of each marker in each serum sample was measured with ELISA kits for Human total MMP-9 (CN Biosciences Company, San Diego, CA USA); TIMP-1, IL-2sRα (R&D Systems, Inc., Minneapolis, MN, USA); Cu/Zn Superoxide Dismutase, and p53-Autoantibody (Oncogene, Cambridge, MA USA).

Laboratory technicians were blind to the subject identities and their associated pathological diagnoses. To determine the assay reliability two identical quality control serum samples were included on each ELISA plate. The quality control serum was obtained from pooled whole blood drawn in 2000 from 22 residents of the same commune.

Standard curves were generated using samples that were serial dilutions of the internal standards provided with each kit and the on-board software from the BIORAD Model 550 Microplate Reader. The mean absorbance for each standard sample was plotted on the ordinate and its concentration was plotted on the abscissa and then a regression line was drawn through these points. All regressions had r^2 between 0.95 and 0.99. Experimental sample concentrations were calculated using the standard curve regression line equation.

Statistical analysis. Individual concentrations for each protein were tabulated and the distributions examined graphically using dot plots for each protein concentration by histologic category. Because most subjects had undetectable concentrations of anti-p53 auto-

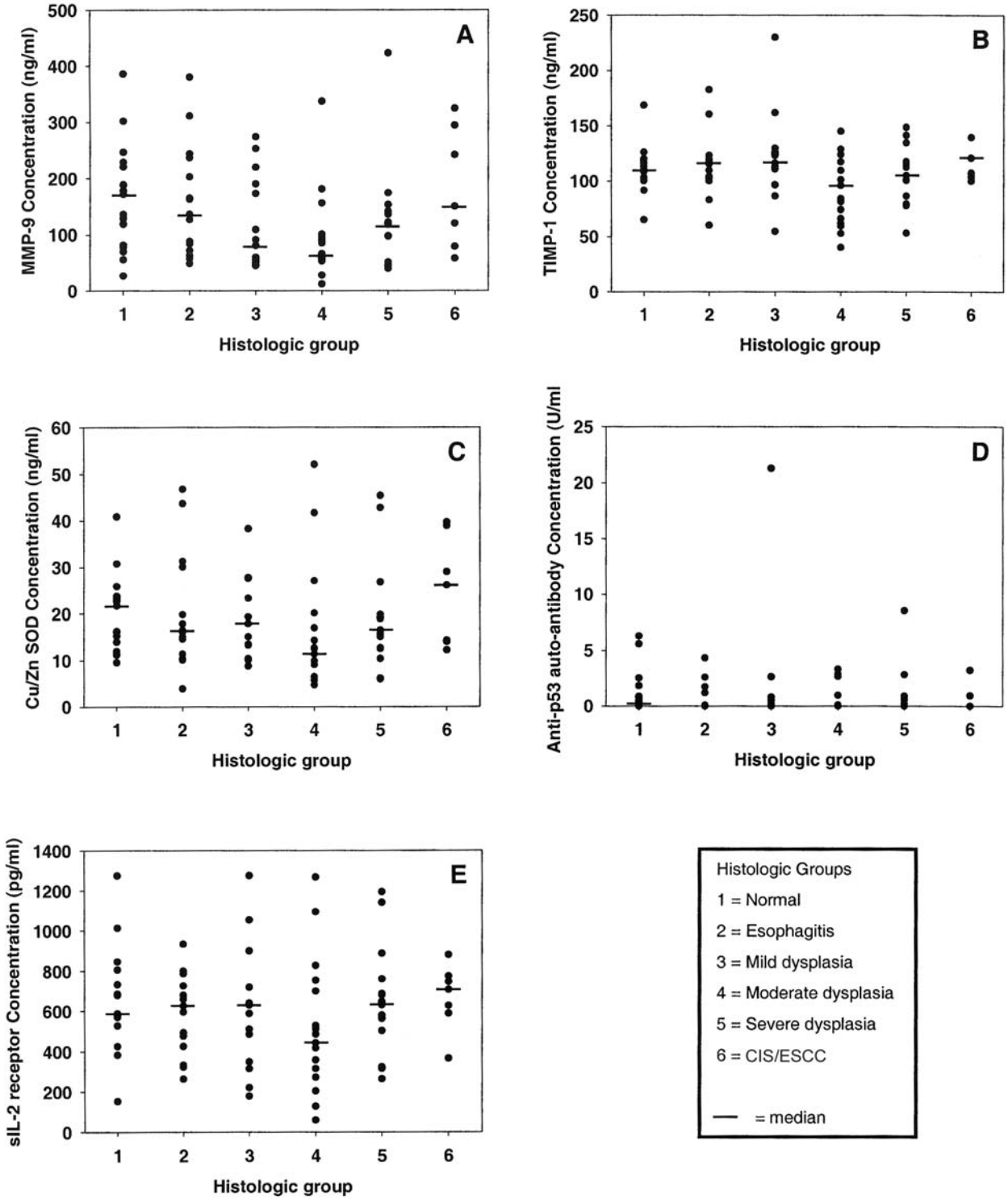

Figure 1. *A. Distribution of serum matrix metalloprotease-9 concentrations by worst histologic diagnosis. The 5 df Kruskal-Wallis p-value for a difference between groups was 0.09. B. Distribution of serum tissue inhibitor of matrix metalloprotease-1 concentrations by worst histologic diagnosis. The 5 df Kruskal-Wallis p-value for a difference between groups was 0.21. C. Distribution of serum copper/zinc superoxide dismutase concentrations by worst histologic diagnosis. The 5 df Kruskal-Wallis p-value for a difference between groups was 0.37. D. Distribution of serum anti-p53 auto-antibodies concentrations by worst histologic diagnosis. The 5 df Kruskal-Wallis p-value for a difference between groups was 0.84. The median for categories 2 through 6 was zero. E. Distribution of soluble serum interleukin-2 receptor concentrations by worst histologic diagnosis. The 5 df Kruskal-Wallis p-value for a difference between groups was 0.40.*

antibody, we also parameterized this as present or absent. We tested for correlations with other subject characteristics using Pearson correlations or the Wilcoxon rank sum test. We tested for a difference in serum marker concentrations between histologic groups using the Kruskal-Wallis test. We also collapsed the histologic categories into no dysplasia or any dysplasia/cancer and tested for differences using the Wilcoxon rank sum test with the normal approximation *p*. A receiver operating characteristic (ROC) curve was generated using the *rocfit* and *rocplot* commands in STATA SE version 8 (College Station, TX, USA). For this, the continuous concentration data was binned into 10 categories. All *p*-values come from two-sided tests.

Results

We selected 84 subjects from a population-based screening study to test the utility of five serum protein markers for detecting esophageal squamous dysplasia or early cancer. The subject characteristics by worst histologic diagnosis are presented in Table I. All characteristics were similar to the underlying cohort of 724 subjects.

We examined whether any of the characteristics in Table I were associated with serum concentrations of the tested markers. The only significant association was a negative correlation between matrix metalloprotease-9 and age (r^2=0.07, p=0.017).

The distributions for matrix metalloprotease-9, tissue inhibitor of matrix metalloprotease-1, copper/zinc superoxide dismutase, anti-p53 auto-antibody, and soluble serum interleukin-2 receptor are presented in Figures 1 (A-E). Because many samples had a value of zero for anti-p53 autoantibody concentration, we also parameterized it as present or absent; prevalences were as follows: Normal=53%, esophagitis=33%, mild dysplasia=36%, moderate dysplasia=38%, severe dysplasia=33%, and CIS/ESCC=43% (5 df Chi-square p= 0.84).

We collapsed all histologic categories into either no dysplasia (normal + esophagitis) or dysplasia/early cancer. The distributions and tests for significance are given in Table II. Only MMP-9 showed a statistically different distribution between these two histologic groups. We generated an ROC curve for MMP-9 for predicting the presence of any dysplasia. The area under the curve (95% confidence interval) was 0.36 (0.24-0.49), which indicates poor performance as a screening test. The simultaneous maximum sensitivity and specificity that could be achieved were 58% and 34%, respectively. If the negative correlation with age is accounted for in these calculations, the potential utility of MMP-9 is further decreased.

Discussion

ESCC is a common cause of cancer death worldwide and in high-risk populations can approach 20% of all deaths. To substantially reduce this burden it will be necessary to devise a primary screening test that identifies subjects before they develop advanced tumors. Currently, endoscopy with Lugol's iodine staining can effectively discern the presence of high-grade dysplasia/early cancer in the esophagus with a sensitivity and specificity of 96% and 63%, respectively (6). However, the practical challenges of endoscopy and biopsy in high-risk populations, which often have few health care resources, demand a simpler, less expensive and patient-acceptable screening program.

The use of serum markers to screen for disease is well understood, but few markers have proven their worth for detecting cancer. Numerous researchers have studied serum markers for their prognostic ability in subjects with documented ESCC, but few studies have examined the ability of these markers to identify ESCC or its precursor, squamous dysplasia, in subjects without known disease. We selected a panel of potential markers based on the ESCC and other squamous tissue cancer literature and tested their utility as screening tests.

MMP-9 is a type 4 collagenase that can play a critical role in cancer metastasis. We had hypothesized that serum MMP-9 concentrations may begin to rise prior to the advent of an invasive tumor. Surprisingly, we found that serum MMP-9 concentrations were significantly lower in subjects with dysplasia/cancer compared to subjects without dysplasia. The side-by-side dot plots reveal that subjects with ESCC have a wider distribution of serum MMP-9 concentrations and a higher median than subjects with dysplasia as a worst diagnosis. The origin of this depression in serum concentration in dysplasia samples cannot be assessed in this data. Although insufficiently sensitive or specific on its own, MMP-9 may contribute to a future panel of early detection markers.

The other markers we evaluated have been previously investigated in ESCC patients or have a plausible connection to ESCC risk including tissue inhibitor of matrix metalloprotease-1 (7-9), copper/zinc superoxide dismutase (10;11), anti-p53 auto-antibodies (12-15), and soluble serum interleukin-2 receptor (16-18). We found that none of these markers was significantly different between subjects with preneoplastic esophageal lesions and those without these lesions.

One notable finding among these other markers was the relatively high prevalence of anti-p53 auto-antibodies in this population. The high sensitivity of endoscopy with Lugol's staining and biopsy for high-grade dysplasia/cancer suggests that the presence of undiagnosed cancer in our ostensibly normal individuals is unlikely. The temporality of acquisition of anti-p53 auto-antibodies with regard to the esophageal squamous dysplasia sequence is unknown, and the presence of this marker in our population and its relationship to cancer risk has not previously been examined.

This study was strengthened by using subjects drawn from a population-based cohort and by using the gold-standard diagnostic procedure to define the disease state of all subjects. We used a relatively small sample size to facilitate rapid screening of each of these potential markers, which reduced our ability to detect statistically significant differences in serum concentration distributions between the histologic categories. But inspection of the distributions presented in the figures suggests that no amount of added precision would be likely to lead us to the conclusion that the markers other then MMP-9 would be clinically useful.

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Received July 20, 2004
Accepted July 30, 2004