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# Clinicopathological implications to micropapillary bladder urothelial carcinoma of the presence of sialyl Lewis X-decorated mucin 1 in stroma-facing membranes

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## Abstract

**Objectives:** Bladder urothelial carcinoma (UC) comprises more than 90% of all bladder cancers. Among several UC variants, micropapillary UC (MPUC) is a rare one with high potential for lymphovascular invasion and subsequent lymph node metastasis. Histologically, MPUC is characterized by the presence of small papillary carcinoma cell clusters surrounded by lacunar spaces. Immunohistochemically, the outer circumference of these clusters, that is, the stroma-facing membrane of carcinoma cells, is reportedly almost invariably positive for mucin 1 (MUC1) protein and to a lesser extent for sialyl Lewis X (sLeX) carbohydrates; however, the clinicopathological implications of these expression patterns have not been fully investigated.

**Materials and methods:** We performed immunohistochemical analysis of MPUC ($n = 11$) and conventional UC ($n = 57$) for MUC1 and sLeX to determine whether these factors immunolocalized. Dual immunofluorescence staining was also carried out to assess MUC1 and sLeX colocalization. We also performed Western blot analysis of Chinese hamster ovary cells misexpressing both recombinant epitope-tagged MUC1 and glycosyltransferases enabling sLeX biosynthesis.

**Results:** MPUC samples preferentially exhibited both MUC1 protein and sLeX carbohydrate expression on the stroma-facing membrane of carcinoma cells. Based on univariate analysis, MUC1 expression in that pattern was positively correlated with tumor extension, lymphovascular invasion, lymph node metastasis, disease stage, and relatively poor patient prognosis. A comparable sLeX expression pattern also correlated positively with tumor extension and nodal metastasis. Based on multivariate analysis, localization of MUC1 and sLeX on the stroma-facing side of the membrane was positively correlated with lymph node metastasis.

**Conclusions:** Overall, our immunofluorescence findings as well as immunoprecipitation analyses of Chinese hamster ovary cell transfectants strongly suggest that MUC1 is a potential scaffold protein for sLeX carbohydrates in MPUC. Both MUC1 and sLeX may cooperatively contribute to MPUC histogenesis and clinicopathological characteristics. 

*Keywords:* Bladder urothelial carcinoma; Micropapillary urothelial carcinoma; Mucin 1; Sialyl Lewis X

## 1. Introduction

Bladder cancer is one of the most common cancers worldwide, and more than 90% of cases are histologically classified as urothelial carcinoma (UC) [1,2]. Several UC histologic variants have been recognized, and among them, micropapillary UC (MPUC), first described by Amin et al. [3],

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is a rare form with high propensity for lymphovascular invasion and subsequent lymph node metastasis [1,4,5]. Reflecting its biological aggressiveness, the clinical stage of MPUC at initial presentation is usually more advanced than that of conventional UC [1,3,6].

Similar to micropapillary adenocarcinomas arising at other anatomical sites, MPUC is histologically characterized by cleft formation around carcinoma cell clusters: specifically, carcinoma cells form small papillary clusters without fibrovascular cores, and these clusters are present within lacunar spaces formed in tumor stroma [3,5]. Immunohistochemically, the outer circumference of the clusters, namely, the stroma-facing side of carcinoma cell membranes, is almost invariably mucin 1 (MUC1)-positive [7,8].

MUC1, also known as epithelial membrane antigen, is a species of membrane-tethered glycoprotein composed of 2 noncovalently bound subunits: a larger N-terminal subunit consisting of a long extracellular domain and a smaller C-terminal subunit containing a short extracellular domain, a transmembrane region, and a cytoplasmic tail [9–11]. The long extracellular domain of the former contains a serine/threonine-rich region termed "variable number tandem repeats" (VNTR), which is usually extensively *O*-glycosylated with a series of Lewis blood group-related carbohydrate antigens, such as sialyl Lewis A (sLeA) and sialyl Lewis X (sLeX) [11–13].

We previously demonstrated that 14 of 78 (17.9%) bladder UC cases expressed 6-sulfated sLeX [2]. In that report, we also noted that, in addition to 6-sulfated sLeX, nonsulfated sLeX was present in all MPUC cases examined ($n = 5$), and similar to the expression pattern of MUC1, sLeX was expressed along the outer circumference of small papillary carcinoma cell clusters formed in MPUC [2]. However, the clinicopathological implications of these expression patterns have not been fully investigated.

This was undertaken to determine whether the presence of MUC1 and sLeX in stroma-facing membranes of MPUC correlates with patients' clinicopathological characteristics. We also performed immunofluorescence and immunoprecipitation analyses to determine whether MUC1 can serve as a scaffold protein for sLeX carbohydrates.

## 2. Materials and methods

### 2.1. Tissue samples and clinicopathological information

Formalin-fixed, paraffin-embedded tissue blocks of surgical specimens of either MPUC ($n = 11$; 10 males and 1 female; mean age = 68.9 years) or conventional UC ($n = 57$; 46 males and 11 females; mean age = 72.2 years) bladder cancer were retrieved from the pathology archives at University of Fukui Hospital. All tissue slides were reviewed by 2 experienced pathologists (Y.S. and M. K.) to confirm the initial diagnosis. Among 11 MPUC cases, 5 were initially diagnosed as conventional UC, and these cases were subsequently rediagnosed as MPUC based on the consensus of the 2 pathologists. Among 11 MPUC cases, 9 were pure MPUC and the other 2 were a mixture of MPUC and conventional UC, with the MPUC component representing 50% in one case and 20% in the other. Among 68 bladder UC cases, 66 received radical cystectomy and 2 received simple (without lymph node dissection) cystectomy. The median follow-up period was 27 months. Supplementary Table 1 shows detailed clinicopathological information of the patients. The Ethics Committee of the Faculty of Medical Sciences, University of Fukui, approved the study protocol to use human bladder cancer tissues and patients' clinicopathological information.

### 2.2. Immunohistochemistry

The following monoclonal antibodies served as primary antibodies: GP1.4 (mouse IgG; Leica Biosystems, Nussloch, Germany) and EPR1023 (rabbit IgG; Abcam, Cambridge, MA) both recognizing human MUC1; and KM93 recognizing sLeX (mouse IgM; Millipore, Billerica, MA). Conventional immunohistochemical staining for GP1.4 and KM93 was conducted as described [2,11]. Dual immunofluorescence staining for EPR1023 and KM93 was also carried out essentially as described [14].

### 2.3. Expression of recombinant epitope-tagged MUC1 in Chinese hamster ovary (CHO) cells expressing sLeX

Chinese hamster ovary (CHO) cells stably expressing CD34 and a set of glycosyltransferases enabling biosynthesis of sLeX attached to core 2-branched *O*-glycans, sialic acidα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→6(Galβ1→3)GalNAcα1→Ser/Thr were established and cultured as described [15]. Cells were transiently transfected with either pcDNA3.1/hygro-MUC1/FLAG harboring cDNA encoding FLAG-tagged MUC1 [11] or pcDNA3.1/hygro (mock) using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, and analyzed 36 hours later.

### 2.4. Immunoprecipitation and Western blot analysis

CHO cell transfectants described earlier were harvested in phosphate-buffered saline supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland) using a cell scraper, and the membrane fraction containing FLAG-tagged MUC1 was obtained as described [14,16]. The membrane fraction was then incubated with anti-FLAG M2 monoclonal antibody (mouse IgG; Sigma-Aldrich, St. Louis, MO) or, as negative control, nonimmune mouse IgG (Beckman Coulter, Indianapolis, IN) at 4°C for 60 minutes on a rotator. Protein G Sepharose Fast Flow (Sigma-Aldrich) was added to the membrane fraction/antibody mix, and then samples were incubated at 4°C for 60