Claudin expression of endometrial and thyroid carcinomas: may we speak of a common marker of the papillary tumors of these organs?

Ph.D. Thesis Synopsis

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Budapest
2009

1. INTRODUCTION

Papillary cancers can be found in different organs of the human body, although they may originate from heterogeneous basic tissues, they show substantively, uniformly, characteristic morphology.

Papillary cancers may derive from the mucosa brushing the cavity of the uterus, namely the endometrium, as well. Endometrial carcinoma is the most prevalent malignancy of the female genital tract in the developed countries. Basically two major pathomechanisms are presumed in the background of the development of endometrial cancers forming the basis of the dualistic model. The two different pathogenetic backgrounds result the development of tumors of different features and clinical behavior. The majority of endometrial cancers are endometrioid carcinomas (type I) which are associated with chronic exposure of the endometrium to estrogen as opposed to a progestin. The precancerous lesion of endometrioid carcinoma is complex atypical endometrial hyperplasia. The other minor, but aggressive behavior group of endometrial cancers is the nonendometrioid carcinomas (type II) to which the serous papillary carcinomas belong.

The other important group of papillary cancers originates from the thyroid gland which, similarly to the endometrium, is an organ stimulated by hormones and its tumors occur predominantly in women. The papillary thyroid cancers can be of different size: microcarcinomas vs. carcinomas measuring >1 cm in greatest diameter; different etiology: Hashimoto vs. non-Hashimoto thyroiditis; different histological subtype: follicular variant vs. conventional and tall cell, oncocytic variant. Papillary thyroid carcinoma most often metastasizes into the regional (cervical) lymph nodes. Besides papillary thyroid cancers, from the thyroid follicular epithelial cells arise the follicular thyroid carcinomas and follicular adenomas, as well. These thyroid tumors have supposedly different molecular pathways.

Claudins, the main transmembrane proteins of tight junctions, were discovered in 1998, with currently 24 known members in the human body. Claudins have been suggested to be involved in the growth and differentiation of cells, in carcinogenesis, and cancer progression in several tumors. Altered expression of claudins (increased or decreased expression) may characterize the invasion and metastasize of different tumors.

The connection between papillary cancers and expression of claudins has only been investigated to some extent. Some organs, for example the kidney, ovary, pancreas, have been analyzed in regard to the connection between papillary tumors and expression of claudins. At the onset of our studies, the endometrium had not yet been investigated in view of claudins.

Our study results of endometrial samples raised the question whether claudin expression similar to that notable in the endometrium could be confirmed in the papillary tumors of other organs, and if so, would it bear the same prognostic significance as in the case of the endometrium?

2. AIM OF THE STUDY

According to the dualistic model of the development of endometrial carcinomas, two major pathomechanisms are assumed between the tumors of different etiology and clinical behavior. The role of claudins, the main tight junction proteins, is being extensively investigated in carcinogenesis, thus the changed expression of claudins has been proved in the development, biological behavior and progression of many epithelial tumors.

Based on these data the questions are raised:

2.1. Does the expression pattern of claudins reflect the hypothetically different pathogenetic mechanisms of endometrial cancers (types I and II)?

2.2. Is different claudin expression characteristic to the serous papillary carcinoma showing aggressive clinical behavior?

The study results of our endometrial samples raised the question of whether the increased claudin-1 expression may also be characteristic to the papillary tumors of other organs. The thyroid gland, similarly to the endometrium, is an organ stimulated by hormones and its tumors occur predominantly in women. Papillary thyroid carcinomas comprise the majority (about 80%) of thyroid follicular epithelial cell tumors. These thyroid tumors have different molecular pathways similar to endometrial carcinomas.

Based on these data we have searched for answers to the following questions:

2.3. What kind of claudin-1 expression is characteristic to papillary thyroid carcinomas and their regional lymph node metastases?

2.4. Is the claudin-1 expression of follicular thyroid carcinomas and follicular adenomas similar to papillary thyroid carcinomas?

3. MATERIAL AND METHODS

3.1. Samples

3.1.1. Endometrial samples

A retrospective series of 70 formalin fixed, paraffin embedded endometrial blocks was used from the archives of the 2nd Department of Pathology and the National Institute of Oncology with permission from the Regional Ethical Committee of the Semmelweis University of Budapest (172/2003). Total abdominal hysterectomy with bilateral salpingo-oophorectomy was performed in all patients, none of whom had received chemo- or radiotherapy prior to surgery (Table 1.).

Table 1: Tested endometrial samples.

Diagnosis	Number of samples (n=70)	Average age (year)
Normal endometrial tissue Proliferative phase	12	46,2±4,3
Normal endometrial tissue Secretory phase	12	46±4,3
Complex endometrial hyperplasia	14	45±6,3
Endometrioid carcinoma (type I)	17	55,3±8,6
Serous papillary carcinoma (type II)	15	63,8±5,6

3.1.2. Thyroid samples

A retrospective series of 63 formalin fixed, paraffin embedded thyroid blocks was used from the archives of the 2nd Department of Pathology and the National Institute of Oncology with permission from the Regional Ethical Committee of the Semmelweis University of Budapest (172/2003). None of the patients received chemo- or radiotherapy prior to surgery (Tables 2., 3.).

Table 2: Tested thyroid samples.

Histology	**Number of samples (n=19)**
Hashimoto's-associated papillary thyroid carcinoma	5
Papillary carcinoma follicular variant within these: oncocytic	5 1
Papillary carcinoma tall cell variant	1
Conventional papillary carcinoma	8

Table 3: Etiology and histological subtypes of papillary thyroid carcinomas.

Diagnosis	**Number of samples (n=63)**
Papillary thyroid carcinoma From these: Lymph node metastases	19 10
Papillary microcarcinoma	8
Follicular thyroid carcinoma	17
Follicular adenoma	19

3.2. Methods

3.2.1. Histology

Tissue blocks were fixed in 10% neutral buffered formalin in PBS (pH 7,0) for 24 h and embedded in paraffin, 3-to 5-µm-thick sections were routinely stained with hematoxylin and eosin (HE).

3.2.2. Immunohistochemistry

Paraffin embedded 3-4 µm thick sections were used for immunohistochemistry. After endogen peroxidase blocking and antigen retrieval, the following primary antibodies were used: claudins-1, -3, -7, were polyclonals rabbit (Zymed Inc. San Francisco, CA, USA), claudins-2, -4, -5, β-catenin, PCNA, p53, estrogen and progesterone-receptor

monoclonals mouse (Zymed Inc. San Francisco, CA, USA; BD Transduction San Diego, CA, USA; Dako Glostrup Denmark; Novocastra Newcastle, UK). The biotinilated secondary antibody and avidin-streptavidin-enzyme conjugate were applied according to the protocol of the automated Ventana system. The secondary antibodies and the reagents were the products of Ventana (Tuscon, AZ, USA). For visualization, diaminobenzidine (DAB) was applied and hematoxiline was used for nuclear staining.

Negative controls for nonspecific binding, incubated with secondary antibodies only, were processed and revealed no signals. Positive controls recommended by manufacturer (Zymed) were used to confirm correct immunohistochemical staining for claudins, that is normal skin for claudin-1, normal colon for claudins-2, -3, -4, -5, and normal breast tissue for claudin-7. For positive control of β-catenin immunoreaction human hepatoblastoma tissue was used, previously reported to show nuclear positivity and analyzed for β-catenin mutation.

Verification of the different hormonal backgrounds of endometrial cancers in endometrioid and serous papillary carcinomas were used as accessory immunreaction with estrogen- and progesterone receptors. In addition, p53 and β-catenin protein reactions and localizations were analyzed in our endometrial carcinoma cases. The differences in the clinical behavior of endometrial tumors were studied using PCNA as proliferation marker. In the thyroid samples, β-catenin was investigated by immunohistochemistry in order to find out whether it plays role in the regulation of claudin-1 expression, similar to colorectal cancers.

3.2.2.1. Evaluation of endometrial samples

Immunoreactions were evaluated by semi-quantitative method. In all, 10 randomly selected areas of each slide were analyzed using high power fields objective (x40) with 100 cells counted per field and the positively stained cells were determined in respect to the total number of cells. For semi-quantitative evaluation, tumorous and nontumorous epithelia were considered negative if less than 5% of the cells reacted. The following further values were given: 1 (6-20% positivity), 2 (21-40% positivity), 3 (41-60% positivity), 4 (61-80% positivity), 5 (81-100% positivity). Claudins-1, -3, -4, -5, and -7 exhibited membranous, whereas claudin-2 cytoplasmatic staining. For β-catenin the mebranous, cytoplasmic and/or nuclear staining, for the estrogen/progesterone receptors,

p53 and PCNA the positive nuclei were counted and expressed in percentage. For the statistical analysis of the immunohistochemical scores, the Mann-Whitney U test (SPSS 15,0, SPSS Inc., Chicago, III, USA) was used to compare the expression of proteins in the different groups. The probability values were: *$p<0.05$; **$p<0.01$; *** $p<0.001$.

3.2.2.2. Evaluation of thyroid samples

The claudin-1 immunoreaction in thyroid samples was evaluated by digital morphometry. The results of claudin-1 immunohistochemical reactions were photodocumented using Mirax MIDI Scanner (3DHistech Ltd., Budapest, Hungary). The quantitative evaluation of claudin-1 immunoreactions was performed with Leica QWin software (Leica Microsystem Imaging Solution Ltd., Cambridge, UK).

Before performing the measurements, a threshold level of colors (RGB- red, green, blue) to be considered as positive was defined by selecting the stained areas on the digitized positive control tissue. Ten non-overlapping representative fields were assessed. Positive area was defined as percentage of pixels above the threshold within a defined area of interest. The threshold for the positive reaction was more than 1 percentage of immunopositive area. Statistical analysis for the comparison of immunopositive areas in the different sample groups was performed using non-parametric Mann-Whitney test (SPSS 15,0, SPSS Inc., Chicago, III, USA. The probability values were: *$p<0.05$, **$p<0.01$,***$p<0.001$.

The intensity of β-catenin immunoreaction in the center of lesions was graded semi-quantitatively as: 0-absent staining, 1-weakly positive, 2-moderately positive and 3-strongly positive. For the statistical analysis of the immunohistochemical grades Pearson χ^2 was used to compare the expression of β-catenin in different groups.

3.2.3. Real-time PCR- mRNA analysis

Five 10-μm sections were cut from each endometrial tissue block (necrosis and bleeding excluded) and total RNA from tissues was isolated with High Pure RNA Paraffin Kit (Roche, 3270289, Manheim, Germany). All purifications were performed according to the manufacturer's protocol. Total RNA was reverse transcribed for 50 minutes at 42°C with MuIV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) in the presence of RNAse inhibitor (Applied Biosystems) using Random

Hexamers (Applied Biosystems). Real-time PCR reactions were performed with 2 µl cDNA template in total volume of 25µl, using the ABI Prism 7000 sequence detection system (Applied Biosystems). Each PCR was conducted in 12,5 µl volume of SYBR Green Supermix (BIO-RAD, Hercules, CA, USA). Rea-time PCR reaction was performed in duplicates in 96-well plates for 2 minutes at 95°C for initial denaturing, then 40 cycles at 95°C for 20 seconds, at 63°C for 30 seconds and at 72°C for 1 minute. Data analysis and statistical evaluation were performed by REST (expression software tool, www.wzw.tum.de/gene-quantification), using the average of β-actin as reference gene for relative quantification.

4. RESULTS

Endometrial samples

4.1. Immunohistochemistry

Positive membranous linear reaction along the cell surface was detected for claudins-1, -3, -4, -5, -7. A granular pattern associated with the cell membranes, occasionally intracytoplasmatically, was seen for claudin-2.

4.1.1. Claudin-1 definitely showed the highest reaction in serous papillary (type II) carcinoma. In the other endometrial samples (endometrioid carcinoma [type I], hyperplasia, secretory and proliferative phase) weak reaction was detected.

4.1.2. Claudin-2 showed strong reaction in type I endometrial cancer, hyperplasia and in secretory and proliferative endometrium, in contrast to type II endometrial cancers where weak reaction was found.

4.1.3. No or only slight differences were detected for claudins-3, -4, -7 in our endometrial samples.

4.1.4. Claudin-5, known as a marker of endothelial cells, showed moderate reaction in the secretory phase, then type II endometrial carcinoma followed type I endometrial carcinoma, and weak reaction was seen in the proliferative phase.

4.1.5. According to our expectancy type I endometrial carcinoma expressed significantly higher estrogen and progesterone receptor levels than type II endometrial carcinoma of nuclear localization. Type II endometrial carcinomas showed a significant increase of p53 and PCNA as compared with type I endometrial carcinomas. Membranous β-catenin was detected in both types of carcinomas. Diffuse cytoplasmic reaction was observed in 4 endometrioid carcinoma cases.

4.2. mRNA expression of claudins

4.2.1. The expression analysis of mRNA in endometrial samples showed similar results for claudin-1 as by immunohistochemistry. The mRNA expression of claudin-1 similarly to protein expression was increased (upregulated) in type II endometrial carcinomas as compared with type I endometrial carcinomas, hyperplasia, secretory and proliferative endometrium samples.

4.2.2. Claudin-2 did not show similar significance with mRNA expression as by immunohistochemistry in the endometrial samples.

Thyroid gland

4.3. Immunohistochemistry/claudin

Claudin-1 showed positive linear reaction along the cell membranes.

4.3.1. Claudin-1 protein expression was increased in every examined papillary thyroid carcinoma, including microcarcinomas, Hashimoto's-associated papillary thyroid carcinomas and different histological subtypes.

4.3.2. As a first description, the lymph node metastases belonging to primary papillary thyroid cancers proved increased claudin-1 expression as well.

4.3.3. By contrast, weak or no expression of claudin-1 was detected in follicular thyroid cancers, follicular adenomas, and in the peritumoral non-malignant thyroid tissues.

4.4. Immunohistochemistry/β-catenin

β-catenin showed membranous and cytoplasmic reaction also in papillary thyroid carcinomas in intranuclear pseudoinclusions.

4.4.1. In peritumoral thyroid tissue of all tumor types, β-catenin was seen primarily on the cell membranes of small follicles. Such small follicles were abundantly seen in thyroid tissue with increased regenerative activity, such as in the peritumoral tissue associated with Hashimoto's-associated papillary thyroid carcinomas.

4.4.2. Strong cell membrane β-catenin immunostaining was observed in follicular adenomas. In follicular thyroid carcinomas cytoplasmic β-catenin reaction appeared in addition to cell surface immunostaining. In papillary thyroid carcinomas both cytoplasmic and membranous β-catenin reactions were detected.

4.4.3. In papillary thyroid cancers, a tendency of decreased membrane staining was proved as compared with follicular thyroid carcinomas and follicular adenomas.

4.4.4. Cytoplasmic β-catenin staining was significantly increased in the malignant tumors (papillary and follicular carcinomas) when compared with adenomas.

4.4.5. Some papillary thyroid carcinomas showed positive reaction in intranuclear pseudoinclusions.

To summarize our results, it can be established that type I endometrial carcinoma, hyperplasia and normal (secretory and proliferative) endometrial tissue show congruous claudin-1, -2 expression pattern that differed sharply from type II endometrial carcinoma. The serous papillary cancers of aggressive behavior of the endometrium were characterized by increased claudin-1 expression.

In addition, it can be stated that in thyroid papillary cancer, independent of etiology, size, histological subtype, significantly increased claudin-1 expression was detected as compared with follicular thyroid carcinomas, adenomas and the peritumoral non-malignant thyroid tissues. Increased claudin-1 reaction was also preserved in lymph node metastases of papillary thyroid carcinomas. Cytoplasmic β-catenin reaction was significantly increased in the malignant tumors (papillary and follicular carcinomas) when compared with adenomas. The role of Wnt/ β-catenin pathway as a regulator of claudin-1 expression could not established in our study.

5. NEW ESTABLISHMENTS

5.1. The pattern of claudin-1, -2 protein expression in serous papillary carcinomas (type II) differs from the endometrioid carcinomas (type I), hyperplasia and normal (secretory and proliferative) endometrial tissues.

5.1.a. Increased claudin-1 expression characterizes the serous papillary carcinomas as compared with endometrioid carcinomas, hyperplasia and normal (secretory and proliferative) endometrial tissues.
5.1.b. By contrast, increased claudin-2 expression was detected in the endometrioid carcinomas and nontumourous endometrial samples when compared with serous papillary carcinomas.

5.2. Claudin-1 mRNA expression was upregulated in serous papillary carcinomas (type II) as a compared with endometrioid carcinoma (type I), secretory, proliferative and hyperplastic endometria.

5.3. With claudins-3, -4, -7 no or only slight differences were detected in our endometrial samples.

5.4. Increased claudin-1 protein expression was seen in every papillary thyroid carcinoma including microcarcinomas, Hashimoto's-associated papillary thyroid carcinomas and different histological subtypes.

5.5. Increased claudin-1 expression was also proved in the lymph node metastases belonging to primary papillary thyroid cancers.

5.6. Contrarily, weak or no expression of claudin-1 was detected in follicular thyroid cancers, follicular adenomas, and in the peritumoral non-malignant thyroid tissues.

5.7. In our thyroid samples, the role of Wnt/ β-catenin pathway as a regulator of claudin-1 expression could not established.

In conclusion, it can be established that increased claudin-1 expression was proved in serous papillary carcinoma of the endometrium, likewise to the papillary thyroid carcinomas. The increased claudin-1 expression in the endometrial tumors associated with aggressive behavior, in turn in the thyroid gland was detected in the primary tumor and their metastases as well.

6. CONCLUSIONS

Our result regarding the different expression of claudins-1, -2 can support the theory of dualistic model in endometrial cancers. Endometrioid cancers show claudin-1, -2 expression pattern similar to precursor lesions of these tumors, namely complex atypical endometrial hyperplasia as well as normal endometrial tissues. These claudin patterns differ sharply from serous papillary carcinoma, thus claudins-1, -2 could be useful differential diagnostic markers of the endometrioid and serous papillary carcinomas of the endometrium.

Claudin-1 as "tightener" of the epithelial barrier was proved in the serous papillary carcinomas of aggressive behavior, both at protein and mRNA level. Based on our results, as in case of other tumors, claudin-1 has prognostic value in endometrial cancers as well.

The increased claudin-1 expression was detected in serous papillary carcinomas, likewise in papillary thyroid cancers and their lymph node metastases. The increased expression of claudin-1 was proved in papillary thyroid cancer associated with better prognosis, however the presence of increased claudin-1 staining both in the primer tumor and metastases refers to the fact that claudin-1 has a role in the metastasizing of these tumors.

Our results on the different claudin-1 protein expression support the varying molecular origin of epithelial thyroid tumors. Based on these data, claudin-1 could help in the differential diagnosis of thyroid follicular epithelial cell-derived tumors.

Summarizing our results, it could be established that claudin-1 could be a useful marker of endometrial and thyroid papillary tumors. In the future, increased claudin-1 expression could be used as targeting therapy in oncology. In addition, we can state that the prognostic value of claudin-1 is different in the serous papillary cancers of the endometrium and papillary thyroid cancers.

7. LIST OF PUBLICATION

Cummulative impact factors (IF): 7,662

Publication related to the Dissertation:

IF: 3,579

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