FSH Levels Are Related to E-cadherin Expression and Subcellular Location in Nonfunctioning Pituitary Tumors

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Context: Gonadotroph pituitary neuroendocrine tumors (PitNETs) can express follicle-stimulating hormone (FSH) and luteinizing hormone (LH) or be hormone negative, but they rarely secrete hormones. During tumor development, epithelial cells develop a mesenchymal phenotype. This process is characterized by decreased membranous E-cadherin and translocation of E-cadherin to the nucleus. Estrogen receptors (ERs) regulate both E-cadherin and FSH expression and secretion. Whether the hormone status of patients with gonadotroph PitNETs is regulated by epithelial-to-mesenchymal transition (EMT) and ERs is unknown.

Objectives: To study the effect of EMT on hormone expression in gonadotroph nonfunctioning (NF)-PitNETs.

Design: Molecular and clinical analyses of 105 gonadotroph PitNETs. Immunohistochemical studies and real-time quantitative polymerase chain reaction were performed for FSH, LH, E-cadherin, and ERα. Further analyses included blood samples, clinical data, and radiological images.

Setting: All patients were operated on in the same tertiary referral center.

Results: NF-PitNET with high FSH expression had decreased immunohistochemical staining for membranous E-cadherin (P < .0001) and increased staining for nuclear E-cadherin (P < .0001). Furthermore, high FSH expression was associated with increased ERα staining (P = .0002) and ERα mRNA (P = .0039). Circulating levels of plasma-FSH (P-FSH) correlated with FSH staining in gonadotroph NF-PitNET (P = .0025). Tumor size and invasiveness was not related to FSH staining, E-cadherin, or ERα. LH expression was not associated with E-cadherin or ERα.

Conclusion: In gonadotroph PitNETs, FSH staining is related to E-cadherin, ERα expression, and circulating levels of P-FSH. There was no association between FSH staining and invasiveness. The abbreviations: EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; FSH, follicle-stimulating hormone; FP, forward primer; IRS, immunoreactivity score; LH, luteinizing hormone; MRI, magnetic resonance imaging; mRNA, messenger RNA; NF, nonfunctioning; PitNETs, pituitary neuroendocrine tumors; qPCR, quantitative polymerase chain reaction; RP, reverse primer; SF-1, steroidogenic factor 1.
pituitary neuroendocrine tumors (PitNETs) are among the most common intracranial tumors, with nonfunctioning (NF)-PitNETs being the most prevalent subtype (1, 2). In contrast to functioning pituitary tumors, NF-PitNETs do not hypersecrete pituitary hormones. However, immunohistochemistry shows that most NF-PitNETs produce hormones indicating their cell lineage (gonadotroph, corticotroph, thyrotroph, somatotroph, or lactotroph), and true negatives for pituitary hormones and transcription factors (null cell) are rare (3). Gonadotroph tumors are the most common subtype of NF-PitNETs and are characterized by positive staining for steroidogenic factor 1 (SF-1) and may also express follicle-stimulating hormone (FSH) and/or luteinizing hormone (LH), but are rarely functional (4). The mechanisms underlying this specter of PitNETs from hormone negative, hormone producing, to hormone hypersecreting is unknown.

PitNETs originate from specialized neuroendocrine epithelial cells. During tumor progression they may de-differentiate to a more mesenchymal phenotype, a process called epithelial to mesenchymal transition (EMT) (5-14). EMT is characterized by altered expression and localization of E-cadherin (15, 16). In normal epithelial tissues, E-cadherin is localized on the plasma membrane where it functions as a cell to cell adhesion molecule (15). In addition, E-cadherin interacts with multiple signaling pathways (6-8) and mediates cell to cell inhibition (17). During EMT, membranous E-cadherin can be cleaved, leading to shedding of the extracellular domain and nuclear translocation of E-cadherin (5, 18-23).

Loss of membranous E-cadherin and gain of nuclear E-cadherin is associated with loss of cell differentiation, high Ki67 index, larger tumors, and increased invasiveness in PitNETs (21, 24, 25). This includes NF-PitNETs (26), somatotroph PitNETs (25, 27-31), corticotroph PitNETs (32), and also prolactinomas (33).

Gonadotroph cells in the pituitary express estrogen receptors (ERs) (34, 35), and estrogen has a direct inhibitory effect on FSH secretion from pituitary cells (36). Estrogen also inhibits FSH synthesis and secretion in pituitary cultures (37-39). Furthermore, estrogen seems to decrease E-cadherin in various tissues (40-46), including NF-PitNETs (26). We have previously found that the absence of estrogen receptor-α (ERα) (47) is a predictor of reintervention after surgery for NF-PitNETs in male patients. How this relates to the levels of FSH in the tumor tissue and the circulating counterpart has not been studied. We therefore investigated whether the degree of EMT, represented by the loss of membranous E-cadherin, is related to hormone expression in gonadotroph NF-PitNETs. Since gonadotroph tumors are mostly nonsecretory, we hypothesized that FSH expression would be higher in well-differentiated epithelial cells, and decrease as the tumors undergo EMT. This effect was expected to be attenuated by ERα expression.

**Materials and Methods**

We investigated 105 patients with NF-PitNETs operated at our tertiary referral center between 1998 and 2009. The majority (>90%) of operations were performed by 3 neurosurgeons. None of the patients had previously undergone surgery or radiation to the pituitary gland. Informed consent was obtained from all participants. These patients have been presented in previous publications (47-49), whereof 105 gonadotroph tumors with available tissue samples were included in this study. Of the included patients, 72 (69%) were males. Of the females, 27 were considered postmenopausal (age 53-84 years), while 6 were considered to be premenopausal (age 32-48 years). The mean age of participants was 59.9 ± 13.0 years. Plasma FSH (P-FSH) and LH (P-LH) were measured with routine laboratory assays. Mean P-FSH in the entire cohort was 6.7 ± 6.6 IU/L, in premenopausal females 7.4 ± 6.1 IU/L (normal range 1.2-21 IU/L), and in males 6.6 ± 6.6 IU/L (normal range 0.7-11.1 IU/L). Blood samples were taken preoperatively and were not timed in regard to the menstrual cycle for premenopausal women.

**Immunohistochemistry**

Paraffin-embedded tissue was available from all the patients. The diagnosis of PitNET was confirmed on hematoxylin and eosin stained sections. All tumors were of gonadotroph cell lineage with positive immunohistochemical staining for transcription factor SF-1. From each tumor, 2 1-mm cores from 2 different representative tumor areas were taken and used to construct tissue microarrays. The immunohistochemical staining of SF-1 (Perseus Proteomics, monoclonal, clone N1665) was performed on DAKO Autostainer Link 48, as previously described (50, 51). FSH and LH staining on samples operated until the first half of 2007 was carried out using a Ventana automatic stainer with anti-FSH (Thermo Scientific, monoclonal, Clone FSH03, Ab-3, 1:3,000) and anti-LH (Thermo Scientific, monoclonal, Clone LH01, Ab-1, 1:3,000), as reported earlier (51). Tumors operated on after the middle of 2007 were stained with anti-FSH (DAKO, monoclonal, Clone C10, Code M3502) and anti-LH (DAKO, monoclonal, Clone C93, Code M3502) on a DAKO Autostainer Link 48 (47).

The expression of FSH and LH varied in intensity from weak to strong among immunolabelled cells in almost all positive tumors. Staining was considered low when less than 10% of the cells were positive independently of the staining intensity, moderate when 10% to 50% were positive, and high when >50% were positive for FSH or LH. FSH and LH expression was usually similar between the 2 tumor cores; however, when
this was not the case the core with the highest percentage of positive cells was taken into consideration. Normal pituitary tissue served as control.

E-cadherin was detected using antibody against the intracellular domain (BD Transduction Laboratories, mouse monoclonal, clone 36/E-Cadherin, dilution 1:300) performed on the DAKO EnVision Flex+ system (K8012; DAKO, Glostrup, Denmark) and DAKO Autostainer as described previously (48, 51). Skin biopsy with keratinocytes was used as the positive control, while the negative control was obtained by omitting the primary antibodies. The extracellular domain of E-cadherin (Abcam ab1416, mouse monoclonal, clone HEC1D, dilution 1:100) has been investigated in earlier papers, but not considered in this study.

ERα was detected with ready-to-use monoclonal antibody SP1 (Ventana-Roche) on a Ventana Bench MarkUltra and the UltraView Universal detection kit as described earlier (47). A sample from a ductal mammary carcinoma served as the positive control. The negative control was obtained by omitting the primary antibody. All positive and negative controls gave satisfactory results.

Staining for membranous E-cadherin and ERα were quantified using an immunoreactivity score (IRS). The IRS is the product of the percentage of positively staining cells (0 = 0%; 1 = 1-10%; 2 = 10-50%; 3 = 50-80%; 4 ≥ 80%) and the staining intensity (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining), and ranges from 0 to 12. The 2 tissue cores for each tumor were assessed as 1 area in the assessment of the IRS score. In the cases with variable staining intensity within the tumor, the most prevalent intensity was used in the score. Nuclear E-cadherin was scored as either positive or negative. All immunohistochemical analyses were performed by the same pathologist (O.C.-B.), blinded to the clinical data.

Real time qPCR. Frozen tissue samples were available from 74 patients. The samples were collected during operation, frozen immediately after resection, and stored at −80°C until assayed. mRNA was extracted and quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed as described previously (52). The following primer pairs were used for PCR analyses: E-cadherin forward primer (FP) 5’-CATTGCCACATACACTCTCTTCTTCT-3’ and reverse primer (RP) 5’-CGGTTCACCGTGATCAAAATCTC-3’; ERα FP 5’-GGGAAGTAGATGGCTATGGAATCTG-3’ and RP 5’-TGGCTGGACACATATGGTCGTT-3’; FSH FP 5’-TTCAAGAGGGATGCTCATTC-3’ and RP 5’- CCTTGACAGGACAAGCTCC-3’. Primers for FSH and LH RT-qPCR results were standardized against the geometric mean of the reference genes GADPH and ALAS1, validated in a previous study (52).

Magnetic resonance imaging. Preoperative magnetic resonance (MR) images scans were available for 47 patients and used to evaluate tumor volume and invasiveness. Tumor volume was calculated as described previously (49). Invasiveness was defined as Knosp score of 3 or higher on either side on preoperative MR images (53). Magnetic resonance imaging (MRI) analyses were performed by 2 investigators (K.A.B.Ø. and G.R.).

Statistics. Comparisons between groups were performed with the Mann–Whitney U-test and the chi-square test. Spearman’s rank correlation was used for correlation analyses. A P < .05 was considered significant. Analyses were performed using Stata 16.0 for Windows (StataCorp LLC, College Station, TX).

Results

FSH staining

FSH staining was absent or low in tissue from 52 (49.5%) patients (27 showed no hormone staining), moderate in 22 (21%) patients, and high in 31 (29.5%) patients (Fig. 1). There was a strong correlation between FSH staining and mRNA gene expression levels (R = 0.69, R < 0.0001). FSH and LH staining were not significantly different between males and females (Table 1). FSH staining in tumors did not correlate with plasma levels of testosterone, estradiol, or prolactin (data not shown). Tumor volume or invasive behavior was not associated with FSH staining.

FSH versus E-cadherin

There was an inverse relationship between FSH staining and membranous E-cadherin IRS (Fig. 2A). The median membranous E-cadherin IRS was 6.0 (IQR 5.0-9.0) in tumors with low FSH staining, 4.0 (IQR 4.0-6.0) in tumors with moderate FSH staining, and 4.0 (IQR 2.0-6.0) in tumors with high FSH staining (R = −0.3842, P < 0.0001).

Figure 1. Examples of FSH immunohistochemical staining: (A) low, (B) moderate, and (C) high score.
Conversely, the presence of nuclear E-cadherin was seen in 56% of tumors with low FSH staining, 86% with moderate staining, and 94% with high staining \((P < .0001)\). Tumors with nuclear E-cadherin had lower levels of membranous E-cadherin (IRS 4.3 ± 1.8 vs 8.2 ± 2.2, \(P < .0001\)). FSH staining did not correlate with E-cadherin mRNA, although there was a trend (\(R = -0.229, P = .0502\)). Neither E-cadherin staining nor mRNA was associated with tumor size or invasiveness (data not shown).

### FSH versus estrogen receptors

FSH staining correlated positively with ER\(\alpha\) IRS and mRNA levels (Fig. 2B). In tumors with low FSH staining, median ER\(\alpha\) IRS was 1.0 (IQR 0.0-2.0), versus 2.0 (IQR 1.0-4.0) in tumors with moderate and 3.0 (IQR 1.0-4.0) in tumors with high FSH expression (\(R = 0.357, P = .0002\)). FSH staining also correlated with ER\(\alpha\) mRNA (\(R = 0.331, P = .0039\)). There was no correlation between ER\(\beta\) mRNA and FSH staining. Further, there was an inverse correlation between ER\(\alpha\) and membranous E-cadherin staining (\(R = -0.31\), \(P = .0014\)). Tumors with nuclear E-cadherin tended to have higher ER\(\alpha\) staining (2.2 ± 2.2 vs 1.5 ± 2.1, \(P = .066\)). Neither ER\(\alpha\) nor \(\beta\) expression was associated with age, sex, P-FSH, or P-LH levels.

### Plasma FSH

Preoperatively, P-FSH was measured in 88 patients and P-LH in 84 patients. Patients taking estrogens/testosterone and postmenopausal women were excluded, and therefore P-FSH from 62 males and 6 females was available for analysis. P-FSH correlated with tumor FSH staining (\(R = 0.37, P = .0004\) (Fig. 3), but not FSH mRNA (\(R = 0.22, P = .12\)). There was no association between P-FSH and age, sex, plasma-estradiol, or plasma-prolactin.

### Table 1. Immunohistochemical data given as median and interquartile range.

|                        | Total (n = 105) | Female (n = 33) | Male (n = 72) | \(P\)-value |
|------------------------|----------------|----------------|--------------|-------------|
| Age (years)            | 59.9 ± 13.0    | 62.2 ± 13.1    | 58.8 ± 13.0  | .223        |
| FSH staining (IRS)     | 1.0 (0.0-2.0)  | 0.0 (0.0-1.0)  | 1.0 (0.0-2.0)| .508        |
| FSH mRNA (mean ± SD)   | 0.56 ± 0.73    | 0.77 ± 0.95    | 0.48 ± 0.62  | .312        |
| LH staining (IRS)      | 0.0 (0.0-2.0)  | 0.0 (0.0-1.0)  | 1.0 (0.0-2.0)| .079        |
| E-cadherin IRS         | 6.0 (4.0-6.0)  | 6.0 (4.0-6.0)  | 6.0 (4.0-6.0)| .940        |
| E-cadherin mRNA        | 1.37 ± 0.74    | 1.50 ± 1.04    | 1.32 ± 0.59  | .990        |
| Nuclear E-cadherin IRS| 73%            | 70%            | 75%          | .568        |
| ER\(\alpha\) IRS       | 1.0 (0.0-4.0)  | 2.0 (0.0-3.0)  | 1.0 (0.0-4.0)| .522        |
| ER\(\alpha\) mRNA      | 1.17 ± 1.63    | 0.87 ± 1.26    | 1.30 ± 1.76  | .277        |
| ER\(\beta\) mRNA       | 0.41 ± 0.24    | 0.40 ± 0.22    | 0.42 ± 0.25  | .670        |
| Tumor volume (cm\(^3\)) | 66.1 (40.9-111.7)| 60.6 (36.9-90.4)| 82.5 (45.9-115.6)| .312        |
| Invasive (Knosp ≥ 3)   | 43%            | 56%            | 35%          | .172        |

mRNA data are given as mean ± standard deviation. Binary data given as percentages. Tumor volume and invasiveness were available from 47 patients. \(P\)-values indicate males vs females.

Abbreviations: IRS, immunoreactivity score; ER, estrogen receptor; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Figure 2. (A) FSH staining correlated inversely with membranous E-cadherin IRS (\(R = -0.384; P < .0001\)). A similar trend was seen between FSH staining and E-cadherin mRNA (\(R = -0.229; P = .0502\)). (B) FSH staining correlated with ER\(\alpha\) IRS (\(R = 0.357; P = .0002\)) and mRNA (\(R = 0.331, P = .0039\)). There was no difference in the expression of ER\(\beta\) (\(R = 0.304; P = .775\)). IRS data shown as median and interquartile range. mRNA data shown as mean ± standard error of the mean. ER, estrogen receptor; IRS, immunoreactivity score.
LH staining was low in 54 (51%) tumors, moderate in 24 (23%), and strong in 27 (26%). Samples with strong LH staining showed lower expression of membranous E-cadherin ($R = -0.20, P = .040$), whereas there was no relationship with E-cadherin mRNA ($P = .2798$) or presence of nuclear E-cadherin ($P = .202$). LH staining did not correlate with ER$\alpha$ IRS or mRNA expression, P-LH, P-testosterone, tumor volume, invasiveness, age, or sex.

**Discussion**

We found that FSH expression in gonadotroph NF-PitNETs was associated with E-cadherin expression and localization, both at the protein level, assessed by using immunohistochemistry, and at the gene expression level. Tumors with high FSH staining showed lower expression of membranous E-cadherin ($R = -0.20, P = .040$), whereas there was no relationship with E-cadherin mRNA ($P = .2798$) or presence of nuclear E-cadherin ($P = .202$). LH staining did not correlate with ER$\alpha$ IRS or mRNA expression, P-LH, P-testosterone, tumor volume, invasiveness, age, or sex.

FSH staining

Figure 3. FSH staining in the tumor correlated with circulating levels of P-FSH. Data shown as mean ± standard error of the mean. Patients taking estrogen, testosterone and postmenopausal women were excluded from analyses.

LH staining

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Our finding of FSH and E-cadherin is in line with studies in rat lactotroph cells (54), where expression of membranous E-cadherin was related to decreased prolactin staining levels, and in studies of pancreatic islet cells where membranous E-cadherin correlates with an increase in insulin secretion (55-57). These studies suggest that hormone secretion is promoted by E-cadherin, and that hormones may accumulate intracellularly in the absence of E-cadherin. However, this does not explain the association we found between FSH staining and P-FSH levels. This could be due to increased production of FSH in dedifferentiated mesenchymal cells, but we did not examine this in our study. Compared with other PitNET subtypes, gonadotroph tumors have lower levels of membranous E-cadherin (27, 28, 32, 48), and are more commonly nonfunctional than other PitNET subtypes (4). These findings support the role of E-cadherin in hormone secretion, but the link between E-cadherin and hormone secretion is not fully understood.

Gonadotroph PitNETs have particularly low extracellular E-cadherin (48), but more frequent nuclear translocation of E-cadherin (21, 48). Loss of membranous E-cadherin and nuclear translocation is associated with changes in $\beta$-catenin and p120 expression and intracellular location (14), both of which interact with several signaling pathways and transcription factors (16-18, 20, 57-61). These changes are associated with larger and more aggressive tumors in somatotroph and corticotroph tumors (21, 24, 26, 28, 32). However, we have previously found that nuclear E-cadherin was associated with less surgical reintervention in gonadotroph PitNETs (48), although the underlying cause could not be determined.

ER$\alpha$ is expressed in both normal gonadotroph cells and in gonadotroph PitNETs (34, 35), and has been implicated in the development of PitNETs (26, 62-64). ER$\alpha$ expression is known to decrease membranous E-cadherin, increase cell proliferation, and contribute to EMT (26, 41, 42, 46), in accordance with our results. This is mediated through the Slug and Snail pathways (26, 43, 46). Estrogen also enhances pituitary tumor growth through pituitary tumor transforming gene ($pttg$), and this effect is counteracted by antiestrogens (62, 63). We lacked the possibility to investigate the gene expression of Slug, Snail, or $pttg$ in this study, but this will be investigated in our newly launched prospective study.

Estrogen has also been shown to inhibit FSH secretion directly on the pituitary gland in humans (36) and in cell cultures (37-39). By inhibiting FSH secretion, estrogen may contribute to intracellular accumulation of FSH. This remains uncertain, however, since the majority of our study population was male patients and p-estrogens were not measured routinely. FSH in itself has also been shown to induce EMT in ovarian cancer cells (65). The FSH effect on ovarian cancer was shown to be dependent on FSH-receptors that was not investigated in this study.
Although circulating levels of P-FSH correlated with tumor staining for FSH, none of the patients were diagnosed with clinically functioning gonadotroph tumors preoperatively. Functioning gonadotroph tumors are considered rare (4, 66), but our data suggest that some of the FSH produced may be secreted. However, whether the circulating P-FSH was biologically active is not possible to investigate with the present study design, and will have to await prospective sampling.

Limitation

The design of the study was retrospective, but was based on a large and well-characterized population including tumor classification by immunohistochemistry for pituitary hormones and pituitary-specific transcription factors. We did not have the opportunity to evaluate the biological activity of the circulating FSH in the present study. Moreover, our cohort included both genders, as well as pre- and postmenopausal women. Tumor volume and invasiveness assessed by MRI were not available for all patients, thus representing the risk of a type 2 error. Another limitation is the tissue microarray technique used for immunohistochemical analyses, since FSH and LH expression may be heterogeneous within a tumor. This limitation has been reduced by using 2 cores from different areas from each tumor, and a high correlation between FSH staining and mRNA expression indicated that the cores were representative of the tumors.

Conclusion

Contrary to our hypothesis, loss of membranous E-cadherin and gain of nuclear E-cadherin were associated with higher staining levels of FSH in the tumors. This may suggest increased production of FSH or accumulation of FSH in gonadotroph tumors when they develop a mesenchymal phenotype. Furthermore, increased expression of FSH was related to high levels of ERTs and may indicate a loss of normal feedback mechanisms. Neither FSH nor E-cadherin expression was associated with tumor volume or invasiveness. Tumor expression of FSH is associated with circulating levels of P-FSH, suggesting that some of the produced hormone is secreted without leading to overt clinical symptoms. Further prospective studies are needed to understand the mechanisms behind and the clinical significance of these findings.

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Additional Information

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Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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