CLAUDINS AND PROGNOSTIC FACTORS IN CERTAIN GASTROINTESTINAL DISEASES

Ph.D. Thesis Synopsis

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1. INTRUDUCTION

Gastrointestinal tumors are among the first ten tumor mortalities worldwide, following the leading pulmonary and breast cancers.

There are particularly high the incidence and mortality of tumors in Hungary. A total of 140 thousand malignant tumors are diagnosed each year, which is much higher than in the Western and surrounding countries. More than 260 thousand inhabitants have died due to malignomas in the year of 2007.

The Barrett's esophagus/metaplasia is known as the premalignant lesion of the esophagus, with a morbidity rate of 18/100 thousand people. Esophageal cancers mostly involve male patients, situated in the 11th place according to the WHO statistics. Twenty-six percent of these tumors are adenocarcinomas (ACC). The incidence of ACCs has elevated in the last decades. Gastric intestinal metaplasia is also known as an important, frequent premalignant disease. Tumors of the small intestines are very rare, such as stromal tumors of the gastrointestinum (GIST). Morbidity of the colorectal epithelial neoplasias has increased in the last 3 years in both sexes. The morbidity of celiac disease is 1/200-300 thousand inhabitants in the world. One percent of the population is affected in Hungary. Long term celiac disease could cause increased presumption of bowel tumors, eg. lymphoma.

During development and progression of gastrointestinal (GI) diseases the morphology and the function of the epithelial cells change. The altered function is usually complicated to the changes of the intercellular junctions, like tight junctions (TJ). Claudins (CLDN), main TJ proteins, appear in different tissues, especially epithelial cells in different quantity and quality as cellular electric resistence and paracellular transport need. A total of 24 different claudin proteins are known in the human body at present, although fifty kinds of them are localised in some species, eg. fish. Epithelia, with different function, have variant TJs with various claudins.

Intercellular junctions have indispensable role of the function of epithelia of the GI tract. According to several studies, different alterations of claudins have been found in individual GI diseases, thus in tumors. The receptor of CLDN3 and -4 is similar to the receptor of enterotoxin of *Clostridium perfringens* (CPE). CPE may cause the lysis and

damage of those cells. Experimental results could conclude the role of CPE in tumor target therapy. Clinical trials are in progress with reassuring results.

The pathomechanism of several diseases, especially neoplasms is unclear despite intensive research. Research data refer to the important role of intercellular junctions in carcinogenesis in the GI tract where those junctions have a role in barrier and permeability.

Intercellular structures and proteins have not been examined in tumors of soft tissues with regular significance. CLDN5 has been confirmed in vessels and tumors of vessel origin. The presence of claudin proteins in undifferenciated sarcomas was explained with epithelial differenciation.

Based on the above we studied changes of certain TJ proteins, principally claudins, which are the main structural molecules of TJ, in gastointestinal epithelial and mesenchymal lesions.

2. AIM OF THE STUDY

Changes of intercellular junction of the gastrointestinal tract are crucial in the development and progression of certain diseases.

We supposed that, certain intercellular junctional - mainly tight junction - proteins, namely claudins express in various degree in non-tumorous and tumorous lesions. According to our hypothesis, the changes of claudin expression are related to progression of the disease.

To confirm the above the following questions were asked and studies conducted:

2.1.Barrett's esophagus (BE), adenocarcinoma (ACC) and squamous cell carcinoma (SQCC)

2.1.1. To define the expression profiles of different claudins in BE and ACC in contrast normal foveolar epithelia.

2.1.2. To compare the claudin expression profiles of SQCC and normal squamous epithelia.

2.2. Intestinal metaplasia (IM), tumors of the small and large bowels

2.2.1. We wish to analyse the claudin expression in tissues which have a potential to intestinal differenciation, eg. IM and bowel eithelia. To perform a comparative study of claudin expression of tumors and normal epithalium.

2.2.2. To compare different colorectal tumors with dissimilar dignity.

2.3. Celiac disease

2.3.1. To define the presence and changes of claudins in celiac disease compared with .

2.3.2. To define the optimal site of biopsy sampling in order to decrease the time of endoscopic examination and mental stress.

2.3.2.1. To perform comparative research of the samples from duodenal bulb and distal duodenum regarding to claudin expression.

2.3.2.2. To search CD3 positive intraepithelial lymphocytes (IEL).

2.4. Gastrointestinal stromal tumours (GIST)

2.4.1. To find claudins in GIST comapred with angiosarcoma (AS), leiomysarcoma (LS) and benign kinds of them, which have some similarities in light microscopy and immunohistochemistry.

2.4.2. To find out whether there are any difference between malignant and benign mesenchymal tumors.

2.4.3. We have participated in a study the on duodenal mesenchymal tumors with **Armed Forces Institute of Pathology (AFIP)**. Earlier it was not define unequivocally if tumors were genuine GIST or had smooth muscle origine. Since Imatinib (Gleevec, Glivec, Novartis, Basel, Switzerland), which is an inhibitor of thyrosin kinase, have been available for the therapy, it was important to *re-analyse* of so-called gastrointestinal stromal tumors. We have participated in the collection of samples, processing and data analysis.

2.4.3.1. To define the incidence and prognosis of GIST.

2.4.3.2. To search the c-Kit mutation.

3. MATERIAL AND METHODS

3.1. Material

Tested samples are totalized in the following table:

Study	Sample	Case	Age (year)	Mean age (year)
Barrett's esophagus – adenocarcinoma – squamous cell carcinoma (n=125)	**SQCC**[3,4,6]	25	48-75	60,6
	ACC[3,4,6]	25	46-63	52
	BE[3,4,6]	25	58-67	63,2
	Control[3,4,6]	25+25	15-75	55,4
Intestinal metaplasia – small and large bowel tumors (n=45)	**IM**[3,4,6]	5	61-84	70,6
	Small bowel ACC[3,4,6]	5	48-78	60,8
	Large bowel ACC[3,4,6]	15	51-80	65,3
	Large bowel adenoma[3,4,6]	20	49-84	66,7
Coeliakia (n=47)	**Coeliakia**[5]	33	2-17	6,4
	Control	14	17-29	22,6
GIST, angiosarcoma, leiomyosarcoma (n=35)	**GIST**[1,2]	13	46-80	67,6
	LS[1]	12	27-49	48,2
	AS[1]	10	27-95	65,8
GIST (AFIP) (n=156)	**GIST**[7]	156	10-88	56

SQCC=squamous cell carcinoma, ACC=adenocarcinoma, BE= Barrett's esophagus, IM=intestinal metaplasia, GIST=Gastrointestinal stromalis tumour, LS=leiomyosarcoma, AS= angiosarcoma

Sources were collections from the 2nd Dept. of Pathology, Semmelweis University; National Institute of Oncology[1] and National Health Center, Dept. of Pathology[2]. Partners were the 1st Dept. of Internal Medicine[3], 1st Dept. of Surgery[4], 1st Dept. of

Pediatrics[5], Ferencváros Health Center[6], AFIP Dept. of Soft Tissue and Orthopedic Pathology[7].

3.2. Methods

3.2.1. Histology, histochemistry

Tissue samples were fixed in 4% buffered formalin for 24 hours, then embedded into paraffin. H&E stain and alcian-blue – PAS reaction were performed on 3-4 μm thin slides. In cases of *GIST*s, histology and morphometry (eg. size, mitotic figures, metastases) were performed in the AFIP Labs. The GISTs were divided into 6 prognostic groups.

3.2.2. Immunohistochemistry

Paraffin sections were used for immunohistochemistry.

Primary antibodies of **CLDN2, -4, -5** were monoclonal, **CLDN1, -3, 7** were polyclonal. Immunohistochemistry was made on 3-5 μm thin slides. First, the slides were treated in a target retrieval solution followed by incubation of the primary antibody in different dilution. For visualization, a standard avidin–biotin peroxidase technique (ABC) was used with diaminobenzidine (DAB) as chromogen. The reactions were carried out in a Ventana ES automatic immunostainer.

The number of positive cells was calculated as follows: 10 randomly selected areas per slide were analyzed using ×20 objective with 100 cells counted in each field. The scoring standardized for each group was as follows: 5=80 to 100%, 4=60 to 80%, 3=40 to 60%, 2=20 to 40%, 1=5 to 20% and 0≤5% of the cells showed positive reactions.

Method of **CD3** immunohistochemistry was similar to that described above. The number of positive cells was calculated as follows: 3 randomly selected areas per slide were analysed using x20 objective with 100 cells counted in each field. The evaluation was performed on the superficial surface of villi, that way the villi realized as inverse letter „U” and count was performed on the median part.

For statistical analysis, Mann-Withney *U*-test and Shapiro-Wilk *W*-test were used - the latter in case of celiac disease.

CD117 (c-Kit). Method of immunohistochemistry was similar to that described for claudins. c-Kit positive GISTs were selected for the study, so c-Kit immunreaction was performed on AS and LS only.

At *AFIP Labs* C-Kit, CD34, α-smooth muscle actin, desmin, CK7, CK18, CK19, neurofilament 68 (NF68), glial fibrillary acid protein (GFAP) and S100 immunohistochemical reactions were performed. For **statistical analysis,** Statview statistical software (SAS) was used.

3.2.3. Immunofluorescent method

Intracellular localisation of CLDN2 and -5 were confirm on 10 μm paraffin slides in GIST, AS and LS samples. After antigen retrieval, similar to that used above, primary antibody incubation was performed. The secondary antibody was IgG conjucated anti-mouse Alexafluor 488. Counterstaining and covering were done with medium containing DAPI.

The slides were estimated by laser confocal microscope (Nikon Eclipse E800 mikroscope; BioRad Radiance 100 Laser scanning System and BioRad Lasersharp 2000 software).

3.2.4. mRNA detection

On BE, ACC and FOV samples CLDN 2, CLDN 3, CLDN 4, CLDN5 and GAPDH mRNA level analysis by real time RT-PCR:

1. Tissue samples: 10 paraffin sections each of ACC, BE and FOV samples.
2. RNA isolation: Total RNA from formalin-fixed paraffin-embedded tissues was isolated with High Pure RNA Paraffin Kit. Depending on the size of the dissected area, two to eight 5-μm-thick sections were cut from each tissue block. Samples from Barrett's and foveolar epithelia were microdissected to select the areas of SIM of BE and the columnar epithelial cells without goblet cells. Proteinase K digestion time was 16 h for each sample. All purifications were performed in accordance with the manufacturer's protocol. After total RNA isolation, samples were kept at -80°C until use.
3. For responsible PCR examination of paraffin sections, 6 amplicons were previously analysed with 6 primer pairs. The optimal size was between 100 and 200 bp.

4. Total RNA (500 ng) was reverse transcribed for 50 min at 42°C in 30 µl with MulV reverse transcriptase in the presence of RNAse inhibitor, using Random Hexamers.

5. Real-time quantitative RT-PCR Real-time PCR reaction was performed with 2 µl cDNA template in a total volume of 25 µl, using the ABI Prism 7000 sequence detection system. Each PCR was conducted in 25 µl volume of SYBR Green Supermix. Primer sequences were as follows:

	forward	**reverse**
CLDN2 GI:9966780	5'-CTCCCTGGCCTGCATTATCTC-3' (273-293)	5'-ACCTGCTACCGCCACTCTGT-3' (344-363)
CLDN3 GI:21536298	5'-CTGCTCTGCTGCTCGTGTCC-3' (732-751)	5'-TTAGACGTAGTCCTTGCGTCGTAG-3' (836-860)
CLDN4 GI:14790131	5'-GGCTGCTTTGCTGCAACTGTC-3' (741-761)	5'-GAGCCGTGGCACCTTACACG-3' (829-848)
CLDN5 GI: 38570041	5'-TTCCTGAAGTGGTGTCACCTGAAC-3' (93-116)	5'-TGGCAGCTCTCAATCTTCACAG-3' (169-190)
GAPDH GI:7669491	5' -CATTGACCTCAACTACATGG-3' (186-205)	5'-GAAGATGGTGATGGGATTTC-3' (287-306)
β-actin GI:15928802	5'-CCTGGCACCCAGCACAAT-3' (1031-1048)	5'-GGGCCGGACTCGTCATAC-3' (1157-1174)

Real Time RT-PCR: primer sequences.
CLDN=claudin, GAPDH= gliceryl-aldehyde 3-phosphate dehydrogenase.

PCR was done in duplicates in 96-well plates, for 2 min. at 95°C for initial denaturing, then 40 cycles at 95°C for 20 s, at 63°C for 30 s and 72°C for 1 min. Data analysis and statistical evaluation were performed by REST (expression software tool, http://www.wzw.tum.de/gene-quantification).

Reference genes were GAPDH and ß-actin.

RT-PCR analysis on **GIST, angiosarcoma and leiomyosarcoma** samples was similar to that described above. We analysed of the mRNA expression of *CLDN2* and *CLDN5* (See table above). Reference genes were GAPDH.

GIST (AFIP). Molecular genetic examination was perform on 30 c-Kit positive GISTs. c-Kit mutations of exon 9, 11, 13 and 17 were confirm by PCR and direct sequencing.

4. RESULTS

4.1. Barrett's esophagus

4.1.1. Barrett's esophagus' claudin profile is different from the fovelar epithelias. Normal foveolar epithelia do not express CLDN2 and -3. BEs express CLDN2 at mRNA level, but not at protein level. CLDN3 expression is detectable in BE both at protein and mRNA levels. Esophageal ACCs express CLDN2 and -3 at protein and mRNA levels.

4.1.2. Comparing the squamous epithelia with carcinoma, carcinoma shows significantly higher CLDN1 expression than the normal control. Expression of CLDN 2 and -3 was very weak or not detectable. CLDN4 and -7 expression is strong and was not show significant difference between carcinoma and controls.

4.2. Intestinal metaplasia (IM), small and large bowel tumors

4.2.1. There are remarkable differences between IM, tumors and control samples regarding claudin expression. Antral foveolar epithelia do not express CLDN2, the IM and small bowel control contain CLDN2 protein. In contrast to large bowel control samples, colorectal adenoma and carcinoma show significantly higher CLDN2 expression. Antral foveolar epithelia do not express CLDN3, but the IM, small bowel control and ACC show higher and the large bowel tissues, both normal and neoplastic, significantly higher CLDN3 protein expression.

CLDN4 and -7 reactions are strongly positive in almost every sample, except for antral epithelia, which express lower CLDN4.

4.2.2. Small bowel ACC show higher CLDN2 and -3 expression than control tissues. Colorectal neoplasias show a somewhat higher CLDN2 expression compared with normal epithelia. Adenoma and ACC do not differ from each other.

4.3. Celiac disease

4.3.1. Different CLDN2 and -3 expressions are observable in celiac disease and normal control. Celiac samples show significantly higher CLDN2 and -3 protein expression as compared with control epithelia. CLDN4 expression does not show any remarkable differences.

4.3.2. Value our results regarding the localisation, we have found that the distal part of the duodenum is more adequate to biopsy test hole.

4.3.2.1. Celiac samples show higher CLDN2 and -3 expression in the distal duodenum.

4.3.2.2. CD3 positive lymphocytes are detected in much higher number in epithelia in case of celac disease as contrasted to normal samples. There was no significance difference between the two sites of duodenum, but we have detected an increasing tendency into distal duodenum.

4.4. GIST, angiosarcoma, leiomyosarcoma

4.4.1. All of the mesenchymal tumors express various types of claudins, so CLDN2 is detectable in our samples. CLDN1 is expressed only in leiomyosarcomas. GIST and leiomyosarcoma show CLDN2, -3, -4, -5 and -7 protein expressions. Leiomyomas exclusively show CLDN2 expression. CLDN2 and -5 expressions are features for benign and malignant vessel tumors. CLDN2 is localized intracellularly near the cytoplasmic membrane in immunofluorescent manner. CLDN5 reaction is granular and attached to the membrane. CLDN2 and -5 expressions are confirmed at mRNA level.

4.4.2. There are differences between benign and malignant leiomyogenic neoplasias, accordingly leiomyomas express only CLDN2, in contrast to sarcomas which express all of the claudins studied. Angiosarcomas show high CLDN5 expression, but lower level of CLDN2 than haemangiomas.

4.4.2. GIST (AFIP)

4.4.2.1. From 167 mesenchymal duodenal neoplasms we diagnosed 156 tumors as GISTs according to c-Kit positivity. Those tumors had worser prognosis which had larger size and more than 5/50 hpf (high power field) mitotic figures.

Coagulative necrosis, epitheloid feature, hyaline vessel necrosis and mucosal infiltration are significant risk factors.

4.4.2.1. c-Kit mutation is detected in 43 percent of GISTs in the AFIP Labs. This finding does not correlate with the previous data.

5. NEW ESTABLISHMENTS

5.1. Barrett's esophagus – Adenocarcinoma:

5.1.1. Barrett's esophagus expressed higher CLDN2 at mRNA level and CLDN3 at both protein and mRNA levels, as compared with the normal foveolar epithelia.

5.1.2. Adenocarcinoma of the lower esophagus expressed higher CLDN2 and -3 both at protein and mRNA levels, in contrast to normal foveolar epithelia and Barrett's esophagus.

5.2. Coeliakia

5.2.1. Epithelia in celiac disease expressed significantly higher CLDN2 protein than the normal control.

5.2.2. CLDN2 and -3 immunohistochemistry suggested that the distal part of the duodenum could be a better target for sampling in cases of less or more severe duodenal atrophy.

5.3. GIST, angiosarcoma, leiomyosarcoma

5.3.1. Mesenchymal tumors expressed various amounts of CLDN1, -2, -3, -4, -5, -7 proteins.

5.3.2. CLDN2 was found expressed in vessel, smooth muscle tumors and GIST.

5.3.3. GIST expressed CLDN2, -3, -4, -5 and -7 proteins.

5.3.4. Benign and malignant angiogenic tumors expressed CLDN2 and -5 proteins.

5.3.5. Differences were detected between benign and malignant leiomyogenic neoplasias, accordingly leiomyomas expressed only CLDN2, in contrast to sarcomas which expressed all of the claudins studied.

5.3.6. In mesenchymal neoplasms, CLDN2 was localized intracellularly near the cytoplasmic membrane in immunofluorescent manner. CLDN5 reaction was granular and attached to the membrane.

6. CONCLUSIONS

6.1. Changes of CLDN2 and -3 expression in Barrett's metaplasia and esophageal adenocarcinoma could be the connection with the carcinogenesis.Results confirm that, alterations of Barrett's metaplasia and foveolar epithelia originate in distorsion of the tight junction and could cause dysplastic and neoplastic changes of the epithelium.

6.2. We confirmed first the higher expression of CLDN2 in celiac disease in contrast to normal epithelium. Higher expression of CLDN2 and -3 correspond with alteration of tight junction. Differences more significant in the distal duodenum. So this location could be the most adequate for biopsy sampling, according to increasing tendency of intrepithelial lymphocytes.

6.3. Antral and previously tested cardial foveolar epithelia express claudins with similar quantity and quality, but dot not express CLDN2. Gastric intestinal metaplasia and the intestinal metaplasia of the Barrett's esophagus have the same profile for claudin expression with the normal small bowel epithelia, since they express higher CLDN2 and -3. These claudins may play an important role in the intestinal differenciation.

6.4. Soft tissue tumours and GIST express diverse claudins. The profile of the claudin expression is revealing the mesenchymal tumours. Similarities of the claudin expression of GIST and leiomyosarcoma may suggest the histogenetic reletionship between them. Based on claudin expression profile, malignant vessel and smooth mucle tumours can be differenciated form the benign neoplasms.

7. LIST of PUBLICATION

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Publications related to the Dissertation

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