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Podoplanin expression in peritumoral keratinocytes predicts aggressive behavior in extramammary Paget's disease

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ABSTRACT

Background: Recent studies have demonstrated podoplanin expression in several tumors, which has been associated with lymph node metastasis and poor prognosis. Podoplanin expression in peritumoral cells such as cancer-associated fibroblasts also correlates with tumor progression in several cancers. However, podoplanin expression and its association with extramammary Paget's disease (EMPD) remain unclear.
Objective: In this study, we examined whether the presence of podoplanin expression in tumor cells or peritumoral basal keratinocytes correlated with aggressive behavior in patients with EMPD and investigated the mechanisms of podoplanin-mediated tumor invasion in this disorder.
Methods: Skin samples of 37 patients with EMPD were investigated by immunohistochemical analysis. The functions of podoplanin in keratinocytes were examined *in vitro* by RT-PCR and with invadopodia gelatin-degradation assays using HaCaT cells.
Results: Podoplanin was not identified in tumor cells in all cases. Podoplanin expression in peritumoral basal keratinocytes was observed in 25 patients (67.6%). In *in situ* EMPD, 50% of cases (9 in 18) exhibited podoplanin-positive keratinocytes, whereas 84.2% (16 in 19) demonstrated positive staining in invasive EMPD ($P < 0.05$). Podoplanin expression in peritumoral keratinocytes was also associated with tumor thickness ($P < 0.005$). By immunohistochemical analysis, podoplanin-positive peritumoral keratinocytes were found to be negative for E-cadherin, one of the major adhesion molecules of keratinocytes, which might contribute to tumor invasion into the dermis through a crack in the basal cell layer induced by down-regulation of cell adhesion therein. We further found that podoplanin-positive keratinocytes exhibited invadopodia, which are thought to function in the migration of cancer cells through tissue barriers, indicating that podoplanin-positive peritumoral basal keratinocytes might assist tumor invasion by degrading the extracellular matrix.
Conclusion: The presence of podoplanin expression in peritumoral keratinocytes correlates with aggressive behavior in EMPD and might therefore serve as a useful prognostic marker for patients with EMPD.

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1. Introduction

Extramammary Paget's disease (EMPD) represents a major life-threatening skin cancer that shows possible differentiation toward the apocrine glands. The prognosis for patients with metastatic disease is extremely poor. Several new therapeutic strategies have been attempted [1,2]; however, surgical excision during early-stage disease remains the most effective treatment and no established treatment is available for the advanced stage of EMPD. Therefore, predictive markers to indicate aggressive behavior of the tumor are desired to identify high-risk patients with EMPD.

Abbreviations: EMPD, extramammary Paget's disease; TGF-β, transforming growth factor-β; ECM, extracellular matrix; IE, *in situ* in the epidermis; MDI, microinvasion into the papillary dermis; DI, deep invasion into the reticular dermis.

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Podoplanin is a transmembrane glycoprotein that is known as a lymphatic endothelial marker. Recently, podoplanin up-regulation was observed in the invasive front of several cancers [3–7] and was shown to mediate invadopodia, which serve as a localized source of matrix proteases and play pivotal roles in cancer cell invasion and extravasation by degrading extracellular matrix (ECM) [8]. In addition, podoplanin expression in peritumoral cells such as cancer-associated fibroblasts also correlates with tumor progression in certain cancers including melanoma [9–11]. These observations suggest that podoplanin plays an important role in tumor invasion.

To determine its potential role as a marker for tumor aggressiveness in EMPD, in this study we examined whether the presence of podoplanin expression in tumor cells or peritumoral keratinocytes correlated with aggressive tumor behavior in patients with EMPD and investigated the mechanisms of podoplanin-mediated tumor invasion in EMPD.

2. Materials and methods

2.1. Subjects

We enrolled a cohort of 37 cases of EMPD tissues that had been surgically removed at our hospital from 2006 to 2015. The study was approved by the local ethical committee of Kyoto Prefectural University of Medicine and conducted in accordance with the Declaration of Helsinki.

2.2. Immunohistochemistry

Immunohistochemistry for podoplanin, E-cadherin, and transforming growth factor (TGF)-β was performed on formalin-fixed, paraffin-embedded tissue with commercially available antibodies (podoplanin: D2-40, mouse monoclonal antibody, ready to use, Nichirei Biosciences, Inc., Tokyo, Japan; E-cadherin: mouse monoclonal antibody, Leica-Novocastra, Wetzlar, Germany; and TGF-β: rabbit polyclonal antibody, Yanaihara, Shizuoka, Japan). The D2-40 antibody was used as supplied after heat-induced epitope retrieval. The E-cadherin antibody was used at a dilution of 1:250 after heat-induced epitope retrieval. The TGF-β antibody was used at a dilution of 1:200. Giemsa staining was used as a counterstain to distinguish melanin granules from positive stain in cases of deep melanin deposition. A specimen was considered positive for podoplanin expression in peritumoral keratinocytes when staining was observed in ≥20% of the lesion. For the evaluation of cytoplasmic podoplanin expression in tumor cells, a specimen was considered positive when ≥5% of tumor cells showed distinct podoplanin expression.

The analyses were performed independently by three observers (Z.C., J.A., and E.K.) blinded to the respective clinical data.

2.3. Tumor thickness measurement

Tumor thickness of the sample was measured in the same manner as proscribed in the latest melanoma classification of the American Joint Committee on Cancer [12] as described previously [13]. The greatest tumor thickness in the section was recorded and used in the experiments.

2.4. Cell culture

Human keratinocyte cells (HaCaT cells purchased from CLS Cell Lines Service GmbH, Eppelheim, Germany) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. siRNA transfection

We transfected 50 nM control or podoplanin siRNA (Hs_PDPN_1, Cat. S103060260, QIAGEN, Düsseldorf, Germany) into HaCaT cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. To confirm the efficacy of siRNA, cells were harvested 24 h after transfection and qRT-PCR was performed as described in Section 2.6. For the invadopodia assay, cells were used 24 h after transfection.

2.6. RNA isolation, cDNA synthesis, and quantitative reverse transcription-polymerase chain reaction amplification (qRT-PCR)

RNA was extracted using ISOGENII (NIPPON GENE, Toyama, Japan) according to the manufacturer's instructions, and processed for cDNA synthesis and qRT-PCR as previously described [14]. Following addition of 10 ng/ml recombinant human TGF-β (Peprotech, Rocky Hill, NJ, USA) to the cultured NHEKs, cells were harvested after 24 h. Podoplanin gene expression levels were normalized based on the levels of 18S RNA, an internal reference gene. The primers used in the study were obtained from QIAGEN.

2.7. Invadopodia gelatin-degradation assays

The gelatin invadopodia assay (EMD Millipore, Billerica, MA, USA) was performed according to the manufacturer's protocol as described previously [8,15]. To assess the ability of cells to form invadopodia and degrade ECM, $3–4 \times 10^5$ cells were plated on 8-well chamber slides coated with red fluorescent gelatin and incubated at 37 °C for 24 h. Then, the cells were stained with phalloidin according to the manufacturer's protocol. The cells were observed with an Olympus confocal laser scanning microscope FV-1000 (Olympus, Tokyo, Japan). Three images from different areas in

Table 1
Clinical data and correlation between podoplanin expression and various clinicopathological parameters as analyzed with the chi-square (χ^2) and Mann–Whitney *U* tests.

Parameter	Number of cases	Podoplanin expression in keratinocytes		
		Positive (%)	Negative (%)	*P* value
Sex				
Male	29	22 (88)	7 (58)	
Female	8	3 (12)	5 (42)	
Median age (y.o.)		74	75	
Location				
Genital	32	24 (96)	8 (67)	0.008
Axillary	4	0 (0)	4 (33)	
Perianal	1	1 (4)	0 (0)	
Tumor invasion				
In situ	18	9 (36)	9 (75)	0.026
Invasive	19	16 (64)	3 (25)	
Tumor thickness				
<0.5 mm	13	5 (20)	8 (67)	0.0028
0.5 to <1.0 mm	13	9 (36)	4 (33)	
≥1.0 mm	11	11 (44)	0 (0)	
Nodules				
Absent	31	19 (76)	12 (100)	0.064
Present	6	6 (24)	0 (0)	
Invasion level				
IE	17	8 (32)	9 (75)	0.028
MDI	13	10 (40)	3 (25)	
DI	7	7 (28)	0 (0)	
LN metastasis				
Positive	4	4 (16)	0 (0)	0.192
Negative	33	21 (84)	12 (100)	

y.o.: years old; IE: *in situ* in the epidermis; MDI: microinvasion into the papillary dermis; DI: deep invasion into the reticular dermis; LN: lymph node.

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