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Original contribution

Whole-proteome analysis of mesonephric-derived cancers describes new potential biomarkers☆,☆☆

Evan Gibbard BSc Hons[a,b], Dawn R. Cochrane PhD[b], Jennifer Pors MD[c], Gian Luca Negri PhD[c,d], Shane Colborne BSc[d], Angela S. Cheng BSc[e], Christine Chow BMLSc[e], David Farnell MD[c], Basile Tessier-Cloutier MD[c], Jessica N. McAlpine MD[f], Gregg B. Morin PhD[a,d], Dietmar Schmidt MD[g], Stefan Kommoss MD[h], Friedrich Kommoss MD[i], Jacqueline Keul MD[j], Blake Gilks MD[c,e,k], David G. Huntsman MD[a,b,c,e], Lynn Hoang MD[c,e,k,*]

[a] *Department of Medical Genetics, The University of British Columbia, Vancouver, BC, V6H 3N1, Canada*
[b] *Molecular Oncology, BC Cancer Agency, Vancouver, BC, V5Z 1L3, Canada*
[c] *Department of Pathology and Laboratory Medicine, The University of British Columbia, Vancouver, BC, V6T 2B5, Canada*
[d] *Michael Smith Genome Sciences Centre, BC Cancer Agency, Vancouver, BC, V5Z 4S6, Canada*
[e] *Genetic Pathology Evaluation Centre, Vancouver General Hospital, Vancouver, BC, V6H 3Z6, Canada*
[f] *Department of Gynecology and Obstetrics, Division of Gynecologic Oncology, The University of British Columbia, Vancouver, BC, V6Z 2K8, Canada*
[g] *MVZ of Histology, Cytology and Molecular Diagnostics, Trier, 54296, Germany*
[h] *Department of Obstetrics and Gynecology, University of Tübingen, Tübingen, 72076, Germany*
[i] *Institute of Pathology, Medizin Campus Bodensee, Friedrichshafen, 88048, Germany*
[j] *Department of Women's Health, Tübingen University Hospital, Tübingen, 72076, Germany*
[k] *Department of Anatomical Pathology, Vancouver General Hospital, Vancouver, BC, V5Z 1M9, Canada*

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Summary Mesonephric carcinomas (MEs) and female adnexal tumors of probable Wolffian origin (FATWO) are derived from embryologic remnants of Wolffian/mesonephric ducts. Mesonephric-like carcinomas (MLCs) show identical morphology to ME of the cervix but occur in the uterus and ovary without convincing mesonephric remnants. ME, MLC, and FATWO are challenging to diagnose due to

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* Corresponding author. Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver General Hospital, 1215 – 910 West 10th Avenue Vancouver, B.C. V5Z 1M9, Canada.
E-mail address: Lien.Hoang@vch.ca (L. Hoang).

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their morphologic similarities to Müllerian/paramesonephric tumors, contributing to a lack of evidence-based and tumor-specific treatments. We performed whole-proteomic analysis on 9 ME/MLC and 56 endometrial carcinomas (ECs) to identify potential diagnostic biomarkers. Although there were no convincing differences between ME and MLC, 543 proteins showed increased expression in ME/MLC relative to EC. From these proteins, euchromatic histone lysine methyltransferase 2 (EHMT2), glutathione S-transferase Mu 3 (GSTM3), eukaryotic translation elongation factor 1 alpha 2 (EEF1A2), and glycogen synthase kinase 3 beta were identified as putative biomarkers. Immunohistochemistry was performed on these candidates and GATA3 in 14 ME/MLC, 8 FATWO, 155 EC, and normal tissues. Of the candidates, only GATA3 and EHMT2 were highly expressed in mesonephric remnants and mesonephric-derived male tissues. GATA3 had the highest sensitivity and specificity for ME/MLC versus EC (93% and 99%) but was absent in FATWO. EHMT2 was 100% sensitive for ME/MLC & FATWO but was not specific (65%). Similarly, EEF1A2 was reasonably sensitive to ME/MLC (92%) and FATWO (88%) but was the least specific (38%). GSTM3 performed intermediately (sensitivity for ME/MLC and FATWO: 83% and 38%, respectively; specificity 67%). Although GATA3 remained the best diagnostic biomarker for ME/MLC, we have identified EHMT2, EEF1A2, and GSTM3 as proteins of interest in these cancers. FATWO's cell of origin is uncertain and remains an area for future research.

1. Introduction

Mesonephric carcinoma (ME), mesonephric-like carcinoma (MLC), and female adnexal tumors of probable Wolffian origin (FATWO) are rare gynecologic tumors, thought to derive from remnants of the Wolffian/mesonephric ducts [1]. Classic ME occurs most commonly in the uterine cervix [2], and tumors of identical morphology can be found in the endometrium and ovary, where they have been given the designation MLC [3]. FATWO are generally found in the broad ligament or ovary but also occur in other tissues − notably the fallopian tube [4]. The morphologic variety and rarity of ME, MLC, and FATWO have caused significant problems in their diagnosis and recognition [5,6]. This is further complicated by the morphologic and histologic heterogeneity of Müllerian/paramesonephric cancers, which they are most often mistaken for [7]. The distinction of ME/MLC from endometrial carcinomas (ECs) is important clinically, as ME/MLC have a high propensity for distant metastasis and poor overall survival [8,9].

In early development, human embryos are sexually dimorphic and contain both mesonephric and paramesonephric ducts [1]. In women, mesonephric ducts regress and paramesonephric ducts develop into the fallopian tubes, uterus, and parts of the vagina [1]. Conversely, male development sees the regression of the paramesonephric ducts and the elaboration of mesonephric ducts into the epididymis, vas deferens, seminal vesicles, and the testis' efferent ducts [1]. Mesonephric ductal remnants are found in up to one-third of hysterectomies [2], highlighting their persistence into adulthood. The current disease paradigm is that ME, MLC, and FATWO are of mesonephric descent or differentiation. MEs are frequently associated with mesonephric remnants as well as mesonephric hyperplasia, and share similar immunohistochemistry with both [1]. However, while MLC immunohistochemistry and morphology are similar to ME, MLCs are not identified in association with mesonephric remnants [1,10]. For their part, FATWO show immunohistochemical (IHC) similarity to a variety of mesonephric remnants [11,12] but lack shared molecular alterations with ME [13].

Although both ME and MLC share similarities [1,10], and are frequently *KRAS*-mutant [14,15], the precise origin of MLC remains unclear. Some MLCs are associated with paramesonephric-derived cancers that harbor identical molecular aberrations, indicating a possible paramesonephric cell of origin [16]. In these cases, it is likely that MLC results from tumor cells differentiating into a mesonephric-like phenotype.

Several studies have identified GATA binding protein 3 (GATA3) as a sensitive and specific IHC marker of ME [17−19]. However, GATA3 expression is often lost in solid, spindled and undifferentiated areas of tumor and does not appear to show expression in ME's related tumor, FATWO [18,19]. Furthermore, GATA3 shows staining in a variety of neoplasms throughout the body (e.g., breast carcinomas, squamous cell carcinomas from various tissues [skin/cervix/larynx/lung], chromophobe renal cell carcinoma), and is not specific to malignancies of mesonephric lineage [20]. This highlights a presently unmet need for diagnostic protein biomarkers of undifferentiated ME and FATWO.

Proteomic analysis offers new insights into tumor biology and the opportunity to identify novel biomarkers that may aid in the diagnosis and development of tumor-specific therapeutic strategies. Analyzing the full complement of proteins of a tissue permits the identification of

differentially expressed proteins [21]. These proteins may act as individual biomarkers of ME, MLC, and/or FATWO, as well as could be used to build an IHC panel for clinical pathology practice. Recent developments in proteomic technologies have allowed for the quantitative analysis of protein levels from small inputs of formalin-fixed tissues, overcoming historic issues with protein crosslinking and low scalability [21]. These technologies have opened the door to the analysis of rare tumors such as ME, MLC, and FATWO, as these are typically archival formalin-fixed and paraffin embedded (FFPE) tissues, whose diagnoses may only be apparent many years later [7]. In this study, we explore the proteomic landscape of a small series of ME, to identify candidate proteins that may be used to distinguish ME from other more common EC (a group of tumors with which they are confused). Using IHC analysis in a validation cohort of ME/MLC, FATWO, EC, and normal tissues, we translated our proteomic findings into practical immunohistochemistry and tested their cross-applicability in the related tumor FATWO.

2. Materials and methods

2.1. Sample collection

Cases of normal tissues, ME, MLC, FATWO, and EC were retrieved from the archives of the Vancouver General Hospital. Several mesonephric tumor and EC cases have been previously described [10,19]. Additional FATWO samples were obtained from Tübingen University Hospital, Germany. Ethics approval was received from the respective institutional review board (H18-01652; 10/1/2018).

2.2. Proteomic profiling and data analysis

Unbiased whole-proteome analysis was performed on FFPE samples of pooled ME/MLC and EC using single-pot solid-phase-enhanced sample preparation-clinical proteomics (SP3-CTP) [21,22].

Acquired spectra were searched against Uniprot human reference proteome (20247 sequences, 2018/08/03) using Sequest HT algorithm through the Proteome Discoverer suite (v2.1.1.21, Thermo Scientific). Precursor and fragment ion tolerance were set to 20 ppm and 0.8 Da, respectively. Dynamic modifications included oxidation (+15.995 Da, M), acetylation (+42.011 Da, N-Term), and static modification included carbamidomethyl (+57.021 Da, C) and a tandem mass tag (+229.163 Da, K, N-Term). Peptide-to-spectrum (PSM) identification false discovery rate (FDR) was calculated using percolator by searching the results against a decoy sequence set and only PSMs with a FDR $< 5\%$ were retained in the analysis.

R software was used for downstream data analysis. Channel abundances were median normalized, PSMs assigned to a unique protein identifier and those that were not part of a common contaminant list were median aggregated into unique peptide sequences. To detect which proteins were differentially expressed between sample groups, peptide-level expression-change averaging (PECA) [23] analysis was performed using the modified t-test parameter.

Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the Proteomics Identifications [24] partner repository (PRIDE: PXD020879).

Candidate protein markers were selected from PECA-identified proteins. Only proteins with a fold change >1.5 in the ME/MLC tumors were kept. Priority for follow-up was given to proteins with greater expression differences, low FDR-adjusted p-values, and prior associations with cancer. The remaining candidates were assessed for their levels of expression in male genitourinary tissues and gynecologic tissues using Protein Atlas [25,26]. Proteins exhibiting high levels of male expression and low levels of female expression were favored; with the final selection made on the basis of the presence of commercially available IHC antibodies. Fold change and adjusted p-values are shown rounded to 3 significant digits.

2.3. Tissue microarrays

Tissue microarrays were constructed using FFPE blocks of normal tissues from various anatomic sites. Duplicate 0.6 mm cores were taken and assembled using the TMArrayer (Pathology Devices, San Diego, CA). A previously published tissue microarray containing a variety of ECs was also included [19].

2.4. Immunohistochemistry and pathological scoring

For each of the candidate biomarkers, combinations of parameters (varying primary antibody titrations, incubations, diluents, and detection kits) were tested as needed to optimize IHC staining. Optimization was conducted on normal tissue microarray sections, with additional stains of 1−3 ME or FATWO used when initial optimizations were inconclusive. Final conditions were selected where specific epithelial staining was strongest, particularly in normal male tissues. Antibodies that showed nonspecific background staining of nonepithelial elements or low-intensity (faint) staining of epithelial elements were eliminated from downstream analyses. Once optimized, IHC staining was performed using the Ventana Discovery Ultra and Ventana Discovery XT systems (Ventana Medical Systems, Tucson, AZ) (Supplementary Table 1) as per the manufacturer's recommendations. Sections were cut onto charged glass slides, air dried for 10 min, and baked at 60 °C for 10 min. Heat-induced epitope retrieval was performed using various methods (Supplementary Table 1).

Immunohistochemistry for candidate proteins was then performed on whole tissue sections of ME, MLC, FATWO,

along with tissue microarrays of both EC and normal tissues. Gynecologic pathologists scored the intensity and proportion of epithelial staining in normal tissues and carcinoma staining in tumors. The intensity of staining was scored as negative (0), weak (1), moderate (2), or strong (3). The percentage of positive cells binned into deciles was used to score the proportion of staining. The product of the intensity and proportion values was used to provide a histoscore (H-score), quantifying each protein target's expression. Values were rounded to the nearest whole integer.

2.5. Sensitivity and specificity analysis

The sensitivity and specificity of individual antibodies was calculated by comparing staining in ME/MLC or FATWO with that in EC. Any H-score above zero was considered positive. ECs were used as the comparison group to assess marker specificity in ME/MLC. Specificity in FATWO was not calculated due to the absence of a comparison group. Values were rounded to the nearest whole integer.

3. Results

3.1. Study cohorts

ME/MLC and EC were used for proteomic analyses, with subsequent IHC validation also incorporating FATWO. Whole-proteome analysis was performed on 9 cases (4 ME of the cervix, 1 mesonephric carcinosarcoma of the cervix, 2 MLC of the ovary, and 2 MLC of the uterus) and 56 EC (44 endometrioid, 8 serous, and 4 carcinosarcoma [malignant mixed Müllerian tumor (MMMT)]). IHC validations were carried out on a group of 14 ME/MLC, 8 FATWO, and 155 EC. The ME/MLC group included 4 ME of the cervix, 3 mesonephric carcinosarcoma of the cervix, 4 MLC of the uterus, 2 MLC of the ovary, and 1 mesonephric adenocarcinoma of the vagina. Mesonephric carcinosarcomas and adenocarcinomas are referred to as ME for the remainder of the article. Four FATWO were of paratubal origin, 3 were ovarian, and 1's primary site was unknown. The EC cohort was composed of a variety of histotypes with a predominance of endometrioid cancers (124 endometrioid, 17 serous, 6 mixed serous and endometrioid, 4 undifferentiated, 2 MMMT, and 2 clear cell). All ME/MLC used in proteomics were also used in the subsequent IHC analysis. Fifteen EC tumors were common between the proteomics and immunohistochemistry cohorts.

3.2. Whole-proteome analysis of mesonephric tumors yields 4 candidate biomarkers

An unbiased whole-proteome comparison of protein expression between 5 ME (4 mesonephric adenocarcinomas and 1 mesonephric carcinosarcoma) and 4 MLCs revealed little difference between these tumors (Fig. 1A), with Immunoglobulin Heavy Constant Mu (IGHM) the only differentially expressed protein (adjusted p-value < 0.05 and absolute fold change > 1.5). Subsequent analyses combined ME/MLC into one group, collectively referred to as mesonephric cancers (MCs).

The comparison of all 9 MC and a separate cohort of 54 ECs revealed distinct differences in protein expression between these two classes (Fig. 1B). Of 5646 proteins identified, 543 showed a greater than 1.5-fold increase in expression in MC relative to EC with an FDR-adjusted p-value < 0.05. From these, 4 proteins were selected for IHC validation using the criteria previously described in section 2.2. Euchromatic histone lysine methyltransferase 2 (EHMT2), also referred to as G9a, is a histone methyltransferase that methylates histone 3 at lysine 9 [27]. Glutathione S-transferase Mu 3 (GSTM3) is a member of the GST family of proteins that conjugates reduced glutathione to numerous hydrophobic electrophiles [28]. Eukaryotic translation elongation factor 1 alpha 2 (EEF1A2) is involved in the elongation stage of mRNA translation [29]. Glycogen synthase kinase 3 beta (GSK3β) is a negative regulator in the hormonal control of glucose homeostasis [30]. The fold differential expression, adjusted p-values, and peptide numbers of each candidate biomarker are listed in Table 1. EHMT2 [27,31], GSTM3 [28], EEF1A2 [29,32], and GSK3β [30] all had prior associations with cancer, as well as increased expression in male genitourinary tissues relative to gynecologic tissues [25,26]. Similarly, each had commercially available IHC-grade antibodies. A fifth protein — frequently rearranged in advanced T-cell lymphomas 2 (FRAT2) — showed high levels of enrichment in MC but lacked commercial antibodies with convincing immunohistochemistry and was not followed-up on.

GSTM3 and EEF1A2 showed a mixture of cytoplasmic and nuclear staining. EHMT2 showed nuclear staining. Optimized staining for GSK3β in 8 MC, 9 FATWO, testis, and epididymis showed weak or negative staining throughout (data not shown) and GSK3β was excluded from subsequent analyses.

3.3. Candidate biomarkers are enriched in mesonephric-derived normal tissues

EHMT2, GSTM3, EEF1A2, and GATA3 IHC were performed on a variety of mesonephric and paramesonephric-derived normal tissues. Representative images are shown in Fig. 2 and average H-scores in Fig. 3. Tissue microarrays contained mesonephric-derived tissues in the female gynecologic tract (20 rete ovarii, 20 mesonephric remnants in the fallopian tube, and 4 mesonephric remnants in the cervix), normal gynecologic tissues (3 ectocervixes, 3 endocervixes, 4 fallopian tubes, 4 ovaries, 3 proliferative endometria, 3 secretory endometria, and 3

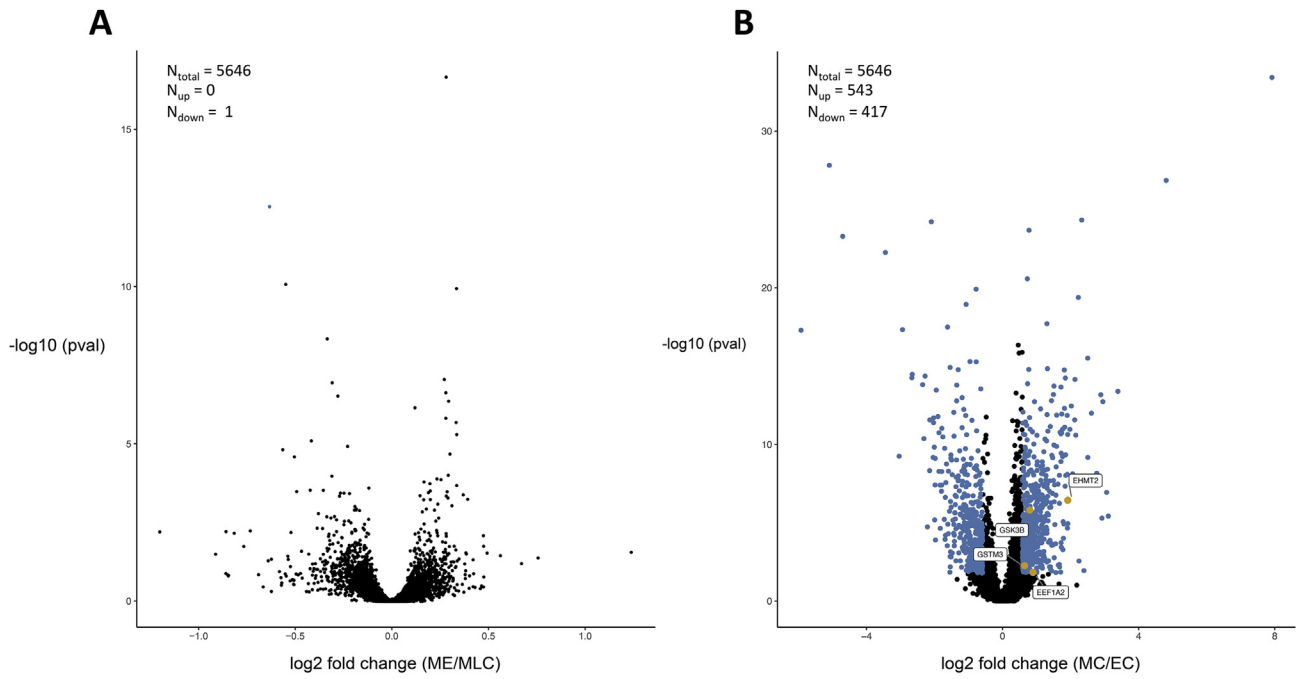

Fig. 1 Volcano plots of protein expression in ME relative to MLC and MC relative to EC. Statistical cutoffs were applied at an FDR-adjusted p-value < 0.05 and an absolute fold change > 1.5. Proteins that met these thresholds for significance are in blue, whereas those that do not are in black. Total number of peptides quantified, as well as the number of significantly upregulated and downregulated (in ME [A] or MC [B]) peptides are indicated. (A) Protein expression in ME relative to MLC. (B) Protein expression in MC relative to EC. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.) ME, mesonephric carcinoma; MLC, mesonephric-like carcinoma; MCs, mesonephric cancers (combined ME and MLC); ECs, endometrial carcinomas; FDR, false discovery rate.

myometria), mesonephric-derived tissues in the male reproductive tract (23 seminal vesicles, 20 vasa deferentia, 23 epididymides, and 20 efferent ducts) and normal male tissues (3 prostate and 3 testis). The number of each tissue with interpretable IHC scores for each marker is shown in Supplementary Table 2.

These stains revealed tissue-specific protein expression patterns for GATA3, EHMT2, GSTM3, and EEF1A2. GATA3 was enriched in fallopian tube mesonephric remnants, cervical mesonephric remnants, and male mesonephric-derived tissues but was absent in the mesonephric remnants in the upper gynecologic tract (rete ovarii) and the efferent ducts (Figs. 2 and 3). Most gynecologic tissues did not express GATA3, but squamous ectocervix showed moderate expression (Fig. 3). EHMT2 expression was present in nearly all tissues tested, with lower H-scores than with GATA3. However, EHMT2 also showed enrichment in mesonephric-derived tissues (Fig. 3). GSTM3 expression was higher in male tissues relative to female tissues, with the exception of prostatic epithelium (Fig. 3). EEF1A2 showed generalized weak staining in normal tissues, with enrichment in both endocervix and cervical mesonephric remnants (Fig. 3). Staining of all markers in other normal tissues appeared variable, and no patterns were identified (Supplemental Fig. 1).

Table 1 Proteomic details of candidate markers.

	EHMT2	GSTM3	EEF1A2	GSK3β
Fold change[a]	3.78	1.56	1.86	1.74
Adjusted p-value[b]	6.12E-06	0.02	0.05	2.14E-05
Peptide number	1	6	2	2

Abbreviations: EHMT2, euchromatic histone lysine methyltransferase 2; GSTM3, glutathione S-transferase Mu 3; EEF1A2, eukaryotic translation elongation factor 1 alpha 2; GSK3β, glycogen synthase kinase 3 beta.

[a] Fold change refers to fold increase in expression in MC relative to EC.

[b] Adjusted p-value refers to FDR-adjusted p-values.

3.4. Candidate biomarkers are enriched in mesonephric tumors and FATWO

GATA3, EHMT2, and GSTM3 IHC was performed in a variety of MC, FATWO, and EC. Representative images are shown in Fig. 4 and a summary of H-scores is shown in Fig. 5.

GATA3 showed a stark enrichment in MC compared with EC and FATWO (Fig. 5). Both EC and FATWO had mean H-scores at or near zero (2.03 and 0, respectively), with the differences between EC and MC (mean = 111), as well as FATWO and MC reaching statistical significance

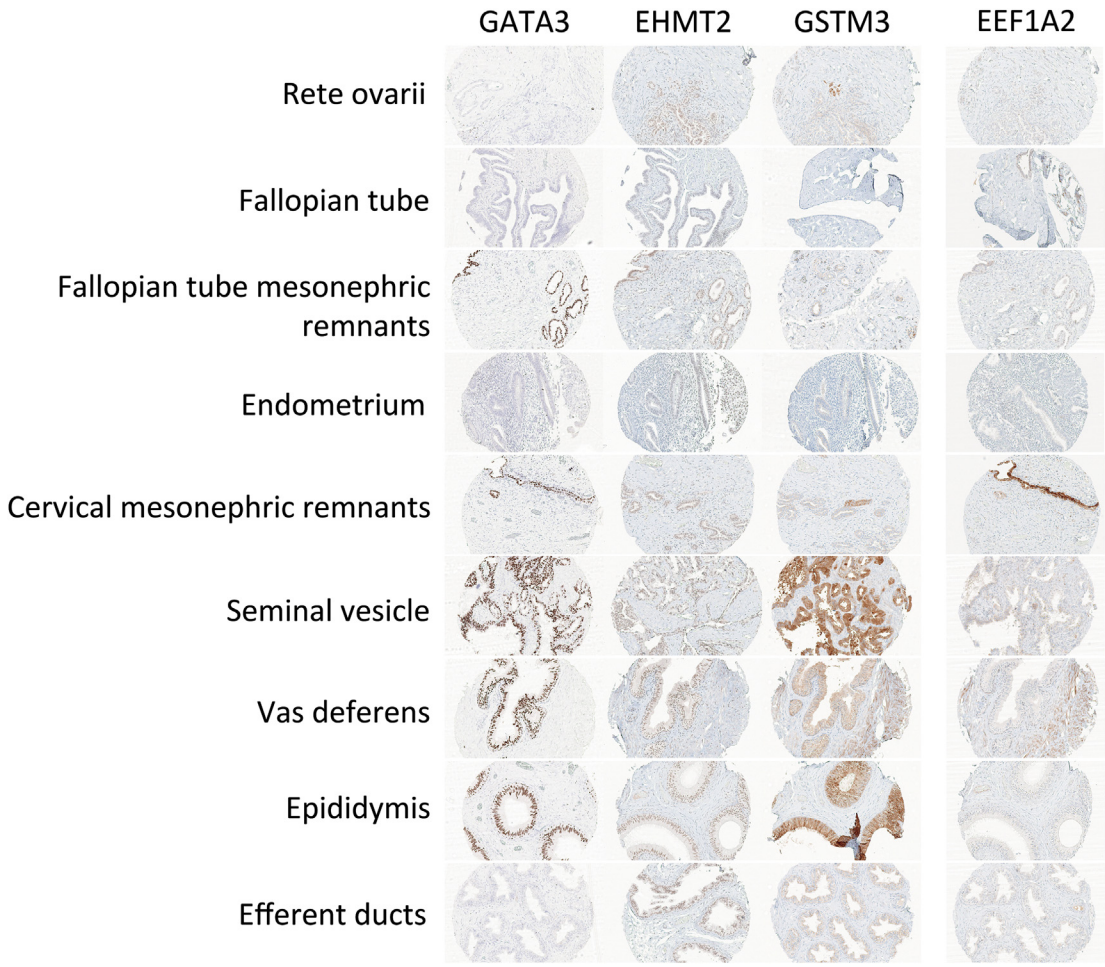

Fig. 2 Representative images of IHC stains of gynecologic tissues, mesonephric remnants, and mesonephric-derived male tissues. Tissues are separated by sex and arranged in descending anatomical order. IHC, immunohistochemical.

(Fig. 5). EHMT2 staining was enriched in both FATWO (mean = 114) and MC (mean = 219) relative to EC (mean = 25). The differences between EC and FATWO, as well as EC and MC reached statistical significance (Fig. 5). GSTM3 had low H-scores across all 3 tumor types (EC mean = 19, MC mean = 53, FATWO mean = 16), with the difference between EC and MC reaching statistical significance (Fig. 5). EEF1A2 showed variable positive expression in all tumor types (EC mean = 51, MC mean = 135, FATWO mean = 85), with a statistically significant difference between EC and MC (Fig. 5). Candidate biomarker enrichment reflected predictions from our proteomic analysis – with EHMT2 showing the highest fold change and enrichment (Table 1 and Fig. 5).

After dividing the IHC stains into positive and negative cases (counts shown in Supplementary Table 3), GATA3 exhibited the highest combination of sensitivity and specificity for MC (93% and 99%, respectively) (Tables 2 and 3). GATA3, however, was completely absent from FATWOs giving it a sensitivity of 0% (Table 2). EHMT2 was 100% sensitive to both MC and FATWO (Table 2) but exhibited staining in almost half of the EC and thus had decreased specificity relative to GATA3 (Table 3). GSTM3 had the poorest performance, being less sensitive for both MC (83%) and FATWO (38%) than EHMT2 (Table 2), as well as being nonspecific (67%) (Table 3). EEF1A2 was reasonably sensitive to MC (92%) and FATWO (88%) (Table 2) but was the least specific (38%) (Table 3). EHMT2, GSTM3, and EEF1A2 staining in EC did not cluster by histotype (Supplementary Table 4).

3.5. Expression in tumors is linked to primary site

Staining varied slightly by anatomical location of the MC (Supplementary Fig. 2). GATA3 showed a dichotomy of strong or weak/negative staining in cervical MC. This dichotomy was not based off of the type of MC: both strong (MC-7/10/11/13) and weak (MC-8/9/12) cases contained a mixture of mesonephric carcinosarcoma and mesonephric adenocarcinoma. Intensity of GATA3 staining decreased dramatically in uterine MC. Of note, none of the 8 FATWO demonstrated GATA3 staining. Relative to GATA3,

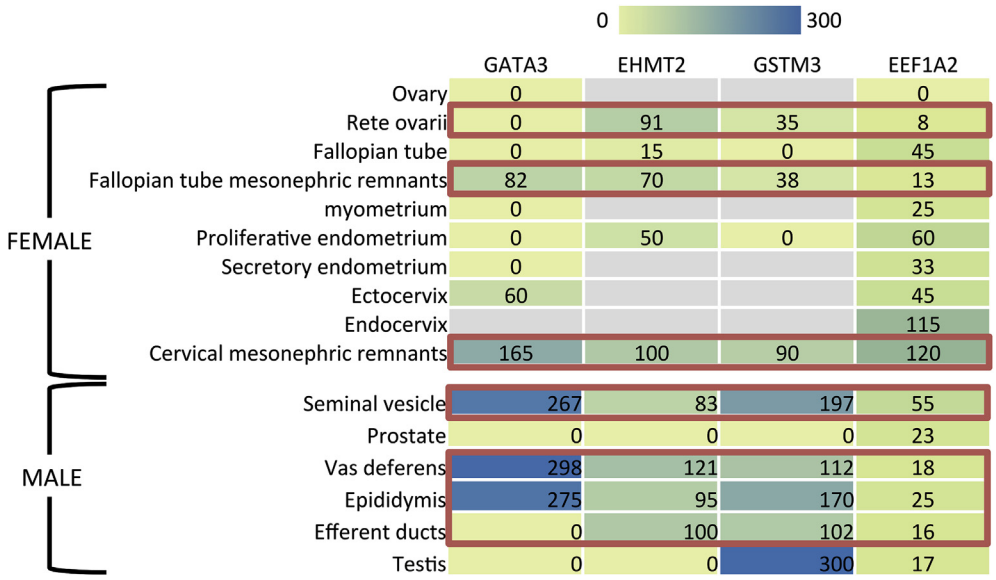

Fig. 3 GATA3 and putative biomarker staining in select gynecologic and genitourinary tissues. Tissues are separated by sex and arranged in descending anatomical order. Mesonephric duct-derived tissues are highlighted by red boxes. Average epithelial H-scores in each tissue are reported. H-score, histoscore. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

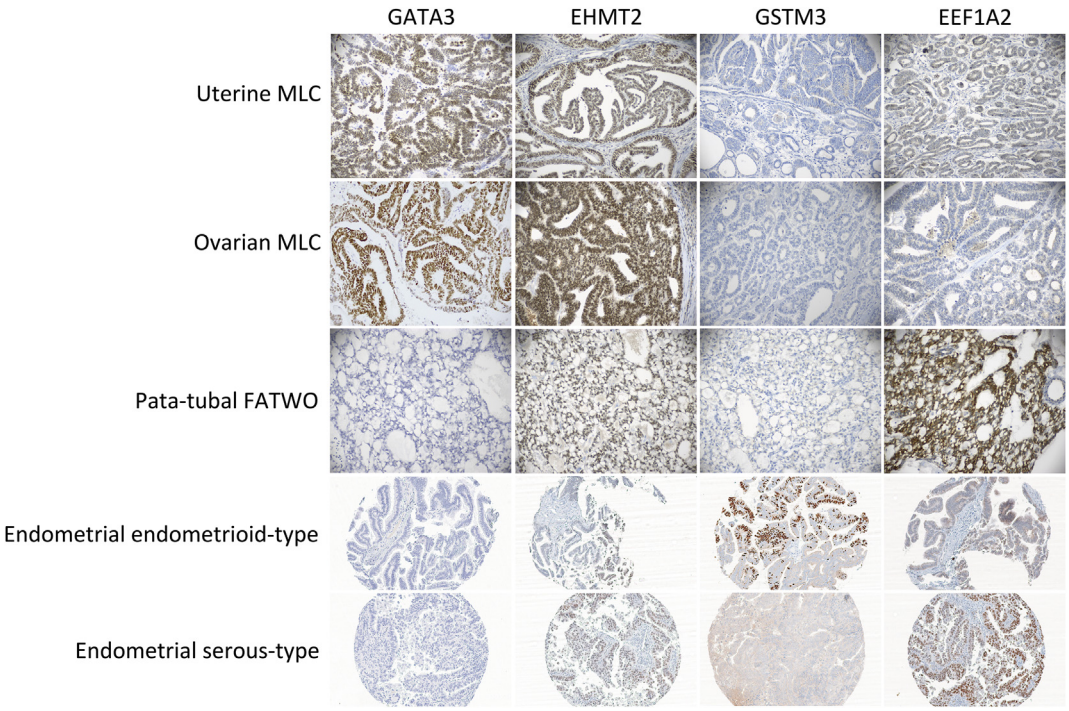

Fig. 4 Representative images of IHC stains of MLC, FATWO, and nonmesonephric carcinomas of the uterine corpus. IHC, immunohistochemical; MLC, mesonephric-like carcinoma; FATWO, female adnexal tumors of probable Wolffian origin.

EHMT2 showed higher H-scores across MC of all sites examined (cervix, endometrium, and ovary). EHMT2 also stained all FATWO. Similar to GATA3, GSTM3's highest H-scores were in MC of the cervix, but it also had the lowest H-scores overall amongst the 4 protein markers. EEF1A2 exhibited a high level of variability in H-score across MC and FATWO, with a mild enrichment in cervical cases.

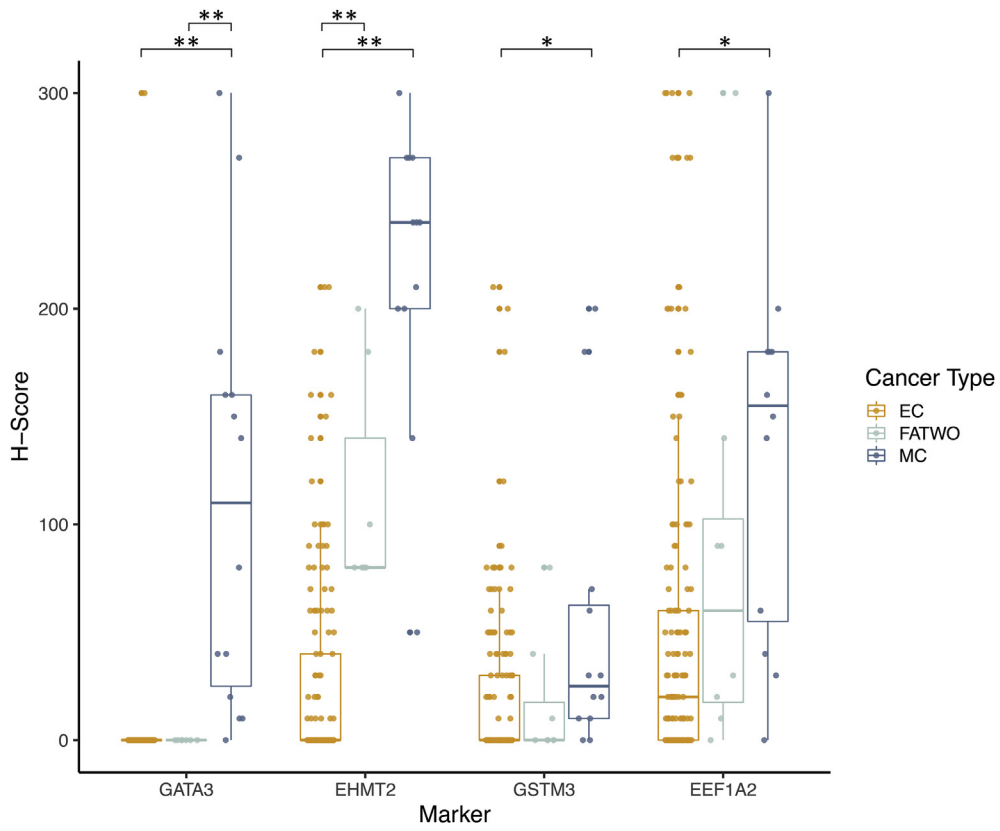

Fig. 5 Quantification of IHC staining. H-scores (intensity multiplied by percent positive tumor cells) are shown. Each point represents an individual case and tumors are grouped by type and biomarker candidate. Boxes indicate 2nd and 3rd quartiles. The middle line indicates medians. Whiskers extend from the edge of the box to the furthest data point still within 1.5 times the interquartile range of the box. H-score means were compared by use of a Kruskal-Wallis and posthoc Dunn test. Statistically significant pairwise comparisons are shown. *Adjusted p-value < 0.05. **Adjusted p-value < 0.001. IHC, immunohistochemical; H-scores, histoscores; ECs, endometrial carcinomas; FATWO, female adnexal tumors of probable Wolffian origin; MCs, mesonephric cancers (combined mesonephric and mesonepric-like carcinomas).

4. Discussion

Unbiased analysis of MC proteomes uncovered several biomarkers that could be used to distinguish MC from EC. Our first exploration into the whole proteomes of MLC and classic cervical ME revealed a high degree of similarity between them. Of 5646 proteins quantified, only IGHM was differentially expressed. Given the lack of other differentially regulated immunoglobulin proteins and an absence of literature linking immunoglobulins to ME/MLC, we concluded that the observed difference was not biologically meaningful. Based on our analysis, we could not find substantial evidence to warrant separation of the two into distinct entities and for the remainder of our analyses they were combined into one category, termed MC. As the cellular proteome is considered a corollary to phenotype, and because both ME and MLC look histologically identical, their similarity at a proteomic level is not surprising.

Table 2 Sensitivity of GATA3 and candidate biomarkers.

Tumor type	GATA3 (%)	EHMT2 (%)	GSTM3 (%)	EEF1A2 (%)
MC	93	100	83	92
FATWO	0	100	38	88

Abbreviations: GATA3, GATA binding protein 3; EHMT2, euchromatic histone lysine methyltransferase 2; GSTM3, glutathione S-transferase Mu 3; EEF1A2, eukaryotic translation elongation factor 1 alpha 2; MC, mesonephric cancers (combined mesonephric and mesonephric-like carcinomas); FATWO, female adnexal tumors of probable Wolffian origin.

Table 3 Specificity of GATA3 and candidate biomarkers in MC.

GATA3 (%)	EHMT2 (%)	GSTM3 (%)	EEF1A2 (%)
99	65	67	38

Abbreviations: GATA3, GATA binding protein 3; EHMT2, euchromatic histone lysine methyltransferase 2; GSTM3, glutathione S-transferase Mu 3; EEF1A2, eukaryotic translation elongation factor 1 alpha 2; MC, mesonephric cancers (combined mesonephric and mesonephric-like carcinomas).

This said, there has been ongoing debate as to whether ME and MLC are distinct. In the original description of MLC by McFarland et al. [3], the authors propose these tumors represent Müllerian carcinomas with secondary mesonephric-like differentiation. Unlike 'classical' ME, which arise from mesonephric remnants in the deep cervical or uterine wall, these tumors occurred in the superficial endometrium, rather than the myometrium, and without evidence of mesonephric remnants [3]. For this reason, these tumors were given the name 'mesonephric-like. Subsequent studies have shown that both ME and MLC harbor KRAS mutations in 75−100% of cases but have moderate differences in other molecular markers [10,14,15,33]. Although there may be some differences on a molecular basis, we were not able to find any from a comprehensive analysis. Our findings add to evidence supporting a mesonephric phenotype for MLC.

Subsequent proteomic comparisons of MC with EC of various histotypes revealed distinct patterns of expression and potential protein biomarkers of mesonephric-derived tumors. We pared-down hundreds of differentially expressed proteins into four candidates: EHMT2, GSTM3, EEF1A2, and GSK3β. However one (GSK3β) could not be optimally translated to immunohistochemistry using commercially available antibodies.

GATA3 is a transcription factor that is associated with breast cancer and is important for normal development [34]. In breast cancer, GATA3 is frequently mutated and in select subtypes its loss of expression appears to worsen prognosis [34]. GATA3 expression is required for the development of the kidney and several other organs [34]. In normal development, the mesonephric duct connects to, and develops in tandem with, the kidney [35]. These findings support GATA3's role in pathways with carcinogenic potential and implicate it in mesonephric ductal development.

Acting in a complex with EHMT1, EHMT2 has diverse functions in processes including cell cycle regulation and differentiation [27]. Interestingly, a study in breast cancer found that EHMT2 is able to form a complex with GATA3 [31], supporting a possible functional link between these proteins.

The GST family of proteins plays a key role in detoxifying chemical substrates in the cell and protecting against oxidative stress [28]. These proteins have been implicated in cancer as they can be used to protect malignant cells against oxidative damage and chemotherapeutics [28].

Alterations in EEF1A2 have been associated with a variety of cancers, including frequent copy number amplifications in ovarian cancer [29] and increased protein expression in breast cancer [32]. EEF1A2 has a wide variety of noncanonical functions including cytoskeletal interactions and inhibition of apoptosis [29,32]. While the exact mechanism underlying EEF1A2's contribution to malignancy remains unclear, its noncanonical functions are thought to be involved [32].

GSK3β was identified as enriched in MC but could not be validated by IHC owing to poor IHC staining. This protein is known to form a complex with FRAT2 [36], which was also identified as being significantly enriched in our data set. Both GSK3β and FRAT2 are involved in Wingless and Int-1 (Wnt) signaling [37]. FRAT2 was not assessed due to a lack of promising commercial antibodies for IHC. Whether or how GSK3β and FRAT2 contribute to MC remains an open question.

IHC staining of normal tissues supported a mesonephric phenotype for MC. GATA3 and EHMT2 expression was increased in mesonephric remnants and mesonephric-derived male tissues. Notably, GATA3 was not expressed in the male efferent ducts, suggesting that either this protein is not ubiquitously expressed throughout all mesonephric-derived tissues or that the efferent ducts may not be entirely of Wolffian origin. Both GSTM3 and EEF1A2 did not appear to preferentially stain Wolffian-derived tissues over other normal tissue types. EEF1A2 also had the lowest staining (H-scores) across gynecologic and male genitourinary tissues. Expression patterns observed in GATA3 and EHMT2 − the most sensitive biomarkers tested − support a mesonephric phenotype.

After elaborate efforts to undercover a more sensitive and specific biomarker for MC, GATA3, the biomarker already in wide use in pathology practice still had the best overall performance. Although EHMT2 had higher overall staining (H-scores), it had inferior specificity to GATA3. GSTM3 had the lowest sensitivity and specificity out of the 4 biomarkers. EEF1A2 was moderately sensitive to MC and FATWO but showed poor specificity—staining more than half of the EC. It would be invaluable to have a biomarker that could distinguish between malignant and benign mesonephric proliferations, such as mesonephric remnants and hyperplasia, where often the cutoff between malignant and benign can be difficult to ascertain. GATA3 and EHMT2 expressions were found in both benign remnants and malignant mesonephric tumors, meaning they would unfortunately not be useful for this purpose.

While both MC and FATWO are thought to be mesonephric-derived, some have hypothesized that differences between these tumors were due in part to their anatomical location. Specifically, that there are biologic differences between the upper and lower parts of the mesonephric ducts [1]. This theory does not fit with our evidence. Although GATA3 expression was slightly higher in mesonephric remnants of the lower tract (ie. cervix), it was still present in mesonephric remnants of the upper tract (ie. Fallopian tube) and ovarian MC. Similarly, EHMT2 expression did not appear to vary substantially with respect to primary site or anatomical 'height' of mesonephric-derived tissues and remnants. The absence of GATA3 staining in FATWO raises the question of whether MC and FATWO are at all related. Limited molecular data have shown an absence of *KRAS* mutations in FATWO, a mutation which is present in almost all ME (as highlighted

previously) [38]. The exact lineage of FATWO remains a mystery to be solved.

Small sample numbers limit the conclusions made by this study. There are also constraints to the proteomics technology, such as batch effects, protein degradation in aged specimens, and not providing topographical expression information due to tissue homogenization during processing [21]. In addition, although GATA3 is used to detect mesonephric neoplasms and TTF1 has been previously reported to stain MLC [19], neither protein was detected by our proteomics analysis. This is perhaps due to the generally low expression levels of transcription factors. Furthermore, our candidate biomarkers were limited to those that had commercially available antibodies, which were not necessarily those proteins that showed the highest differential expression between MC and EC. Some of these proteins, particularly FRAT2 and GSK3β, could be re-evaluated as candidates for further IHC validation if antibodies become available.

Herein, we examined MC using an unbiased systematic whole-proteome analysis. This identified ME and MLC as equivalent on the proteome level. We found that GATA3 and EHMT2 preferentially stained normal mesonephric-derived tissue and MC. GATA3 had the best overall performance as a biomarker of MC. The lineage of FATWO remains a potential area for future research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2020.10.005.

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