Claudin7 and moesin in endometrial Adenocarcinoma; a retrospective study of 265 patients

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Abstract

Background: Metastasis is the main cause of death in cancer and is a multistep process. Moesin (MSN), a member of the ezrin-rodixin-moesin family and Claudin7 (CLDN7), a tight junction protein, both play a role in tumor cell metastasis. Previously, we found an over-expression of MSN and under-expression of CLDN7 at the mRNA level in uterine serous carcinoma in comparison to uterine endometrioid adenocarcinoma. The purpose of this study is to determine the protein expression of MSN and CLDN7 in endometrial cancer (EC) and to evaluate their prognostic value. Two hundred sixty-five patients with EC were retrieved from the archives. MSN and CLDN7 immunostaining were performed on the tissue paraffin sections. The expression of each antibody was reported and then correlated with clinicopathological prognostic factors including age, tumor grade, tumor stage, lympho-vascular involvement, depth of myometrial invasion, overall survival (OS), disease free survival (DFS) and death of disease (DOD).

Results: MSN and CLDN were expressed in 46% and 52% of overall cases. We observed an association between MSN+ staining and tumor grade, and serous and clear cell carcinoma subtypes (p < 0.001 each). There was an association between CLDN7+ staining and low tumor grade and endometrioid adenocarcinoma subtype (p < 0.001 and 0.001 respectively). However, no association between MSN and CLDN7 expression and outcome including OS, DOD, and DFS was found.

Conclusion: A significant prognostic value of MSN and CLDN7 in predicting disease outcomes in patients with EC was not demonstrated. Nevertheless, the high percentage of EC cases with MSN and CLDN7 immunexpression, and their association with tumor grade and subtypes, suggests that these proteins might play a role in tumorigenesis of endometrial adenocarcinomas. Future studies are needed to shed light on their mechanistic properties in EC cells.

Keywords: Moesin, Claudin7, Endometrial adenocarcinoma, Clinical outcome
metastasis were found to be differentially expressed at the mRNA levels in USC in comparison to EAC, with the first (moesin) overexpressed and the second (claudin7) under-expressed.

Metastasis is the primary cause of death and it is a multistep process that requires invasion of the basement membrane by tumor cells, streaming through the blood/lymph vessels and extravasation and growth in distant locations. Moesin or MSN (membrane organizing extension spike protein) is a member of the ERM (ezrin-radixin-moesin) cytoskeleton-associated protein family. This family of proteins acts as a linkage between the cell membrane and the underlying actin cytoskeleton and it has been implicated in maintenance of cell shape, cell motility and membrane trafficking and tumor metastasis [7-11]. EMR proteins share 75% sequence identity and thus it is logical to hypothesize that like ezrin, radixin and moesin might be involved in tumor cell migration [9]. Furthermore, recent studies showed that moesin knock-down increased migration, invasion and metastasis in pancreatic and gastric carcinomas [12,13]. Claudin7 (CLDN7) belongs to the tight junction protein family. This family is composed of 24 proteins and it is critical for maintaining cell polarity and signal transductions. Loss of cell-cell junction is one critical step in tumor cell metastasis [14]. Loss of claudins has been reported in several malignancies and their expression seems to be a prognostic marker in several cancer types [14-16]. More specifically, loss of CLDN7 was reported in breast carcinoma, oral squamous cell carcinoma and colorectal carcinoma where it was found to be associated with poor prognosis in these tumor types [17-19]. The first objective of our study was to explore the MSN and CLDN7 protein expression in a large series of human endometrial cancers. The second objective was to examine the prognostic value of MSN and CLDN7. The extent of immunochemical reactivity of the primary antibody were included in all assays. The stain was membranous and cytoplasmic for both MSN and CLDN7. The extent of membrane-associated with poor prognosis in these tumor types. All pathology specimens were reviewed by one pathologist (PMF), and tumors were classified according to World Health Organization (WHO) criteria [20]. All slides were examined by an expert gynecologic pathologist for confirmation of the histologic type, tumor size, tumor grade, depth of myometrial invasion (MI) and presence of lymphovascular invasion (LVI).

Immunohistochemistry
Four μm thick sections were deparaffinized with xylene, and washed with ethanol. Sections were cooled 20 min and incubated 10 min with 3% H2O2 to quench endogenous peroxidase activity. Blocking was performed using serum-free protein block, Dakocytomation (Carpenteria, CA) for 30 min. The sections were pretreated with an EDTA buffer saline solution, and microwaved for 20 min and then sections were incubated with MSN antibody (monoclonal; 1:20000 dilution; LifeSpan Biosciences, Seattle, WA, USA) and with Claudin7 (polyclonal; 1:50 dilution; Zymed, San Francisco, CA, USA) for 1 h at room temperature. The diaminobenzidine complex was used as a chromogen. Positive control used for MSN was lung adenocarcinoma and for Claudin7 was breast adenocarcinoma. Negative control slides omitting the primary antibody were included in all assays. The stain was membranous and cytoplasmic for both MSN and CLDN7. The extent of immunohistochemical reactivity was graded based on intensity as follows: 0 (negative), 1+ (weak), 2+ (moderate), 3+ (strong). For the sake of statistical analysis, negative and weak stains were grouped as group I (negative) and moderate and strong as group II (positive). Examples of positive and negative cases are illustrated in Figures 1A-B.

Methods
Patients population
The pathology archives were searched for endometrial adenocarcinoma cases from January 2000-December 2010. IRB approval (I-75206) was obtained. A chart review was conducted with extraction of clinical information including the patients' age at the time of diagnosis, the surgical stage, the post-operative therapy, the disease free survival (DFS), the site of recurrence, the cause and the date of death. All patients underwent a surgical staging procedure including a total hysterectomy with bilateral salpingo-oophorectomy, with or without pelvic and para-aortic lymph node dissection and pelvic washings, depending on the tumor grade and the clinical tumor stage. Patients were treated according to the National Comprehensive Cancer Network (NCCN) guidelines (http://www.cancer.gov).

Histological evaluation
All pathology specimens were reviewed by one pathologist (PMF), and tumors were classified according to World Health Organization (WHO) criteria [20]. All slides were examined by an expert gynecologic pathologist for confirmation of the histologic type, tumor size, tumor grade, depth of myometrial invasion (MI) and presence of lymphovascular invasion (LVI).

Statistical analyses
Statistical analyses were performed by R (http://www.r-project.org/). The clinical parameters used for modeling are age, tumor size, histologic subtypes, myometrial depth of invasion, LVI, FIGO grade, recurrence, status, and survival time. To test the association between the biomarker and the clinical parameters, Fisher’s exact test was performed for categorical parameters and Welch t-test was used for the continuous ones. For survival analysis, Kaplan-Meier method with log-rank test was used to calculate the cumulative survival time, and check both the overall survival (OS) and disease free survival (DFS) differences between the patients with the different biomarker status. Multivariate cox proportional hazard model was used to determine the hazard ratio
that represents the relative risk of death among patients with each of MSN$^+$ and CLDN7$^+$ compared with those with MSN$^-$ and CLDN7$^-$. All reported p values were two sided.

**Results and discussion**

The clinical and pathologic features of 265 patients with endometrial adenocarcinoma are summarized in Table 1. All patients had surgery for endometrial cancer with...
no previous chemotherapy or radiation therapy, and all had complete follow-up information with median of 2.7 years. The distribution of clinical factors in relation to the status of MSN and CLDN7 expression is illustrated in Table 2. There was a strong association between MSN+ and high tumor grade (p < 0.001) and with tumor subtype (p < 0.001). Specifically, MSN was more likely to be expressed in USC, CCC and carcinosarcoma subtypes, than in endometrioid adenocarcinomas. As for CLDN7, there was a strong association between CLDN7+ and low tumor grade (p < 0.001) and endometrioid subtype (p = 0.002). There was no association between each of MSN and CLDN7 and disease outcome such as DOD, OS, or DFS (recurrence) (Figure 2A, 2B). Finally, MSN and CLDN7 did not show any association with lymph node metastasis.

We further explored the impact of MSN and CLDN7 on chemotherapy response. Of the 72/265 patients who received radiation + chemotherapy or chemotherapy alone, 39 were MSN+ while 33 were MSN-, and 33 were CLDN7+ while 39 were CLDN-. Based on our analysis, there was no significant independent value of MSN and CLDN7 in predicting OS, DFS, or DOD between these two groups (data not shown).

Metastasis is the primary cause of fatality in endometrial cancer. The ERM and claudins are two families of proteins involved in the multistep tumor metastasis process[8,9,14]. Much is known regarding ezrin and claudins1-4 in endometrial cancer but the role of MSN and CLDN7 is yet to be explored [15,21-24]. Based on previous DNA microarray analysis, we showed an up-regulation of the MSN gene and a down-regulation of the CLDN7 gene in USC in comparison to EAC. Their mRNA expressions were validated by qRT-PCR [5,6]. In addition, MSN was under-expressed and CLDN7 normally expressed in HEC1A and RL95-2 cell lines (data not shown). The present study was done in continuity to our previous work, aiming to determine the protein expression of each of MSN and CLDN7 in EC and to explore their prognostic significance in a large series of patients.

Ezrin is the only gene in the ERM family that has been widely explored in various malignancies. Ezrin was evaluated in EC where it was implicated in the process of invasion in endometrial cancer cell lines [25].

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**Table 1 Clinical and pathologic features of patients.**

| Feature                        | Value         |
|-------------------------------|--------------|
| No. of evaluable patients     | 265          |
| Follow time, year             |              |
| Median                        | 2.74         |
| Age, year                     |              |
| Median                        | 65           |
| Range                         | 29-97        |
| Stage                         |              |
| I                             | 174(66)      |
| II                            | 36(14)       |
| III                           | 36(14)       |
| IV                            | 19(7)        |
| Subtype                       |              |
| Endometrioid                  | 192(73)      |
| USC+CCC                       | 54(20)       |
| Carcinosarcoma                | 19(7)        |
| Grade (FIGO)                  |              |
| 1                             | 113(43)      |
| 2                             | 49(18)       |
| 3                             | 103(39)      |
| Grade (Nuclear)               |              |
| 1                             | 88(33)       |
| 2                             | 70(26)       |
| 3                             | 107(40)      |
| Tumor size, cm                |              |
| Median (Range)                | 4.58(0-33)   |
| < 2                           | 53(20)       |
| > 2                           | 212(80)      |
| Depth of myometrial invasion  |              |
| Median (Range)                | 38.52(0-100) |
| < 50                          | 170(64)      |
| > 50                          | 95(36)       |
| Lympho-vascular involvement   |              |
| no                            | 192(72)      |
| yes                           | 73(28)       |
| Lymph Node Status             |              |
| yes                           | 41(15)       |
| no                            | 119(45)      |
| Not examined                  | 105(40)      |
| Recurrence                    |              |
| no                            | 209(79)      |
| yes                           | 42(16)       |
| persistent                    | 10(4)        |
| progression                   | 4(1)         |
| Status                        |              |
| ANED                          | 202(76)      |
| AWED                          | 28(11)       |
| DOD                           | 22(8)        |
| DNED                          | 6(2)         |
| DWED                          | 4(2)         |
| others                        | 3(1)         |
| MSN                           |              |
| negative                      | 143(54)      |

**Table 1 Clinical and pathologic features of patients.** (Continued)

| Feature                        | Value         |
|-------------------------------|--------------|
| Claudin7                      |              |
| negative                      | 127(48)      |
| positive                      | 138(52)      |

(Data in parentheses are percentages)
addition, strong ezrin immunoexpression was related to poor prognosis in FIGO stage I EAC [26]. On the other hand, MSN is less well studied in malignancies and the few published data showed that alteration of MSN was present in pancreatic cancer, lung adenocarcinoma and oral squamous cell carcinoma [12,27,28]. To the best of our knowledge, MSN expression has never been reported in human EC. Previous studies showed that claudin 3 and 4 were overexpressed in USC and they were associated with higher tumor grade [15,23]. However, these studies failed to show an independent value for claudin3 and 4 in predicting disease outcome. In our current investigation, we showed that MSN and CLDN7 proteins were expressed in a high percentage (almost 50%) of EC cases. Furthermore, we found a strong association of MSN and CLDN7 expressions with two important histologic prognostic factors - tumor grade and tumor subtypes. Specifically, MSN protein expression was associated with type II carcinomas and high tumor grade (G3), while CLDN7 protein expression was associated with low tumor grade (FIGO G1 and G2) and with type I carcinomas. Even though no prognostic value of MSN and CLDN7 expression in predicting EC patient outcome was found, the above data nevertheless led us to suggest that MSN and CLDN7 proteins might be involved in the tumorigenesis of endometrial cancer.

One limitation of our study was that the majority of our tumors were endometrioid type, well differentiated, and presented at an early stage, which is a frequent occurrence in endometrial cancer. Because these tumors have favorable outcome in general, it is expected that the majority of the patient population will be alive at the time of last follow-up. The resulting fewer numbers of unfavorable outcome in these patients might limit our statistical power in predicting survivals.

Conclusions
Using immunohistochemical stains, our work is the first to comprehensively study the protein expression of MSN and CLDN7 in correlation with the clinical characteristics in a large series of patients with endometrial cancer. Although we did not find a significant prognostic value of MSN and CLDN7 in predicting disease outcome in patients with endometrial cancer, our data suggests that MSN and CLDN7 protein

### Table 2 Association between MSN and claudin7 immunoexpression and the clinical variables

| Variables (group 1 vs group2) | MSN | Claudin7 |
|-------------------------------|-----|-----------|
|                               | Pvalue | Odds Ratio | CI | Pvalue | Odds Ratio | CI |
| Age*                          | 0.206 | 1.401 | 0.741-2.664 | 0.196 | 1.033 | 0.546-1.962 |
| Stage*                        | 0.29  | 1.018 | 0.472-2.200 | 1.018 | 0.472-2.200 |
| Tumor size*                   | 0.013 | 1.401 | 1.168-4.724 | 0.759 | 0.876 | 0.456-1.673 |
| Lymph-vascular involvement*   | 0.098 | 0.616 | 0.344-1.096 | 0.413 | 0.125 | 0.707-2.236 |
| Lymph node status             | 0.305 | 1.323 | 0.776-2.262 | 0.442 | 0.797 | 0.467-1.358 |
| Grade FIGO*                   | < 0.001 | 2.561 | 1.504-4.385 | 0.001 | 0.419 | 0.245-0.711 |
| Grade nuclear*                | < 0.001 | 2.557 | 1.504-4.385 | 0.001 | 0.419 | 0.245-0.711 |
| Subtype*                      | < 0.001 | 0.311 | 0.154-0.609 | 0.002 | 0.262 | 1.353-2.523 |
| Recurrence*                   | 0.867 | 0.935 | 0.456-1.925 | 0.309 | 0.714 | 0.447-1.501 |
| Status*                       | 0.567 | 0.847 | 0.462-1.554 | 0.112 | 1.623 | 0.885-3.007 |
| Status*                       | 1.018 | 1.018 | 0.468-2.226 | 0.278 | 1.53  | 0.705-3.386 |

\* The P-values by Fisher exact test to test the correlations between the biomarker and the clinical factors.

\* The P-value is calculated by the two sample welch t-test

Stage: Group1: stages I and II; Group 2: stages III and IV
Tumor Size: Group 1: < = 2 cm; Group2: > 2 cm
LVI: Group 1:yes; Group2: no
Depth of myometrial invasion: Group1:< = 50; Group2: > 50
Grade: Group1: grade 1 and 2; Group2: grade 3
Subtype#: Group1:CCC & USC; Group 2: Endometrioid; Group3: MMMT
Recurrence#: Group1:yes; Group2:no; Group3:others
Recurrence^: Group1:yes; Group2: no &others
Status#: Group1: ANED; Group2: all others
Status^: Group1:ANED&AWED; Group2: all others
Interprete the Odds Ratio:
> 1, the proportion of p and n biomarkers status in Group 2 is greater than the proportion in Group 1
< 1, the proportion of p and n biomarkers status in Group 1 is greater than the proportion in Group 2
immunoexpression might be involved in the development and progression of carcinoma of the endometrium.

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