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SHORT COMMUNICATION

Overexpression of protease nexin-1 mRNA and protein in oral squamous cell carcinomas [☆]

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Summary Protease nexin-1 (PN-1) belongs to the serpin family of serine protease inhibitors. It is the phylogenetically closest relative of plasminogen activator inhibitor-1 (PAI-1). Whilst there are numerous studies of the occurrence and functions of PAI-1 in cancer, a possible tumour biological role of PN-1 has been almost totally neglected. We have now compared the level of PN-1 mRNA in 20 cases of oral squamous cell carcinomas and in matched samples of the corresponding normal oral tissues. We found that the average PN-1 mRNA level in tumours and normal tissues was significantly different, being increased up to 13 fold in tumour samples compared with the average level in normal tissues. The PN-1 mRNA level was significantly higher in tumours from patients with lymph node metastasis than in tumours from patients without. We could conclude that PN-1 is frequently overexpressed in oral squamous cell carcinomas and that its level may correlate with the occurrence of lymph node metastasis. We hypothesise that PN-1 may have a tumour biological function similar to that of PAI-1.

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Introduction

The growth and spread of solid tumours do not only depend on the proliferative and invasive properties of the genetically abnormal malignant cells. It is also important that the surrounding stroma provides the tumour with a connective tissue framework, including a type of extracellular

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matrix specific for tumours, as well as cells such as fibroblasts and endothelial cells.¹ It is well known that extracellular proteolytic enzymes, for example serine proteases and metalloproteases (MMPs), are involved in the alteration of the microenvironment and in invasion of solid tumours. However, their detailed mechanism of action still remains unclear, as different components of proteolytic enzyme system may have different biological and physiological functions. Several components of proteolytic enzyme systems are upregulated in human cancers and have been shown to be markers of a poor prognosis, including urokinase-type plasminogen activator (uPA), its receptor uPAR, and its inhibitor plasminogen activator inhibitor 1 (PAI-1 or serpin E1).^{2–4} A high level of PAI-1 in primary tumours is one of the most informative biochemical markers of a poor prognosis in many different cancer types, including breast, prostate, colon and oral carcinomas, providing evidence to support its important roles in tumour invasion and metastasis.^{5–10} PAI-1's closest phylogenetic relative protease nexin-1 (PN-1), also known as glia-derived nexin (GDN) or serpinE2,¹¹ has been much less studied in human cancers. A couple of recent studies^{12–14} do suggest a tumour-promoting role of PN-1. In order to initiate further studies of the role of PN-1 in cancers, we have now compared PN-1 expression in oral carcinomas and the corresponding normal oral tissues.

Patients and methods

Frozen surgical specimens of oral squamous cell carcinoma from 20 patients were obtained from Odense University Hospital, Denmark. Their informed consent and approval by the Ethics Committee were obtained according to Danish legislation. The median age of the 20 patients with oral squamous cell carcinoma was 63 years (range 48–94 years); there were six women and 14 men. There were 11 patients with lymph node metastases and nine without. The location of the carcinomas was six at the tongue, nine at the floor of the mouth, and five at the alveolar ridge. Firstly, hematoxylin–eosin staining of the sections was performed, the ratio between cancer and normal tissues were examined on histological sections. In 14 cases in which the cancer cells contributed 80% or more, the tissue blocks were used directly for RNA preparation, and the biopsy of the corresponding normal oral tissues from a region distant from the carcinoma was collected as control. In six cases, a laser microdissection system (P.A.L.M.) was used to separate tumour cells from adjacent normal tissue. Total RNA was extracted by routine procedures using the RNeasy Mini Kit (Qiagen). On-column DNase digestion by RNA-free DNase I (Qiagen) was performed during the RNA purification procedure. For detecting the PN-1 mRNA expression, reverse transcription reaction was performed with 2 µg DNA-free RNA using First-strand cDNA Synthesis Kit (Amersham). Q-real time PCR was performed as described previously.¹⁵ The primers were PN-1 QRT-Forward, AGCCTCTGCCTGTGATTCCATCA, PN-1 QRT-Reverse, ACCCTTGAAATACACTGCGTTGAC (136 bp product, from 638 to 773 bp). GAPDH mRNA was amplified as an internal control to normalise the data of the PN-1 mRNA level.¹⁵ The annealing temperatures were 59 °C for PN-1 and 62 °C for GAPDH. All PN-1 mRNA levels were

expressed relative to the PN-1 mRNA in the HT-1080 cell line, which has a high PN-1 expression and was run as a positive control in each analysis. Each sample was analysed three times for PN-1 mRNA and presented as the mean $\pm$ SD. Paired *t* test, Wilcoxon rank sum test and Fisher's exact probability test were performed.

To produce recombinant human PN-1, full length PN-1 cDNA was amplified from HT-1080 cell mRNA, using the primers PN-WF (TGGTGGAAGGAACCATGAACT) and PN-WR (GTAGTCGTTGCTTTGCATGG) (from +187 to +1434 bp relative to the start site of transcription). The cDNA was subcloned into TOPO TA cloning vector pCR2.1 (Invitrogen, Copenhagen). The sequence was verified and the PN-1 cDNA was cloned into pcDNA3 and expressed in HEK293 cells by standard methods. The PN-1 was purified from the conditioned medium by chromatography on a heparin-Sepharose column (Kousted, unpublished). A commercial affinity-purified goat anti-human Serpin E2 (PN-1) antibody was obtained from R&D Systems, Inc. (Minneapolis, MN 55413, USA). Available tissues from six oral carcinomas and matched normal control tissues were taken from -80°C and homogenised immediately in 0.1 mol/l Tris, pH 8.1, 0.5% Triton X-100, 10 mmol/l ethylenediaminetetraacetic acid, and 10 µg/ml aprotinin (10 µl/mg tissue). The homogenate was centrifuged at 10,000g for 10 min to remove cell debris and nuclei. The supernatants will be referred to as "tissue extracts". Samples each corresponding to ~ 80 µg of total protein were subjected to SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride filters (Immobilon-P; Millipore, Bedford, MA).⁵ Fifteen nanograms of purified recombinant human PN-1 were electrophoresed in parallel in the gels, as positive control. The filters were incubated with the anti-PN-1 antibody in a concentration of 0.1 µg/ml in 10% human plasma. The primary antigen–antibody reactions were visualised with peroxidase-conjugated rabbit anti-goat antibody (P0160, DakoCytomation) and the ECL system (Amersham Pharmacia, Copenhagen). A parallel negative control immunoblotting analysis was performed by omitting the primary antibody.

Results

We found that the average PN-1 levels in tumours and normal tissues were significantly different, 20.2 ± 20.1 vs. 6.73 ± 5.31 ($p < 0.0001$ by paired *t* test) (Fig. 1). It was increased up to 13 fold in tumour samples compared with the average level in normal tissues. The PN-1 mRNA level was significantly higher in tumours from patients with lymph node metastasis than in tumours from patients without, presenting median values as 29.1 and 8.9, respectively ($p = 0.031$ by Wilcoxon rank sum test). No difference was found between the PN-1 levels of the corresponding normal tissues from these two patient groups. Furthermore, we divided patients into two groups according to optimised threshold for the PN-1 mRNA level, above or below 15. Correlation between lymph node metastasis and PN-1 mRNA level was analysed using Fisher's exact probability test. A significant correlation was found ($p = 0.008$) (Fig. 2).

In order to estimate PN-1 protein levels in the tissue samples, an immunoblotting analysis was performed with an anti-PN-1 polyclonal antibody. In four of six available pa-

Figure 1 PN-1 mRNA expression level in 20 oral carcinomas and matched normal tissues. PN-1 mRNA levels were determined by Q-real time RT PCR and normalised against the GAPDH mRNA level. The PN-1 level was given as a percentage of the level in HT-1080 cells. The figure shows means $\pm$ SD for three independent determinations. The level in tumours (black bars) was significant elevated compared with the level in corresponding normal tissues (grey bars) ($p < 0.0001$, as evaluated by a paired t test). The patients are organised into two sub-groups in the figure, according to lymph node involvement, as indicated. The location of the carcinomas is indicated: tongue (T1–6), the floor of the mouth (F1–9), and alveolar ridge (A1–5).

Figure 2 Comparison of tumour PN-1 mRNA level in patients with and without lymph node metastasis. A significant difference was found between the two groups with (grey circles) or without (black circles) lymph node metastasis, as evaluated by Wilcoxon rank sum test ($p = 0.031$). The short horizontal bars indicate the median values. A significant correlation was also found between lymph node metastasis and PN-1 mRNA level, as evaluated by Fisher's exact probability test ($p = 0.008$). A dot-line represents the optimised threshold for the PN-1 mRNA level as 15. "n" indicates number of patients.

tient samples, the analysis showed a band co-migrating with recombinant human PN-1. The band was strong in tumour extracts but weak or undetectable in the corresponding normal tissue extracts. No specific signal could be detected in

the negative control blotting. An example of an immunoblotting analysis was shown in Figure 3a. By using the purified PN-1 as a standard, the relative level of PN-1 in the tissue samples was calculated from the intensity of the

Figure 3 Comparison of PN-1 protein level in tissue extracts from tumours and extracts of matched normal tissue of oral carcinoma patients. (a) Protein M_r marker (Fermentas, Helsingborg, Sweden), 15 ng of purified recombinant PN-1(HEKPN-1), extracts from samples of normal mucosa and tumour (80 μ g total protein for each) from patient T2, as indicated, were resolved by SDS-PAGE and then transferred electrophoretically to polyvinylidene difluoride filters, which were analysed for PN-1 by a primary goat anti-human PN-1 antibody and a secondary peroxidase-conjugated rabbit anti-goat IgG antibody. (b) The density between blotting bands from six patient samples of tumour (black bar) and normal tissue (gray bar) was analysed by programme Quantity One, and presented as average OD. The case numbers were indicated in accordance to Figure 1.

bands by scanning the films. The variation in PN-1 protein levels in tumour extracts and normal tissue extracts agreed with the variation in PN-1 mRNA levels (Fig. 3b).

Discussion

PN-1 is an inhibitor not only of uPA and tPA, but also of thrombin, plasmin and trypsin.¹¹ It may be hypothesised that it, like PAI-1, promotes tumour growth, cancer cell-directed tissue remodelling, and tumour invasion by locally stabilising specific extracellular matrix structures in an environment with a high uPA activity. A functional overlap between PAI-1 and other inhibitors such as PN-1 could perhaps explain a lack of an effect of PAI-1 gene deficiency in the MMTV-PyMT transgenic breast cancer model.¹⁶ PN-1 is expressed in a wide variety of normal tissues, in central and peripheral nervous system, muscle, platelet, and placenta.^{17–20} Recently, published findings suggest a tumour biological function of PN-1. A gene profile analysis showed a consistent overexpression of PN-1 in a number of cultured murine cell types transformed by a tumourigenic adenovirus 12 strain, whilst cells transformed by a non-tumourigenic adenovirus 12 had negligible PN-1 expression.¹⁴ Buchholz et al.¹² showed that PN-1 expression was weak or non-detectable in normal pancreas and chronic pancreatitis, but strong in the majority of a small number of investigated pancreatic carcinomas as well as gastric and colorectal cancer samples. Using an animal model, these authors also pre-

sented evidence that PN-1 may promote local invasion of pancreatic tumours by favouring extracellular matrix deposition as they also found the PN-1 overexpressing tumours contained larger amounts of extracellular matrix proteins and more prominent fibrous bundles covering tumour cell areas than non-PN-1 expressing tumours. The surrounding fibers showed positive staining for type I collagen and fibronectin as well as laminin. Candia et al.¹³ found an elevated PN-1 mRNA level in 21 out of 26 human breast carcinomas. The PN-1 mRNA level correlated directly with the PAI-1 and uPA mRNA levels.

Our results of overexpression of PN-1 mRNA in oral carcinomas are in good agreement with the recent reports on human pancreatic, gastric, colorectal, and breast cancers.^{12–14} We also found that the increased PN-1 level was correlated with positive lymph node metastasis, a correlation which has not been found in previous uPA and PAI-1 studies in oral carcinomas.^{8,9} In addition, we found an increased PN-1 protein level in oral carcinomas, indicating the overexpression of PN-1 may have a tumour biological function similar to that of PAI-1, by interacting within uPA system.

We could conclude that PN-1 is frequently overexpressed in oral squamous cell carcinoma and may correlate with lymph node metastasis. One way of understanding better for the roles of PN-1 in tumour progression would be the development to an antibody of PN-1 with sufficient affinity and specificity to detect PN-1 expressing cell types in different kinds of carcinomas by immunohistochemical staining.

Conflict of Interest Statement

We have no conflict of interest.

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