Review

Role of podoplanin expression in squamous cell carcinoma of upper aerodigestive tract

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Summary. Podoplanin, a type-1 transmembrane glycoprotein, was originally named due to its expression in renal podocytes of rats. It was subsequently detected in a variety of normal human tissues, including lymphatic endothelium. Although podoplanin has been identified as the endogenous ligand of C-type lectin-like receptor 2 (CLEC-2) on platelets, its physiological functions and pathways remain largely unknown. A role in lymphangiogenesis has been suggested, since podoplanin-deficient mice were found to die at birth with a phenotype of dilated, malfunctioning lymphatic vessels and lymphedema. Podoplanin is invariably expressed in some tumors, such as lymphangioma, seminoma and follicular dendritic cell tumor, but tumor cell expression of podoplanin is highly variable in squamous cell carcinoma (SCC). It has been found that high podoplanin expression is associated with lymph node metastasis and poor prognosis in SCC of the upper aerodigestive tract. Now there is growing evidence that podoplanin is also involved in carcinogenesis, cell motility, tumor invasiveness, platelet aggregation and hematogenous metastasis. Additionally, animal studies confirmed some *in vivo* effects of podoplanin-overexpressing tumors, including formation of more tumor lymphatic vessels, larger lymph node metastases, more platelet aggregation, and more pulmonary metastases. Several recently developed anti-podoplanin antibodies, such as NZ-1, P2-0 and hP2-0, have been shown to attenuate podoplanin-induced platelet aggregation and prevent experimental hematogenous metastasis in nude mice. These antibodies may be applied in preclinical and clinical studies to evaluate the possibility of podoplanin-targeted therapy.

Key words: Podoplanin, Squamous cell carcinoma, Upper aerodigestive tract, Prognosis, Targeted therapy

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Basic facts and physiological roles of podoplanin

Podoplanin (T1α, aggrus and gp36) is a 38kDa type-1 transmembrane glycoprotein (Wicki and Christofori, 2007), which was originally named due to its expression in renal podocytes of rats (Breiteneder-Geleff et al., 1997). It was subsequently detected in various normal human tissues, including lymphatic endothelial cells, renal podocytes (Breiteneder-Geleff et al., 1999), skeletal muscle, lung, heart, placenta (Martin-Villar et al., 2005), myoepithelial cells of glands (Kanner et al., 2010), osteoblasts, mesothelial cells (Ordonez, 2006), follicular dendritic cells (Yu et al., 2007), Schwann cells (Jokinen et al., 2008), and occasionally in basal layer of epidermis and esophageal mucosa (Schacht et al., 2005; Chuang et al., 2009) (Figs. 1, 2a).

Although podoplanin has been identified as the endogenous ligand of C-type lectin-like receptor 2 (CLEC-2), a tyrosine kinase-dependent receptor present most abundantly in megakaryocytes and platelets, as well as in some hematopoietic cells (Suzuki-Inoue et al., 2007; Suzuki-Inoue, 2011), its physiological functions and pathways remain largely unknown. Podoplanin is probably involved in the regulation of podocyte foot process shape and glomerular filtration (Koop et al., 2008). Moreover, it has been suggested to play an important role in lymphangiogenesis (Schacht et al., 2003). Podoplanin is highly expressed not only in lymphatic endothelial cells throughout the body, but also in their bone marrow progenitors capable of lymphatic neovascularization (Lee et al., 2010). It is also essential for normal development of lymphatic vessels, since podoplanin knockout mice were found to die at birth with a phenotype of dilated, malfunctioning lymphatic vessels and lymphedema (Schacht et al., 2003). Specifically, the critical separation of blood and lymphatic circulation during embryo development depends on interaction between endothelial podoplanin of the developing lymph sac and circulating platelets (Uhrin et al., 2010). Using primary human lung

microvascular lymphatic endothelial cells, it has been demonstrated that small interfering RNA-mediated silence of podoplanin gene expression dramatically blocks capillary tube formation in Matrigel (Navarro et al., 2008), supporting a critical role of podoplanin in lymphangiogenesis.

Podoplanin and squamous cell carcinoma

Podoplanin as a lymphatic endothelial marker

Because podoplanin is expressed by lymphatic endothelium but not by blood vessel endothelium (Breiteneder-Geleff et al., 1999; Kahn and Marks, 2002) (Fig. 1a), it is widely used as a specific marker of lymphatic vessels to evaluate lymphatic vessel invasion (LVI) (Fig. 2b) and lymphatic microvessel density (LMVD). Both LVI and LMVD have been shown to correlate with lymph node metastasis, advanced stage or short survival in various cancers, including non-small cell lung carcinoma (Kadota et al., 2008), colorectal adenocarcinoma (Matsumoto et al., 2007), invasive breast carcinoma (El-Gohary et al., 2008), oral squamous cell carcinoma (SCC; Michikawa et al., 2012) and esophageal SCC (Mori et al., 2007; Nakayama et al., 2007).

Podoplanin expression in tumor cells

Podoplanin expression was also found in a variety of tumor cells, including vascular tumors (Breiteneder-Geleff et al., 1999), malignant and benign soft tissue tumors (Xu et al., 2011), mesothelioma (Kimura and Kimura, 2005), seminoma (Idrees et al., 2010), brain
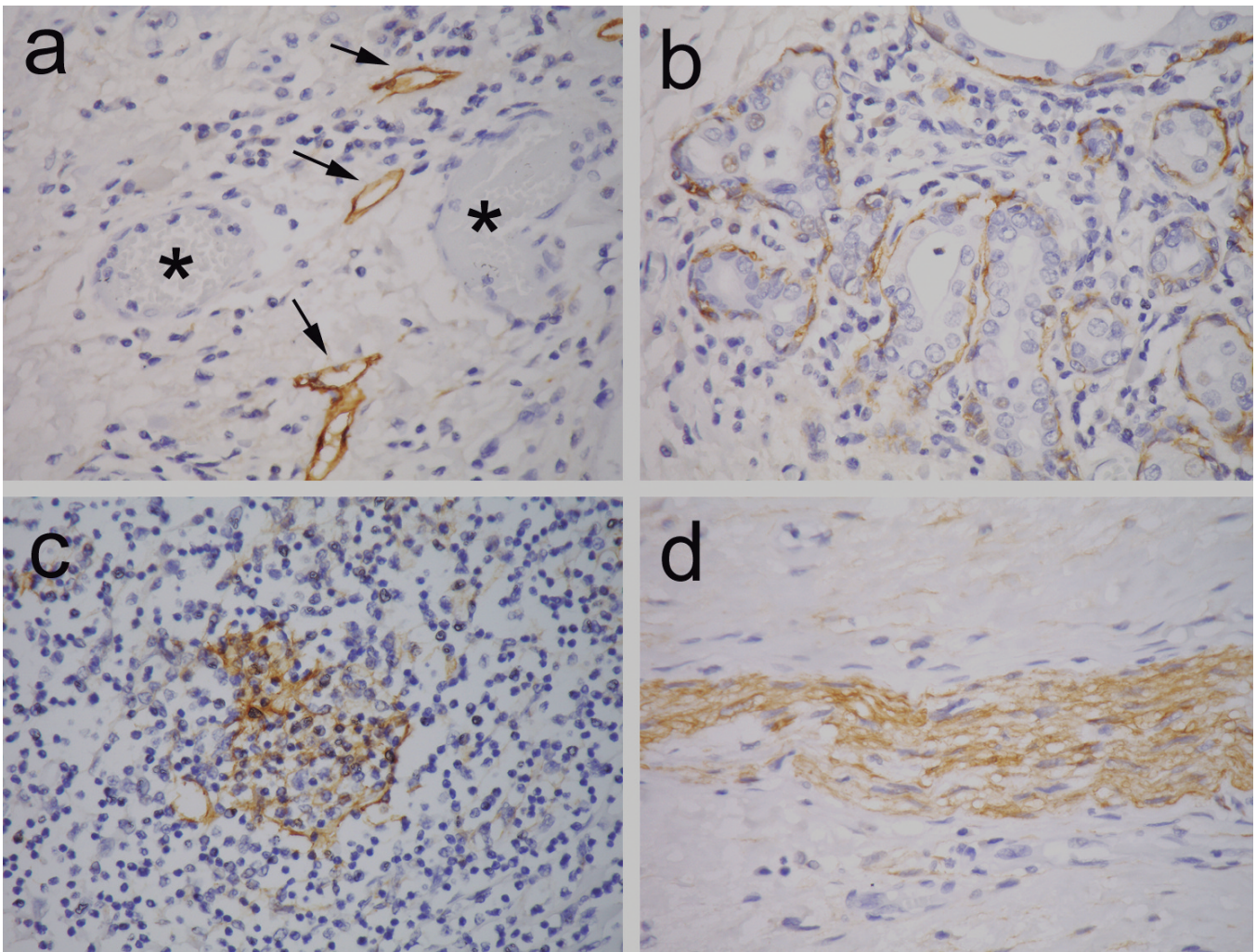

Fig. 1. Podoplanin expression in lymphatic endothelial cells (**a**, arrows), myoepithelial cells of glands **(b)**, follicular dendritic cells of a lymphoid follicle **(c)**, and Schwann cells of a nerve **(d)**. Note that the endothelial cells of blood vessels (a, asterisks) do not express podoplanin. x 400.

tumors (Shibahara et al., 2006; Mishima et al., 2006), follicular dendritic cell tumor (Yu et al., 2007), and SCC of the oral cavity, esophagus, larynx, skin and uterine cervix (Dumoff et al., 2005; Wicki et al., 2006; Yuan et al., 2006). Some of these tumors, such as lymphangioma, seminoma and follicular dendritic cell tumor, were found to invariably express podoplanin, which could then serve as a useful marker to confirm the diagnosis.

Podoplanin and SCC of upper aerodigestive tract

SCC of the upper aerodigestive tract (UADT), including head and neck and esophagus, is one of the most common types of cancer. Despite a wide range of primary sites, SCCs of UADT share similar risk factors, such as consumption of alcohol, tobacco and betel quid

(Lee et al., 2011). Synchronous and metachronous SCCs are also common in UADT (Wind et al., 2000). These findings suggest similar pathogenesis and tumor biology in SCCs of UADT.

Remarkable variations in podoplanin expression have been observed among cases of SCC of UADT (Yuan et al., 2006; Chuang et al., 2009; Huber et al., 2011; Chao et al., 2012) (Fig. 2c,d). Tumor cell expression of podoplanin in oral SCC is associated with lymph node metastasis and poor prognosis (Yuan et al., 2006). It also correlates with sentinel lymph node metastasis in early SCC of oral cavity and oropharynx (Huber et al., 2011).

Previously, we first found that high podoplanin expression in esophageal SCC strongly correlates with clinical nodal metastasis, which is associated with short survival (Chuang et al., 2009). Specifically, in a large
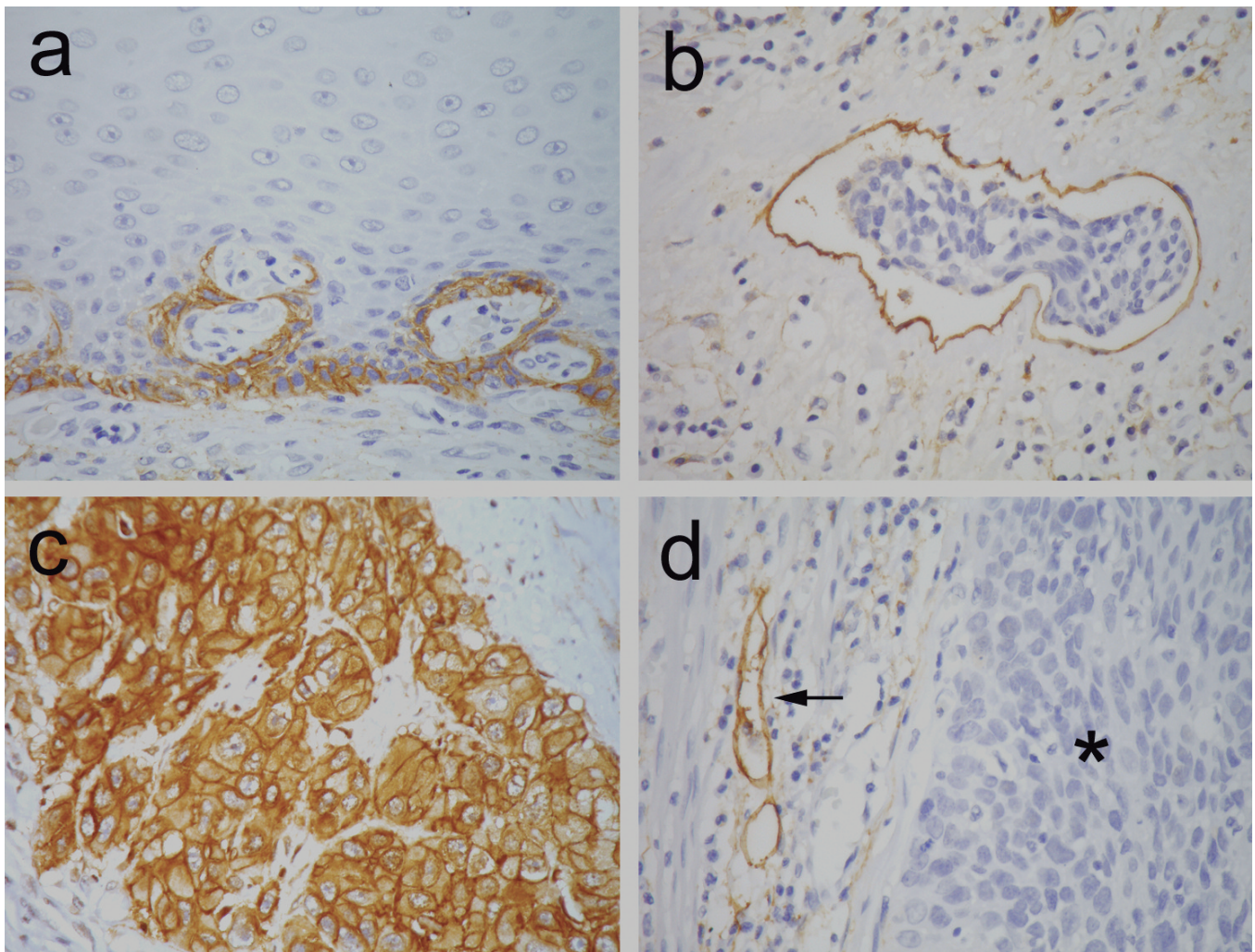

Fig. 2. Podoplanin expression in basal layer of esophageal mucosa **(a).** Immunostaining for podoplanin is helpful to identify lymphatic vessel invasion **(b).** Squamous cell carcinoma with high **(c)** or low **(d,** asterisk) podoplanin expression in tumor cells. Lymphatic endothelial cells **(d,** arrow) serve as an internal positive control. x 400.

cohort of ypT3N0 esophageal SCC patients after chemoradiotherapy, high podoplanin expression correlates with LVI and is the most important independent prognostic factor in a multivariate analysis (Chao et al., 2012). Since esophageal SCC is a highly lethal disease with an overall survival rate of 10-20% (van Meerten and van der Gaast, 2005), podoplanin expression as a prognostic factor could help us to select patients for more aggressive and/or targeted therapy.

Possible mechanisms of podoplanin in SCC of UADT

Clinical evidence supports a role of podoplanin in the tumorigenesis, spreading and metastasis of SCC of UADT (Yuan et al., 2006; Kawaguchi et al., 2008; Chuang et al., 2009; Rodrigo et al., 2010; Huber et al., 2011; Chao et al., 2012). However, the mechanisms of its action are largely unclear. Previous studies have shown that podoplanin induces tumor formation and progression both *in vitro* and *in vivo,* and a number of mechanisms have been suggested.

Lymphangiogenesis and lymph node metastasis

In an animal study using a human MCF7 breast carcinoma xenograft model in nude mice, the podoplanin-overexpressing cell clones induced formation of more tumor lymphatic vessels and larger metastatic foci in draining lymph nodes (Cueni et al., 2010). This study provides *in vivo* evidence that tumor cell expression of podoplanin promotes lymphangiogenesis and nodal metastasis.

Podoplanin and carcinogenesis

Oral leukoplakia is a premalignant lesion harboring an increased risk of developing SCC. In a series of 150 oral leukoplakia patients with long-term follow-up, podoplanin expression was strongly associated with cancer development (Kawaguchi et al., 2008). Of note, in a multivariate analysis using podoplanin (positive vs. negative) and histology (dysplasia vs. hyperplasia) as cofactors, podoplanin was the only independent risk factor for oral cancer development. A similar association of podoplanin expression and cancer development has also been observed in premalignant lesions of the larynx (Rodrigo et al., 2010).

Oncogenic transcription factor activator protein 1 (AP-1; mainly composed of Jun and Fos proteins) is required for neoplastic transformation of epidermal squamous cells *in vitro* and tumor promotion and progression *in vivo* (Durchdewald et al., 2008). In a well established mouse model of skin carcinogenesis (K5-SOS-F transgenic mice), the podoplanin gene was identified as a novel direct Fos target gene, supporting an important role of podoplanin in neoplastic transformation and tumor progression (Durchdewald et al., 2008). Podoplanin has also been suggested as a cancer stem cell marker in a human SCC cell line A431 (Rahadiani et al., 2010).

Cell motility and tumor invasiveness

Epithelial to mesenchymal transition (EMT) is a phenotypic conversion of epithelial cells, which lose their polarity and cohesiveness and acquire an increased motility characteristic of fibroblasts. EMT is not only required for morphogenesis during embryo development, but is also involved in pathological situations, including wound healing, tumor invasion and metastasis (Bissell and Radisky, 2001; Thiery, 2002).

Transfection studies using cultured normal or cancer cells have been performed to evaluate the *in vitro* effect of podoplanin expression. In human keratinocytes and MCF7 breast carcinoma cells, forced expression of podoplanin has been demonstrated to result in a dramatic change of cell morphology with a decrease of cellular stress fibers and formation of filopodia-like protrusions (Scholl et al., 1999; Wicki et al., 2006). In MCF7 cells, podoplanin is capable of promoting invasion of tumor cell sheets into neighboring tissue, a process called collective cell migration, without initiating the pathway of EMT (Wicki et al., 2006). It was later found that podoplanin can also promote a complete EMT in another cell type, Madin-Darby canine kidney (MDCK) type-II epithelial cells (Martín-Villar et al., 2006). In addition, podoplanin was shown to associate with CD44 to promote directional migration in SCC cells (Martín-Villar et al., 2010). These findings support a role of podoplanin in increasing cell motility and tumor invasiveness.

Platelet aggregation and hematogenous metastasis

It has long been recognized that platelets are involved in cancer spread (Suzuki-Inoue, 2011; Nash et al., 2002). Different kinds of tumors have been found to aggregate platelets and thus facilitate tumor metastasis (Kitagawa et al., 1989; Katagiri et al., 1991; Kato et al., 2003). Platelet aggregates surround tumor cells, protect them from shear stress and natural killer cells, and facilitate tumor cell nest formation in the blood stream (Nieswandt et al., 1999; Suzuki-Inoue, 2011). Activated platelets also release growth factors, which stimulate angiogenesis or tumor growth (Suzuki-Inoue, 2011).

Podoplanin is an endogenous ligand of CLEC-2, a signaling receptor expressed on the surface of platelets (Suzuki-Inoue et al., 2007). Binding to CLEC-2, podoplanin can induce platelet aggregation through a mechanism similar to that of rhodocytin, a platelet activating snake venom. In an animal study, podoplanin expression in Chinese hamster ovary (CHO) cells was noticed to induce platelet aggregation and promote pulmonary metastases (Kunita et al., 2007), providing strong *in vivo* evidence that tumor expression of podoplanin facilitates hematogenous spread.

Podoplanin as a therapeutic target

Since podoplanin is involved in carcinogenesis, tumor invasiveness, lymphatic spread and hematogenous metastasis, it is conceivable to see podoplanin as a potential target of treatment in cancer patients, especially for tumors overexpressing podoplanin. Various monoclonal antibodies against human or mouse podoplanin have been established for potential therapeutic use, but most of them cannot interfere with the interaction between podoplanin and its receptor, CLEC-2. Previously, a rat anti-human podoplanin antibody, NZ-1, was found to neutralize this interaction, inhibit podoplanin-induced platelet aggregation, and dramatically inhibit experimental pulmonary metastasis of podoplanin-overexpressing CHO cells in nude mice (Kato et al., 2006, 2008). However, since NZ-1 is a rat antibody, it cannot be precisely examined in universally used mouse cancer models due to species barrier (Nakazawa et al., 2011). Recently, a new monoclonal mouse anti-human podoplanin antibody, P2-0, was established (Nakazawa et al., 2011). P2-0 recognized the conformation near the bioactive O-glycosylation site at the Thr^{52} residue of podoplanin and attenuated its binding to CLEC-2 and platelet aggregation. Of note, P2-0 also prevented the experimental metastasis of human podoplanin-overexpressing CHO cells in a mouse model. Nakazawa et al. (2011) also developed a murine/human chimeric antibody, hP2-0, which can be used in further preclinical tests in monkeys.

Conclusions

Tumor cell expression of podoplanin in SCC of UADT is associated with lymph node metastasis and poor prognosis. There is growing evidence that podoplanin plays important roles not only in lymphangiogenesis and nodal metastasis, but also in carcinogenesis, cell motility, tumor invasiveness, platelet aggregation and hematogenous metastasis. The successful prevention of pulmonary metastasis using a monoclonal anti-podoplanin antibody in a mouse model suggests its potential usefulness in targeted therapy in the future.

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