Up-Regulation of Connexin 30 in Intestinal Phenotype Gastric Cancer and Its Reduction during Tumor Progression

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Short title: connexin 30 in gastric cancer

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Abstract

Aims: The mucin phenotype is associated with clinicopathological findings and tumorigenesis in gastric cancer (GC). The aim was to search for a novel marker regulating the intestinal phenotype of GC. ***Methods and Results:*** We performed microarray analyses, and *GJB6* (encoding connexin 30) was identified as a gene associated with the intestinal phenotype. Immunostaining of connexin 30 in 169 GC cases revealed that 47 (28%) cases were positive for connexin 30, while connexin 30 was negative in non-neoplastic gastric tissue. Connexin 30-negative GC cases showed more advanced T grade, N grade, and tumor stage than connexin 30-positive GC cases. Six (13%) GC cases positive for connexin 30 were histologically of the differentiated type. In addition, expression of gastric and intestinal phenotypes of GC was examined by immunostaining for MUC5AC, MUC6, MUC2, and CD10. Connexin 30 expression occurred more frequently in the intestinal phenotype (48%) than in other phenotypes (21%) of GC. ***Conclusion:*** These results indicate that expression of connexin 30 is a novel differentiation marker mediating the biological behavior of the intestinal phenotype GC.

Introduction

According to the World Health Organization, gastric cancer (GC) is the fourth most common malignancy world wide, with approximately 870,000 new cases occurring yearly. Mortality due to GC is second only to that of lung cancer [1]. Cancer develops as a result of multiple genetic and epigenetic alterations [2, 3]. Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment are major goals in this field [4]. Array-based hybridization [5] and serial analysis of gene expression (SAGE) [6] are currently the most common approaches to identify potential molecular markers for cancer.

GCs have been classified into two histological types, an intestinal type and a diffuse type, by Lauren [7], or a differentiated type and an undifferentiated type by Nakamura et al. [8], based on the tendency towards gland formation. It has been suggested that these two types involve distinct pathways during carcinogenesis [7-10]. Recent studies have demonstrated that GCs are also classified as having a gastric, gastric and intestinal mixed, or intestinal phenotype depending on the expression of mucin phenotypic markers [11-18]. The mucin expression and phenotype of tumors are associated with clinicopathological findings and tumorigenesis in GCs. However, the clinical importance of intestinal mucin in GCs is still controversial and no definite conclusions have been reached [12-18]. Candidate genes controlling gastric and intestinal phenotypes include several transcription factors [19]. The caudal-related homeobox 2 gene   (CDX2) is an intestine-specific transcription factor that is expressed in non-neoplastic mucosa from the duodenum to the distal colon, and is detected in GC with the intestinal phenotype [20]. SOX2, an Sry-like high mobility group box gastric transcription factor, is expressed in normal gastric mucosa and GC with the gastric phenotype

[21]. By performing microarray analyses, we recently discovered that expression of connexin 30 was observed in intestinal phenotype GC.

Connexins, a family of 20 trans-membrane proteins in humans, comprise the main subunits of gap junctions, which are specialized clusters of intercellular channels that allow adjacent cells to directly share ions and hydrophilic molecules of up to 1 kDa in size [22]. Gap junctional intercellular communication (GJIC) is thought to control tissue homeostasis and to coordinate cellular processes such as proliferation, migration and differentiation [23, 24]. Neoplastic transformation is frequently associated with a loss of GJIC and with a reduced expression of connexins in various tumors [25, 26]. Forced expression of connexins in connexin-deficient cell lines results in the inhibition of tumor growth and the induction of apoptosis *in vitro* as well as the prevention of tumor formation *in vivo* [27, 28]. On the other hand, accumulating evidence indicates that connexin 26, a connexin family member, is overexpressed in carcinomas including those of the head and neck, colon, and pancreas [29-32]. Increased connexin 26 expression has been observed in invasive breast carcinomas and metastatic lymph nodes [33, 34]. Together, these strands of evidence appear to contradict the conventionally held view of the role of connexins as tumor suppressors. The localization of connexin 30 has been observed in normal skin [35], cochlea [36] and brain [37]. Connexin 30 gene mutations cause dominant non-syndromic hearing loss [38, 39], and they have been identified in Clouston syndrome (hidrotic ectodermal dysplasia) [40]. Little is known about the role of connexin 30 in human neoplasia, While the expression of connexin 30 is decreased in human head and neck cancer [41] and in cervical dysplasia of the uterus [42], connexin 30 is up-regulated in human skin tumors [43]. Thus, the exact pathogenic mechanisms associated with connexin 30 in carcinogenesis remain obscure.

The present study represents the first detailed analysis of connexin 30 expression in GC. To clarify the pattern of expression and localization of connexin 30 in GC, we performed

immunohistochemical analysis of surgically resected GC samples. In addition, we investigated the association between connexin 30 and various markers determining the gastric/intestinal phenotypes (MUC5AC, MUC6, MUC2 and CD10).

Materials and Methods

Tissue Samples

Primary tumor samples and corresponding non-neoplastic gastric mucosa were collected from 169 patients with GC (123 men and 46 women; age range, 29-88 years; mean, 70 years). Patients were treated at the Hiroshima University Hospital or affiliated hospitals. For RNA extraction, tissue samples obtained at the time of surgery were immediately embedded in O.C.T. compound (Sakura Finetechnical, Tokyo, Japan), frozen in liquid nitrogen and stored at -80˚C. For quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis, 18 GC samples and corresponding non-neoplastic mucosa samples were used. The samples were obtained during surgery at the Hiroshima University Hospital. We confirmed microscopically that the tumor specimens were predominantly (>50%) cancer tissue. Samples were frozen immediately in liquid nitrogen and stored at -80˚C until use. Samples of normal brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, uterus, bone marrow, spleen, peripheral leukocytes, and trachea were purchased from Clontech (Palo Alto, CA). For immunohistochemical analysis we used archival formalin-fixed, paraffin-embedded tissues from 169 patients who had undergone surgical excision for GC. The 169 GC cases were histologically classified as 102 of the differentiated type and 67 of the undifferentiated type, according to the Japanese Classification of Gastric Carcinomas [44]. Tumor staging was carried out according to the

TNM classification [45]. Because written informed consent was not obtained, identifying information for all samples was removed before analysis for strict privacy protection. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Quantitative RT-PCR

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ). Quantitation of *Connexin 30* mRNA levels in human tissue samples was done by real-time fluorescence detection as described previously [46]. *Connexin 30* primer sequences were 5'-CAG TTG CCT TCT CTC CGA GG-3' and 5'-CAT GGG ATG TTA CAC ACG CC-3'. PCR was performed with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously [47]. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Antibodies

Anti-connexin 30 antibody was purchased from Invitrogen/Zymed Laboratories Inc. (San Francisco, CA, USA). We used four antibodies for analysis of the GC phenotypes: anti-MUC5AC (Novocastra, Newcastle, UK) as a marker of gastric foveolar epithelial cells, anti-MUC6 (Novocastra, Newcastle, UK) as a marker of pyloric gland cells, anti-MUC2 (Novocastra, Newcastle, UK) as a marker of goblet cells in the small intestine and colorectum, anti-CD10 (Novocastra, Newcastle, UK) as a marker of microvilli of absorptive cells in the

small intestine and colorectum.

Immunohistochemistry

A Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical analysis. In brief, sections were pretreated by microwave treatment in citrate buffer for 15 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako, Carpinteria, CA, USA) for 20 min to block non-specific antibody binding sites. Sections were incubated with the following primary antibodies: anti-connexin 30 (diluted 1 : 50), anti-MUC5AC (1 : 50), anti-MUC6 (1 : 50), anti-MUC2 (1 : 50) and anti-CD10 (1 : 50). Sections were incubated with primary antibody for 1 h at 25 ∘C, followed by incubations with biotinylated mouse anti-rabbit IgG and peroxidase labeled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin.

Connexin 30 staining was classified according to the percentage of stained cancer cells. Expression was considered to be "negative" if <10% of cancer cells were stained. When at least 10% of cancer cells were stained, the result of immunostaining was considered "positive."

GC cases were classified into four phenotypes: gastric phenotype, intestinal phenotype, gastric and intestinal mixed phenotype, and unclassified phenotype. The criteria [20] for classification of gastric phenotype and intestinal phenotype were as follows. GCs in which more than 10% of the cells displayed the gastric or intestinal epithelial cell phenotype were gastric phenotype or intestinal phenotype cancers, respectively. Those sections that showed both gastric and intestinal phenotypes were classified as gastric and intestinal mixed phenotype, and those that lacked both the gastric and the intestinal phenotypes were classified

as the unclassified phenotype.

Double Immunofluorescence Staining

Double-immunofluorescence staining was performed as described previously [48]. Alexa Fluor 488-conjugated chicken anti-rabbit IgG and Alexa Fluor 546-conjugated goat anti-mouse IgG were used as secondary antibodies (Molecular Probes, Eugene, OR, USA).

Stastical Methods

Correlations between clinicopathologic parameters and connexin 30 staining were analyzed by Fisher's exact test. A *p* value of less than 0.05 was considered statistically significant.

Results

Expression of Connexin 30 in Systemic Normal Tissues and GC Tissues

Quantitative RT-PCR was performed to investigate the specificity of *connexin 30* expression in 16 normal organs. As shown in Fig. 1, *connexin 30* expression was clearly detected in the brain and the spinal cord and to a lesser extent in the bone marrow and uterus. However, expression of *connexin 30* was detected at low levels, or not at all, in other normal organs, including stomach. These results are consistent with those of a previous report [37]. Next, we analyzed quantitative RT-PCR in 18 GC samples. High levels of *connexin 30* (tumor/normal ratio > 2) were observed in 4 of the 18 GCs (22%).

Immunohistochemical Analysis of Connexin 30 in GC

Quantitative RT-PCR revealed obvious *connexin 30* expression in GC, although the levels were not very high. We therefore performed immunohistochemical analysis of connexin 30 in GC (Fig. 2A-2E). At first, we tested the specificity of the anti-connexin 30 antibody. Immunohistochemical analysis of normal skin tissue was performed, and connexin 30 was detected in the keratinocytes of the upper spinous layers and in those of the stratum granulosum (Fig. 2F). This result was consistent with a previous report [49]. Using this antibody, we performed immunostaining of connexin 30 in 169 GC and corresponding non-neoplastic gastric mucosa. As a result, connexin 30 expression was detected in 47 of the 169 GC (28%) and was seen on the cell membrane, especially the apical membrane (Fig. 2A, 2B). However, we sometimes observed its cytoplasmic accumulation (Fig. 2C). There was no difference in connexin 30 expression levels between intratumor areas and infiltrative margins.

Connexin 30 was scarcely expressed in any corresponding non-neoplastic gastric mucosa or intestinal metaplasia. Next, we analyzed the relationship between connexin 30 expression and clinicopathologic characteristics. Expression of connexin 30 was observed more frequently in the differentiated type of GC than in the undifferentiated type ($P <$ 0.0001)(Table 1). Localized distribution of connexin 30 positive GC cells in tumors that had more than one histological component were also often observed in differentiated GC components, rather than in undifferentiated components.

Furthermore, connexin 30 staining showed a significant inverse correlation with the depth of invasion ($P < 0.0001$), lymph node metastasis ($P = 0.0123$) and TNM stage ($P = 0.0014$). There was no significant association between connexin 30 staining and other parameters (age, gender or M grade).

Association between Connexin 30 Expression and Gastric/intestinal Mucin Markers

We next investigated the association between connexin 30 expression and various

markers determining the gastric/intestinal phenotypes. Out of the 169 cases examined, each molecule was detected in 80 (47%) cases for MUC5AC, 33 (20%) cases for MUC6, 60 (36%) cases for MUC2, and 35 (21%) cases for CD10. 169 GC cases were classified into four phenotypes: 50 (30%) were the gastric phenotype, 41 (24%) were the gastric and intestinal mixed phenotype, 42 (25%) were the intestinal phenotype, and 36 (21%) were the unclassified phenotype. Positive expression of connexin 30 was significantly more frequent in MUC2-positive cases than MUC2-negative cases ($p < 0.0001$) (Table 2). In immunohistochemical staining, localized distribution of connexin 30 and MUC2 was partly contiguous (Fig. 2C, D). Double-immunohistochemical staining, however, showed coexpression of connexin 30 with MUC2 in no tumor cells (Fig. 2E). On the other hand, there was no clear relationship between expression of connexin 30 and other markers (MUC5AC, MUC6 and CD10) (Table 2). Connexin 30 expression occurred more frequently in the intestinal phenotype (48%) than in other phenotypes (21%) of GC ($p = 0.0015$) (Fig. 2G).

Discussion

Evidence of altered connexin expression in various human malignancies has been accumulating. With regard to the function of connexin in carcinogenesis, there have been several reports of inhibitory effects on the growth of cancer cells [50-53], and transfection and forced expression of connexin 30 in glioma cell lines has been reported to induce the suppression of tumor growth *in vitro* [54, 55]. In the present study, we found that approximately 30% of GC cases displayed connexin 30 expression, while non-neoplastic gastric mucosa did not express connexin 30. Furthermore, there was a significant inverse association between connexin 30 expression and tumor progression. Once malignant

formation is completed, connexin 30 might inhibit GC cell growth and invasion. In addition, we observed a significant inverse association between connexin 30 expression and the presence of metastasis in the regional lymph nodes. Saunders et al. [56], studying the correlation between the metastatic potential of breast cancer cells and gap junctional communication, showed that disruption of homospecific or heterospecific GJIC contributes to metastatic potential, but mechanisms by which altered connexin expression and GJIC might contribute to this process are unclear and require future studies. Based on our results, we suggest that aberrant expression of connexin 30 in GC might not play a role in the metastatic efficiency of malignant cells. The present study showed the higher expression of connexin 30 in the differentiated type of GC compared with the undifferentiated type. This may reflect a loss of ability to produce this protein along with a decrease in histological differentiation in neoplastic cells. Furthermore, in some cases of GC, we observed a cytoplasmic staining pattern of connexin 30. Previous studies reported that connexin 26 translocated from the cell membrane to the cytoplasm in tumor cells [30, 33]. Furthermore, human connexin 26 and connexin 30 were reported to form functional heteromeric and heterotypic channels [57]. These findings suggest that altered expression of connexin 30 such as a decrease in functional gap junctions and changed localization of connexin 30 are an early event during the development of GC. Although the precise function of cytoplasmic connexin 30 is as yet unclear, one possibility is that the cytoplasmic accumulation of connexin 30 may be a prerequisite for the execution of its role in the cell membrane, contributing to GJIC as needed.

In the present study, positive expression of connexin 30 showed significant correlation with positive expression of MUC2. However, there is no previous report showing direct association between connexin 30 and MUC2. Goblet cells in intestinal metaplasia were positive for MUC2, but scarcely expressed connexin 30. Yamamoto et al. previously reported that connexin 32 might be controlled at the transcriptional level via CDX2 [58]. Therefore,

connexin 30 might also be regulated by CDX2, and displayed the intestinal phenotype of GC. Further studies should be performed in the near future to elucidate a role for CDX2 in regulation of connexin 30 in GC.

In summary, we revealed that GC with connexin 30 expression demonstrates a intestinal phenotype that is significantly MUC2-positive in expression. Connexin 30 may be a novel differentiation marker mediating the biological behavior of the intestinal phenotype of GC.

Acknowledgements

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Table 1. Relationship between connexin 30 expression and clinicopathologic parameters in 169 GC cases

| | Connexin 30 expression | | |
	Positive (n = 47)	Negative (n = 122)	*p* value
Age			
≤65 years (n = 46)	13 (28%)	33	NS
>65 years (n = 123)	34 (28%)	89	
Gender			
Male (n = 123)	33 (27%)	90	NS
Female (n = 46)	14 (30%)	32	
Histology			
Differentiated (n = 102)	41 (40%)	61	< 0.0001
Undifferentiated (n = 67)	6 (9%)	61	
T grade			
T1 (n = 83)	35 (42%)	48	< 0.0001
T2/T3/T4 (n = 86)	12 (14%)	74	
N grade			
N0 (n = 107)	37 (35%)	70	0.0123
N1/N2/N3 (n = 62)	10 (16%)	52	
M grade			
M0 (n = 163)	47 (29%)	116	NS
M1 (n = 6)	0 (0%)	6	
Stage			
Stage 0/I (n = 104)	38 (37%)	66	0.0014
Stage II/III/IV (n = 65)	9 (14%)	56	

GC, gastric cancer; NS, not significant. *P*-values were calculated by Fisher's exact test.

[1] Tumor stage was classified according to the criteria of the International Union Against Cancer TNM classification of malignant tumors.

[2] Histology was according to the Japanese Classification of Gastric Carcinomas.

Table 2. Relationship between connexin 30 expression and gastric/intestinal mucin markers in 169 GC cases

	Connexin 30 expression		
	Positive (47)	Negative (122)	*p*-value
MUC5AC			
Positive	17 (21%)	63	NS
Negative	30 (34%)	59	
MUC6			
Positive	13 (39%)	20	NS
Negative	34 (25%)	102	
MUC2			
Positive	33 (55%)	27	< 0.0001
Negative	14 (13%)	95	
CD10			
Positive	14 (40%)	21	NS
Negative	33 (25%)	101	

GC, gastric cancer; NS, not significant. *p*-values were calculated by Fisher's exact test.

Fig. 1. Quantitative RT-PCR analysis of *connexin 30* in various human normal tissues and gastric cancer (GC) tissues. Clear *connexin 30* expression is present in normal brain, spinal cord, bone marrow, uterus and so on. High levels of *connexin 30* were observed in some GCs. The units are arbitrary and *connexin 30* expression was calculated by the standardization of 1.0 µg of total RNA from normal stomach as 1.0

Fig. 2. Immunohistochemical staining of connexin 30 and MUC2 in gastric cancer (GC) tissues (A-E). Connexin 30 was detected in apical membranes of both well differentiated GC (A) and poorly differentiated GC (B), but not in non-cancerous epithelium. Serial sections showed that expression of connexin 30 (C) was partly adjacent to cytoplasmic expression of MUC2 (D). Double-immunostaining of connexin 30 (red) and MUC2 (green) revealed no colocalization of both molecules (E). Nuclei are stained with 4; 6-diamidino-2-phenylindole (DAPI; blue). Immunostaining of human epidermis as a positive control showed that connexin 30 was distributed in the keratinocytes of the upper spinous layers and the granular layers (F). Summary of connexin 30 expression and expression of the GC mucin phenotype (G). Expression of connexin 30 was observed more frequently in I-type and GI-type GC than in other (G and N) GC types. *p* values were statistically analyzed by Fisher's exact test.

Fig1

Fig2

A Connexin 30

B Connexin 30

C Connexin 30

D MUC2

E Connexin 30/MUC2/DAPI

F Connexin 30   Epidermis

G

| Connexin 30 positive | I 20/47 (43%) | GI 19/47 (40%) | G 3/47 (6%) | N 5/47 (11%) |
| Connexin 30 negative | I 22/122 (18%) | GI 22/122 (18%) | G 47/122 (39%) | N 31/122 (25%) |
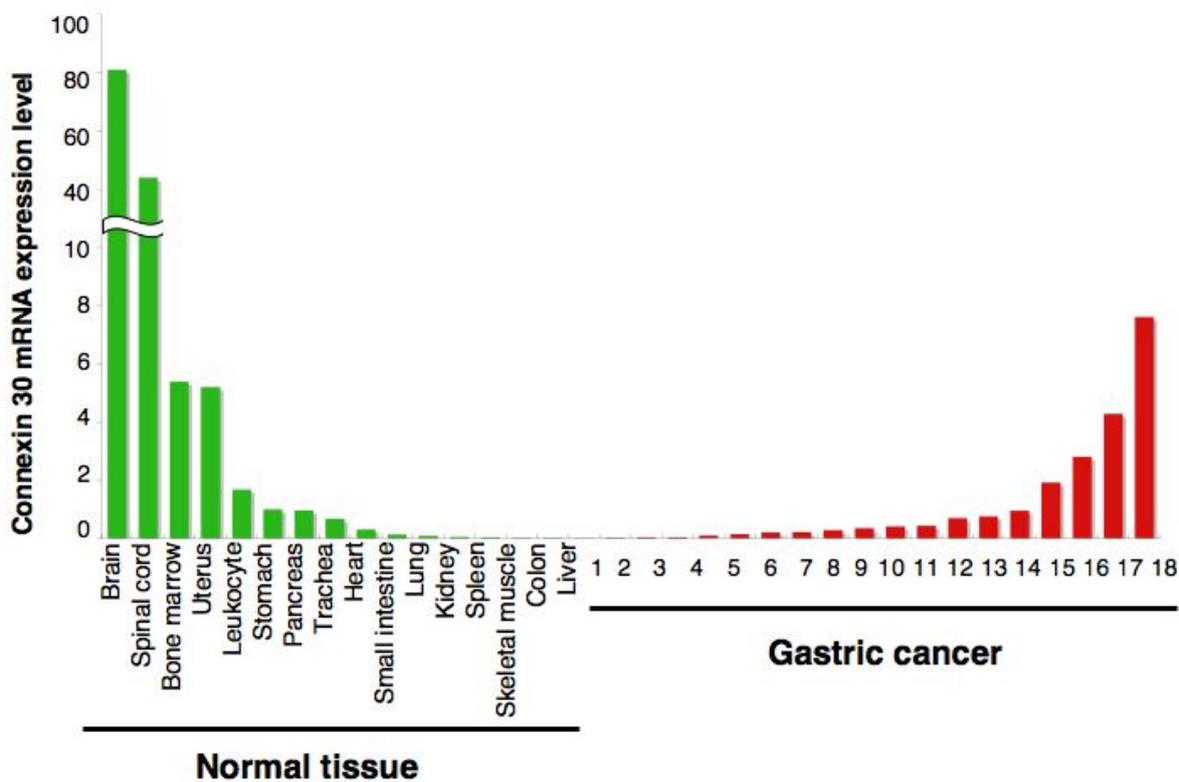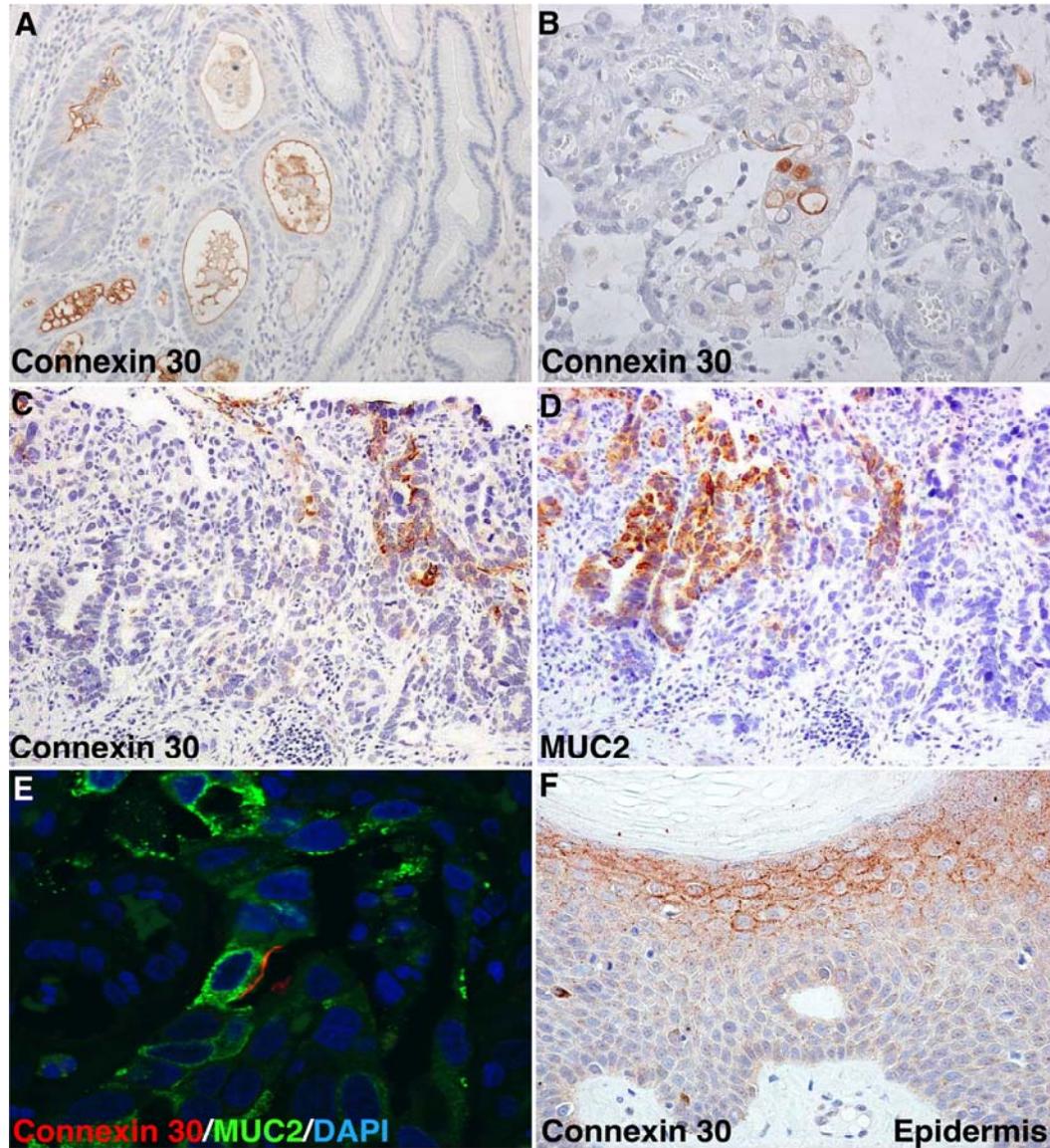
* *P* = 0.0015 (I type vs GI, G, and N types)
  P = 0.0045 (GI type vs I, G, and N types)
  P < 0.0001 (G type vs I, GI, and N types)
  P = 0.0375 (N type vs I, GI, and G types)