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Type I and III collagen degradation products in serum predict patient survival in head and neck squamous cell carcinoma

Sini Nurmenniemi [a,b], Marja-Kaisa Koivula [b,c], Pia Nyberg [a], Taina Tervahartiala [d], Timo Sorsa [d], Petri S. Mattila [e], Tuula Salo [a,f], Juha Risteli [b,*,f]

[a] Department of Diagnostics and Oral Medicine, Institute of Dentistry, University of Oulu, Oulu University Central Hospital, FIN-90014 Oulu, Finland
[b] Department of Clinical Chemistry, Institute of Diagnostics, University of Oulu, Oulu University Central Hospital, FIN-90014 Oulu, Finland
[c] Central Hospital of Kainuu region, FIN-87100 Kajaani, Finland
[d] Department of Oral and Maxillofacial Diseases, Institute of Dentistry, University of Helsinki, Helsinki University Central Hospital, FIN-00014 Helsinki, Finland
[e] Department of Otorhinolaryngology, University of Helsinki, Helsinki University Central Hospital, FIN-00014 Helsinki, Finland

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SUMMARY

Cancer invasion induces extracellular matrix remodeling and collagen degradation. The aim of this study was to assess whether serum levels of type I and III collagen degradation products were associated with patient survival in head and neck squamous cell carcinoma (HNSCC). A novel enzyme immunoassay was developed for measuring type III collagen N-terminal telopeptide (IIINTP) in human serum samples. In addition, type I collagen C-terminal telopeptide (ICTP), matrix metalloprotease-8 (MMP-8) and tissue inhibitor of metalloproteases-1 (TIMP-1) were assessed in 205 blood samples from HNSCC patients. High levels of serum ICTP and IIINTP and plasma TIMP-1 were associated with poor survival. The concentration of ICTP was associated with levels of IIINTP and TIMP-1. The plasma concentration of MMP-8 was associated with tumor stage, but not with survival or levels of ICTP, IIINTP or TIMP-1 suggesting that other collagenases/proteases are responsible for the cleavage of type I and type III collagens. The rate of type I and type III collagen degradation is associated with patient survival and can be used as a prognostic marker in HNSCC.

Introduction

Cancer progression is accompanied by remodeling of the extracellular matrix.[1–4] This remodeling occurs by enhanced synthesis and degradation of extracellular matrix components, including fibrillar collagens. Type III collagen is often associated with type I collagen fibers and it is expressed in human soft tissues, such as skin, blood vessels, internal organs, and placenta. Type III collagen degradation releases a cross-linked N-terminal telopeptide (IIINTP) which has been assessed in tissue digests as a marker of mature type III collagen.[5,6] Mature type III collagen has been found in benign uterine leiomyomas, and in ovarian,[5] endometrial,[7] and colonic[8] tumors. Type I collagen is the most abundantly expressed collagen in the human body and a major component in the interstitial extracellular matrix. Degradation of type I collagen leads to the release of the carboxyterminal telopeptide, ICTP.[9] Serum concentrations of ICTP have been found to correlate with bone metastasis in prostate[10–12] and breast cancers,[13] as well as with osteolytic bone destruction in multiple myeloma.[14,15] Serum concentrations of ICTP have also been shown to correlate with survival in lung,[16–18] prostate,[19] esophageal,[20] ovarian,[21,22] and breast cancers,[23] as well as with survival of multiple myeloma.[24]

Prior to this study, collagen degradation and its potential use as a prognostic marker have not been assessed in head and neck squamous cell carcinoma (HNSCC) patients, and therefore we measured serum concentrations of type I and type III collagen degradation products, ICTP and IIINTP, respectively. For measuring the serum concentration of IIINTP, a novel human enzyme immunoassay was developed. Several proteinases, such as MMPs, are responsible for collagen degradation during cancer invasion. Paradoxically, some MMPs can be protective in cancer: we have previously found that tissue expression of MMP-8 is associated with a favorable outcome in tongue cancer.[25] We now wished to evaluate if there was any association between serum ICTP and IIINTP and plasma MMP-8 concentrations and the clinical outcome of patients with HNSCC. We also measured the blood levels of tissue inhibitor of metalloproteases-1 (TIMP-1) which, in addition to being able to inhibit MMP-8 and other MMPs, functions as a growth factor and the plasma levels of which associate with a poor outcome in several cancers, including HNSCC.[26]

* Corresponding author. Address: Institute of Diagnostics, P.O. Box 5000, 90014 University of Oulu, Finland. Tel.: +358 8 315 4431; fax: +358 8 315 4451.
E-mail address: juha.risteli@oulu.fi (J. Risteli).
[f] The last two authors corresponded equally to the supervision of this work.

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Patients and methods

Patients

The 205 study patients were recruited among patients who had been diagnosed with HNSCC at the Helsinki University Central Hospital between January 1990 and December 2007. Only those patients were included who had donated both plasma and serum samples for our cancer research program and whose samples were available for this study. The patients had given a written consent to participate in our cancer research program. This study was approved by the Research Ethics Board of the Helsinki University Central Hospital and it was carried out in accordance with the Declaration of Helsinki.

Blood samples

Peripheral blood samples were drawn at the time of HNSCC diagnosis. Serum samples were collected in glass tubes without a clot activator and plasma samples in tubes containing an anticoagulant. The samples were allowed to coagulate at room temperature (RT) for 30 min. After centrifugation at 1600 g for 10 min, the supernatants were collected and stored at −70 °C.

IIINTP serum concentrations

A novel indirect enzyme immunoassay (EIA) was developed for measuring the concentrations of N-terminal telopeptide of type III collagen (IIINTP) from 202 serum samples. The level of patient IIINTP was elucidated by measuring the ability of patient serum to inhibit binding of rabbit IIINTP antibody to a synthetic IIINTP peptide that was attached to the solid phase. The IIINTP rabbit anti-serum was produced by immunizing New Zealand White rabbits with a synthetic IIINTP peptide QNYSPQYDSYDVKSGVAVGG (NeoMPS, Strasbourg, France) conjugated to thyroglobulin.

The inhibition reactions were performed by incubating undiluted patient serum samples with IIINTP rabbit anti-serum at RT for 30 min. The reaction mixtures were transferred to washed, streptavidin-coated, 96-well plates (BioBind Assembly, Thermo Fisher Scientific, Vantaa, Finland) that had been pretreated with a biotinylated synthetic IIINTP peptide pQYDSYDVKSGVAVGGGGGK (NeoMPS) at a concentration of 1 μg/well in assay/wash buffer (20 mM Tris–HCl, 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Tween; pH 7.5) at RT for 2 h. After incubation at RT for 1 h, the plates were washed and 100 μl of anti-rabbit-IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA) diluted 1:80 000 in guardian peroxidase conjugate stabilizer/diluent (Pierce) was added. After 1 h at RT, the plates were washed and the bound antibodies were detected with 3,3′-5,5′-tetramethylbenzidine (Sigma–Aldrich, St. Louis, MN, USA) 10 μg/100 μl per well in 100 mM sodium acetate trihydrate, 1.5 mM citric acid monohydrate, and 0.0015% hydrogen peroxide. After 30 min, the reaction was stopped by adding 100 μl of 2 M sulphuric acid per well. The absorbances at a wavelength of 450 nm (A450) were read in a Victor^2 instrument (Wallac, Turku, Finland). The signal obtained with IIINTP rabbit anti-serum only (blank) was defined as 0% inhibition and the extent of inhibition by the human serum samples was defined by the formula [A450(blank)-A450(sample)]/A450(blank)∗100%. All samples were analyzed in duplicate. The intra- and inter-assay coefficients of variation were 4% and 7%, respectively.

ICTP serum concentrations

Concentrations of C-terminal telopeptide of type I collagen (ICTP) were determined from 203 serum samples by a radioimmunoassay kit (UniQ ICTP RIA, Orion Diagnostica Oy, Finland). All samples were diluted 1:4 and analyzed in duplicate. The intra- and inter-assay variations were 5% and 7%, respectively.

MMP-8 and TIMP-1 plasma concentrations

MMP-8 concentrations were determined from 198 plasma samples by a time-resolved immunofluorometric assay (Medix Biochemica, Kauniainen, Finland) according to previously published protocol.[26,27] For MMP-8, the samples were diluted 1:5 and analyzed in duplicate, and the inter-assay variation was 7%. TIMP-1 levels were determined from 198 plasma samples using an enzyme-linked immunosorbent assay kit (GE Healthcare UK Limited, Buckinghamshire, UK). For TIMP-1 the samples were diluted 1:300 and analyzed in duplicate. The intra- and inter-assay variations were 9% and 2%, respectively.

Statistical analysis

Pearson product moment correlation coefficients were calculated for analyses of correlations between concentrations of peripheral blood proteins. Analysis of variance was used to test associations of geometric mean concentrations. The Kaplan–Meier method was used to calculate survival rates and log-rank tests were used to test differences in survival. Cox regression analyses were used to assess adjusted hazard ratios (HR) and 95% confidence intervals (95% CI) of overall survival. The analyses were adjusted by age and gender of the patient as well as the stage of the disease. p values <0.05 were considered significant. Pearson product moment correlation coefficients were calculated using R software.[28] Analysis of variance and survival analyses were performed using Statview software (SAS Institute Inc., North Carolina, USA).

Results

Most of the 205 patients (78%) had oral or oropharyngeal cancer (Table 1). One hundred and twenty-nine of the patients were male, and the mean age of the patients was 61 years (range 26–98 years).

Table 1
Patient characteristics.

Characteristics	*N*	Percentage
Total	205	
Mean age (years)	60.5	(Range 26.0–98.6)
Gender		
Female	76	37.1
Male	129	62.9
Primary site		
Oral	84	40.0
Oropharynx	78	38.0
Larynx	22	10.7
Hypopharynx	18	8.8
Nasopharynx	3	1.5
Tumor stage		
Stage I	56	27.3
Stage II	30	14.6
Stage III	31	15.1
Stage IV	88	42.9
Treatment		
Surgery with postoperative radiotherapy	86	42.0
Surgery without radiotherapy	61	29.8
Chemoradiotherapy	29	14.1
Radiotherapy	7	3.4
Radiotherapy with palliative intent	22	10.7