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PRINCIPAL INVESTIGATOR: Pamela Pollack

CONTRACTING ORGANIZATION: The Translational Genomics Research Institute
Phoenix, AZ 85004

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Pamela Pollock, Ph.D.
Email: ppollock@tgen.org

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Objective: Ovarian cancer is the leading cause of death from gynecologic malignancies in the Western world. Fibroblast growth factor receptor (FGFR) signaling has been implicated to play a role in ovarian tumorigenesis. Given our recent report of activating mutations in FGFR2 in endometrioid endometrial tumors and the similarities in the molecular genetics of ovarian and endometrial cancer, we hypothesized that activating FGFR2 mutations may also occur in a subset of ovarian tumors, particularly in the endometrioid subtype. Methods: Six exons of FGFR2 were sequenced in 120 ovarian tumors representing the various histotypes of ovarian cancer. Results: Mutation of FGFR2 was detected at low frequency in endometrioid (1/46, 2.2%) and serous (1/41, 2.4%) ovarian cancer. No mutations were detected in clear cell, mucinous, or mixed histology tumors or in the ovarian cancer cell lines tested. Functional characterization of the FGFR2 mutations confirmed that the mutations detected in ovarian cancer result in receptor activation. Conclusions: Despite the low incidence of FGFR2 mutations in ovarian cancer, the development and validation of anti-FGFR agents in other cancer types may allow for the future use of these agents in the small subset of ovarian cancer patients whose tumors possess activating FGFR2 mutations.

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Introduction.

The molecular pathology of ovarian carcinomas is heterogenous with multiple precursor lesions and multiple pathways of tumor development. The four most common histological subtypes are serous, endometrioid, mucinous and clear cell carcinoma and the different etiologies of these various histotypes is supported by the presence of different underlying molecular genetic alterations eg PTEN mutations are common in endometrioid but not serous or mucinous ovarian carcinomas. Similar histological subtypes are observed in carcinoma of the endometrium and there is a high degree of similarity regarding the molecular genetics of endometrial carcinoma and ovarian carcinoma with mutations in TP53, PTEN, KRAS, PIK3CA and b-catenin identified in both tumor types, albeit sometimes at different frequencies in the different histological subtypes of both cancers. The endometrioid subtype of ovarian carcinoma bears close histological resemblance to endometrioid carcinoma of the endometrium, indeed clear cell and endometrioid carcinomas of the ovary are often histologically associated with endometriosis. Similar molecular genetic alterations have been reported in adjacent endometriosis and synchronous endometrioid carcinomas of the ovary, supporting endometriosis as a possible precursor for both endometrioid and clear cell carcinomas of the ovary. Moreover, population based cohort studies have indicated a higher incidence of ovarian cancer in women with endometriosis (1). We identified mutations in FGFR2 in endometrioid endometrial cancer (2) and demonstrated oncogene dependence as inhibition of FGFR2 resulted in growth arrest and cell death (3). As FGF signaling had been implicated in the pathogenesis of ovarian cancer (4) we hypothesized that FGFR2 might be activated in a subset of ovarian cancers, most likely those demonstrating an endometrioid or clear cell histology.

Body.

Specific Aim 1A. Determine the mutation frequency of FGFR2 in a panel of ovarian cancer cell lines and ovarian carcinomas representing the different histological subtypes of ovarian cancer

The four most common histological subtypes of ovarian cancer are serous (80-85%), endometrioid (10%), clear cell (5%), and mucinous (3%) [2]. 120 fresh frozen ovarian tumor samples from multiple institutions were used in this study, including 46 endometrioid, 41 serous, 14 mucinous, 12 clear cell, and 7 mixed histology ovarian tumors. Ovarian tumor samples were obtained from Ian Campbell at the Peter MacCallum Cancer Institute (26 endometrioid, 32 serous, 10 mucinous, 5 clear cell, and 3 mixed histology ovarian tumors), Michael Birrer at the National Cancer Institute (5 endometrioid, 9 serous, 4 mucinous, and 7 clear cell, 4 mixed histology), and Paul Goodfellow at Washington University (15 endometrioid tumors). The latter 15 tumors from Dr Goodfellow were sequenced prior to the initiation of this DoD funded study. The majority of specimens used in this study contained >80% tumor epithelial cells, as determined by H&E staining of multiple sections. In some tumors where this was not the case, samples were microdissected from serial fresh frozen sections to provide specimens with >80% neoplastic cellularity. Ovarian tumor DNA provided by Dr Ian Campbell underwent whole genome amplification (WGA) using the Repli-G Phi-mediated amplification system (Qiagen, Hilden, Germany). To minimize the potential for generation of artifacts, WGA was carried out in triplicate, using 25 ng of primary DNA, and the products were pooled. DNA from six ovarian cancer cell lines (CAOV3, SKOV3, ES-2, OVCAR-3, SWS-26, TOV-21G) was also screened. Additional attempts were made to obtain additional ovarian tumors with a clear cell histology but these were unsuccessful.

120 ovarian tumor samples were screened for FGFR2 mutations in exons 7, 8, 9, 10, 13, and 15, as previously described [20]. All sequencing was performed at the Translational Genomics Research Institute Sequencing Core. Mutation of FGFR2 was detected at low

frequency in endometrioid (1/46, 2. 2%) and se rous (1/41, 2.4%) ovarian cancer. S252W, the most common mutation observed in endometrioid endometrial cancer [20], was ide ntified in an endometrioid ovarian tumor and the Y376C mut ation was id entified in a serous ovarian tumor. No mutations were detected in clear cell, mucinous, or mixed histology tumors or in the ovarian cancer cell lines tested.

Specific Aim 1b. Evaluate the expression of FGFRs and a subset of FGF ligands in normal ovary and a panel of ovarian carcinomas.

We have optimized IHC of FGFR1-FGFR4. All four FGFRs are expressed in many ovarian tumors. We initially proposed to look at a subset of the relevant FGF ligands and correlate the expression of the FGF ligands with the cognate receptor. We originally looked at FGF1, FGF2 and FGF7 however the staining pattern seen with FFG1 and FGF7 was identical suggesting that these antibodies demonstrated crossreactivity. Further investigation revealed that many of the FGF antibodies available, while capable of neutralizing the activity of the ligand to which they had been raised, had not been assessed for specificity. We therefore purchases 18 myc-tagged expression constructs (Origene) for ech of the FGF ligands. These were then sequence verified and transduced into BaF3 cells (that express no endogenous FGFs or FGFRs) and polyclonal

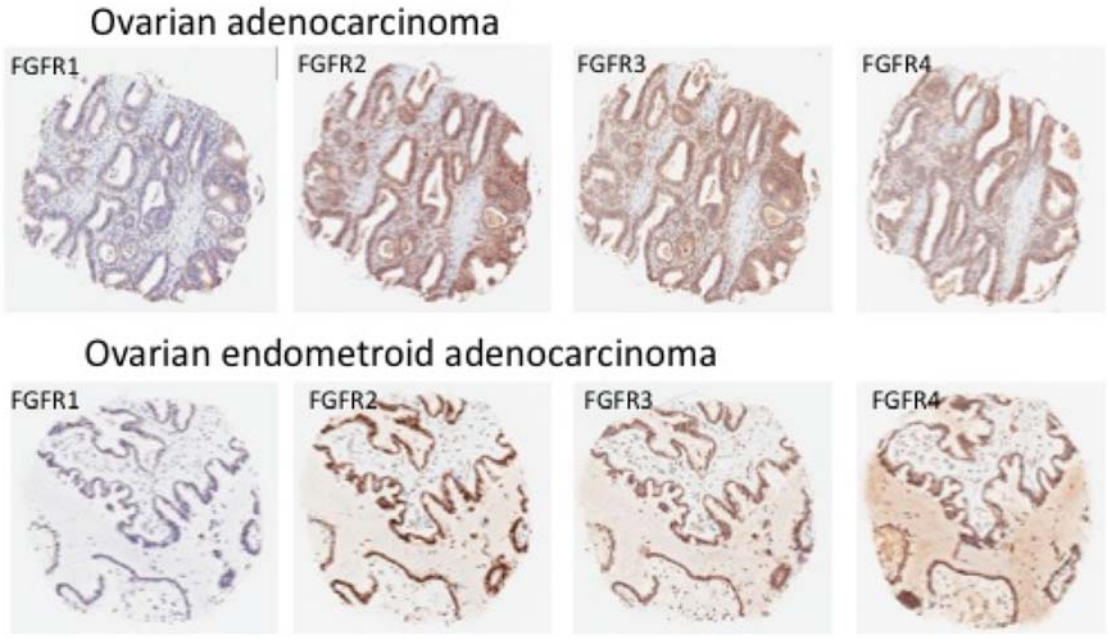

Figure 1. IHC detects expression of multiple FGFRs in ovarian tumors.

stable cell lines were selected with 1200ug/mL G418.

We have now generated total cell lysates from all of these cell lines and studies to confirm antibody specificity have been initiated. We have currently evaluated antibodies against FGF1 (n=2), FGF2 (1), FGF3 (2), FGF4 (1), FGF7 (2), FGF9 (1) and FGF10 (2). As shown in Figure 1A, suitable antibodies against FGF1, 2, 3 & 4 have now been identified. No cross reactivity is observed in cell lines transduced with related FGF ligands, evidenced by the expression of the myc-tagged ligands. As part of the optimization process, new antibodies are run against lysates from stably transfected BaF3 lines expressing the individual FGF ligands. Antibodies are acceptable for subsequent IHC only if they meet ***all*** the following criteria: 1) they detect the correct molecular size band; 2) they do not cross react with other proteins and 3) they

do not cross react with other members of the FGF family. Figure 1B shows examples of antibodies that failed the screening process for a number of different reasons. The antibody against FGF3 does not detect FGF3 but cross reacts with a number of non-specific high molecular weight proteins; the 2 antibodies against FGF7 do not detect any protein by Western blotting; and the antibody against FGF10 does detect FGF10 but also cross reacts with other proteins making it unsuitable for IHC.

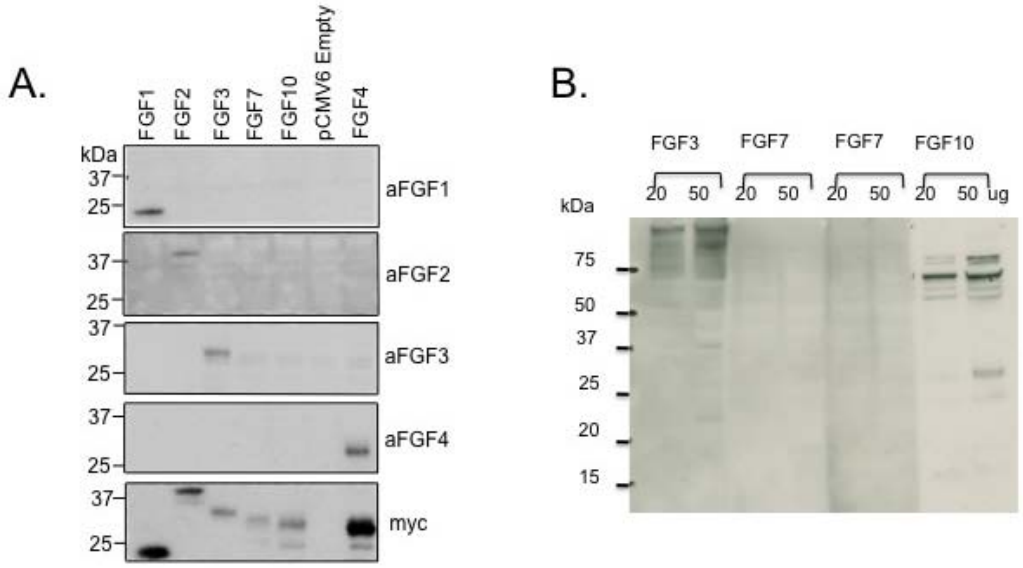

Figure 2. Western blot showing specificity of antibodies raised to detect FGF ligands in total cell lysates from BaF3 cells stably transduced with each myc-tagged FGF ligand. We have identified antibodies that specifically detect FGF1, FGF2, FGF3 and FGF4 (Panel A). To date we have been unable to identify a good antibody to detect FGF7 and FGF10 (Panel B). This work is ongoing.

Specific Aim 2a. Test the hypothesis that FGFR2 activation drives transformation of primary ovary surface epithelial (OSE) cells by transducing immortalized OSE cells with wildtype and constitutively activated FGFR2 and assaying for changes in proliferation and anchorage independent growth.

As the rate of FGFR2 mutations was very low in ovarian cancer, we chose to determine whether the mutations we detected resulted in receptor activation using the BaF3 proliferation assay rather than focus on OSE cells. These cells do not express endogenous FGF ligands or FGFRs and introduction and activation of FGFRs has been shown to substitute for IL-3 to promote cell proliferation [29]. Mitogenic assays in the IL-3-dependent BaF3 cells could therefore be used to determine if the Y376C mutation in FGFR2 resulted in ligand independent or dependent receptor activation. The S252W and Y376C mutations were first introduced to the pEF1a.FGFR2b.IRES.neo plasmid (NM_022970.3) using the Quikchange XL Site Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. After restriction enzyme screening, the entire coding sequence of FGFR2 was sequenced for each clone to confirm the presence of the intended mutation and to ensure that no other mutations were introduced during the mutagenesis process. BaF3 cells were transduced with empty vector, wildtype FGFR2b and mutant FGFR2b using Amaxa nucleofection and stably selected in 1.2 mg/mL Geneticin in the presence of 5 ng/ml IL-3 for 14 days. For the proliferation assays, cells were washed in PBS to remove IL-3, and plated at 1 x 10^4 cells per well in triplicate in a 96 well plate in IL-3 free media

containing 1nM FGF7 and 5 mg/mL heparin. Cells had a 50% volume media change on day 3 to provide fresh FGF ligand. On day five, bioluminescent measurement of ATP was assessed as an indicator of cell number using the ViaLight Plus Cell Proliferation/Cytotoxity Kit (Lonza Rockland, Inc.). Experiments were performed twice in each of two independent sets of stable cell lines, with triplicate wells measured for each assay.

As shown in Figure 3 A, the Y37 6C mutation result s in ligand ind ependent re ceptor activation, a s evidenced by BaF3 proliferation in the absence of FGF ligand. Stimulation with FGF7 resulted in increased proliferation in the Y376C FGFR2b BaF3 cells compared to wildtype FGFR2b (Figure 3B), d emonstrating that this mutant receptor can be further act ivated by the addition of ligand. In comparison, the S252W mutation does not lead to BaF3 proliferation in the absence of ligand, similar to wildtype FGFR2b (Figure 3A) but does lead to in creased BaF3 proliferation in response to its cog nate ligand (Figure 3B), consisten t with publish ed literature

Figure 3. Effects of S252W and Y376C mutations on FGFR2b activity in BaF3 cells (A) Proliferation of BaF3 cell s e xpressing wil dtype or mutant (S252 W, Y376C) F GFR2b in the absence of FGF ligand. (B) Proliferation of BaF3 cells expressing wildtype or m utant (S252W, Y376C) F GFR2b in response to FGF7, a cognate FGFR2b ligand. (C) P roliferation of BaF 3 cells expressing wildtype or mutant (S252W) FGFR2b in response to FGF2, a cognate FGFR2c ligand.

[22].

Specific Aim 2b. Evaluate FGFR2 as a viable therapeutic target in ovarian cancer by inhibiting FGFR2 in an ovarian cancer cell line expressing activated FGFR2 via shRNA knockdown of gene expression or a pan FGFR kinase inhibitor and assaying for decreased proliferation and induction of apoptosis.

Based on the low frequency of FGFR2 mutations identified in ovarian tumors and the lack of an ovarian cancer cell line carrying an activating mutation in FGFR2, we did not complete this aim. Although we only sequenced six cell lines, we performed extensive searches on the COSMIC (Catalog of somatic mutations in Cancer) website (http://www.sanger.ac.uk/genetics/CGP/cosmic/) and did not identify an ovarian cell line with an FGFR2 mutation. We did however determine that the mutations we identified resulted in receptor activation (see Aim 2A). We allocated the resources from this aim to Aim 1b as the optimization of antibodies to detect FGF receptors and ligands was much more difficult than expected.

Key Research Accomplishments

- We screened 120 ovarian tumor for mutations in FGFR2
- Mutation of FGFR2 was detected at low frequency in endometrioid (S252W, 1/46, 2.2%) and serous (Y376C, 1/41, 2.4%) ovarian cancer. No mutations were detected in clear cell, mucinous, or mixed histology tumors or in the ovarian cancer cell lines tested.
- IHC revealed expression of all four FGFRs in ovarian carcinomas. Each carcinoma frequently expressed more than one FGFR.
- We determined that the sensitivity and specificity of many of the existing antibodies detecting many of the FGF ligands was poor.
- We have made stable BaF3 cell lines expressing each of the 18 FGF ligands to enable proper evaluation of the specificity of FGF antibodies
- We have identified antibodies against FGF1, FGF2, FGF3 and FGF4 that are specific.

Reportable Outcomes

Sara A. Byron, Michael G. Gartside, Candice L. Wellens, Paul J. Goodfellow, Michael J. Birrer, Ian G. Campbell, Pamela M. Pollock. FGFR2 Mutations are Rare Across Histologic Subtypes of Ovarian Cancer. (Submitted, Gynecologic Oncology)

18 BaF3 Stable cell lines each expressing a myc-tagged FGF ligand (FGF1-10, 16-22)

Conclusion

FGFR2 mutations are rare in ovarian carcinoma, even in those with an endometriod histology.

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Appendices

See Byron et al. manuscript.

FGFR2 Mutations are Rare Across Histologic Subtypes of Ovarian Cancer

Sara A. Byron[1], Michael G. Gartside[1], Candice L. Wellens[1], Paul J. Goodfellow[2,3], Michael J. Birrer[4], Ian G. Campbell[5,6], Pamela M. Pollock[1]*

[1]Cancer and Cell Biology Division
Translational Genomics Research Institute
Phoenix, Arizona 85004

[2] Departments of Obstetrics and Gynecology and [3]Surgery
Washington University School of Medicine
St. Louis, Missouri 63110

[4] Cell and Cancer Biology Department
Center for Cancer Research
National Cancer Institute
Bethesda, MD 20892

[5] Victorian Breast Cancer Research Consortium
Cancer Genetics Laboratory
Peter MacCallum Cancer Centre
East Melbourne, Victoria, Australia.

[6] Department of Pathology
The University of Melbourne
Melbourne, Victoria, Australia

*Corresponding Author

445 North 5th Street

Phoenix, AZ 85004

Phone: 602-343-8815

Fax: 602-343-8840

e-mail: ppollock@tgen.org

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Running Title: FGFR2 Mutations in Ovarian Cancer

Key Words: Ovarian Carcinoma, FGFR2, Mutation

ABSTRACT

Objective: Ovarian cancer is the leading cause of death from gynecologic malignancies in the Western world. Fibroblast growth factor receptor (FGFR) signaling has been implicated to play a role in ovarian tumorigenesis. Given our recent report of activating mutations in FGFR2 in endometrioid endometrial tumors and the similarities in the molecular genetics of ovarian and endometrial cancer, we hypothesized that activating FGFR2 mutations may also occur in a subset of ovarian tumors, particularly in the endometrioid subtype.

Methods: Six exons of FGFR2 were sequenced in 120 ovarian tumors representing the various histotypes of ovarian cancer.

Results: Mutation of FGFR2 was detected at low frequency in endometrioid (1/46, 2.2%) and serous (1/41, 2.4%) ovarian cancer. No mutations were detected in clear cell, mucinous, or mixed histology tumors or in the ovarian cancer cell lines tested. Functional characterization of the FGFR2 mutations confirmed that the mutations detected in ovarian cancer result in receptor activation.

Conclusions: Despite the low incidence of FGFR2 mutations in ovarian cancer, the development and validation of anti-FGFR agents in other cancer types may allow for the future use of these agents in the small subset of ovarian cancer patients whose tumors possess activating FGFR2 mutations.

INTRODUCTION

The late stage of diagnosis, resistance to chemotherapy, and heterogeneous nature of ovarian cancer make it a clinically challenging disease, with an overall 5-year survival rate of only 46% [1]. The four most common histological subtypes of ovarian cancer are serous (80-

85%), endometrioid (10%), clear cell (5%), and mucinous (3%) [2]. The different etiologies of these various histotypes are supported by the presence of different underlying genetic alterations. For example, *TP53* mutations are most prevalent in serous and high-grade endometrioid ovarian carcinomas [3], whereas *PTEN*, *PIK3CA*, and *CTNNB-1* (β-catenin) mutations are more common in low-grade endometrioid ovarian cancer [4-6]. However, despite their underlying genetic differences, most advanced ovarian carcinomas are currently treated with a standard approach involving surgical cytoreduction and carboplatin and paclitaxel combination chemotherapy [7, 8].

Fibroblast growth factor (FGF) signaling has been previously implicated in ovarian tumorigenesis. The fibroblast growth factor family includes 18 ligands (FGF1-FGF10 and FGF16-FGF23) which signal through four transmembrane receptor tyrosine kinases (FGFR1-FGFR4) and their alternatively spliced isoforms [9]. Alternative splicing of the exons that encode the third immunoglobulin domain of FGFR is the primary determinant of both the patterns of redundancy and specificity in FGF/FGFR binding and signaling. This splicing event is tissue specific and gives rise to the "b" and "c" receptor isoforms for FGFR1-FGFR3, which possess distinct ligand specificities [9, 10]. For FGFR2, cells of an epithelial lineage typically only express the "b" isoform (FGFR2b) encoded by exon 8 while mesenchymally derived cells only express the "c" isoform (FGFR2c) utilizing exon 9.

Ovarian carcinomas are thought to arise from cells of the ovarian surface epithelium (OSE), a layer of poorly committed mesodermally derived epithelial cells surrounding the ovary. During the process of malignant transformation, OSE cells become more committed to an epithelial phenotype, gaining expression of epithelial-specific markers such as E-cadherin and CA125 [11]. Normal ovarian surface epithelial cells have been reported to lack FGFR2b

expression and instead express FGFR 2c, a finding that is in contrast to the expression pattern in most other epithelial cell types and may be a consequence of the pluripotent nature of the OSE [12, 13]. Interestingly, a majority of epithelial ovarian cancers express FGFR2b [14], consistent with the increased epithelial differentiation of this tumor type during malignant transformation.

In the NCI-60 cancer cell line panel, ovarian cancer cell lines are unique with an almost universal expression of all four FGFRs and the highest incidence of detectable expression of FGFR2 mRNA [15]. FGF-stimulated activation of FGFR in epithelial ovarian cancer cell lines contributes to multiple aspects of the malignant phenotype, including proliferation, motility, cell survival, and reorganization of the actin cytoskeleton [13]. Clinically, mRNA and proteins levels of FGF1, the universal FGF ligand, are associated with poorer overall survival in patients with high-grade advanced stage serous ovarian tumors [16]. FGF9 has also been implicated as playing a key role in ovarian endometrioid adenocarcinomas carrying defects in the Wnt/β-catenin pathway [17].

Activating mutations in FGFR2 have been reported in various cancer types, including ovarian cancer [18]. As part of the Cancer Genome Project, 26 ovarian tumors were screened for mutations in FGFR1-4 [19]. A single mutation in FGFR2, G272V, was identified in a serous ovarian tumor, for a mutation frequency of 1 out of 20 (5%) serous ovarian tumors screened. No mutations were identified in endometrioid, clear cell or mucinous ovarian tumors, though only small numbers of tumors of these histological subtypes were evaluated. No mutations were detected in FGFR1, FGFR3, or FGFR4.

We and others recently identified activating mutations in FGFR2 in endometrial cancer, predominantly in the endometrioid histologic subtype [20, 21]. Interestingly, ovarian cancer and endometrial cancer display similarities in their underlying histology and molecular genetics. The

endometrioid subtype of ovarian carcinoma bears close histological resemblance to endometrioid carcinoma of the endometrium, and both of these cancer types exhibit mutations in PTEN, PIK3CA, and CTNNB-1 (β-catenin).

The previous reports implicating FGF signaling in ovarian tumorigenesis, the shared molecular genetics between endometrioid ovarian cancer and endometrioid endometrial cancer, and the identification of activating mutations in FGFR2 in endometrioid endometrial cancer led us to hypothesize that FGFR2 mutations may occur in a subset of ovarian cancers.

MATERIALS AND METHODS

Clinical specimens and cell lines

120 fresh frozen ovarian tumor samples from multiple institutions were used in this study, including 46 endometrioid, 41 serous, 14 mucinous, 12 clear cell, and 7 mixed histology ovarian tumors (Table 1). Ovarian tumor samples were obtained from Ian Campbell at the Peter MacCallum Cancer Institute (26 endometrioid, 32 serous, 10 mucinous, 5 clear cell, and 3 mixed histology ovarian tumors), Michael Birrer at the National Cancer Institute (5 endometrioid, 9 serous, 4 mucinous, and 7 clear cell, 4 mixed histology), and Paul Goodfellow at Washington University (15 endometrioid tumors). The majority of specimens used in this study contained >80% tumor epithelial cells, as determined by H&E staining of multiple sections. In some tumors where this was not the case, samples were microdissected from serial fresh frozen sections to provide specimens with >80% neoplastic cellularity. All samples were deidentified and the study approved by the Western Institutional Review Board (WIRB). Ovarian tumor DNA provided by Dr Ian Campbell underwent whole genome amplification (WGA) using the Repli-G Phi-mediated amplification system (Qiagen, Hilden, Germany). To minimize the

potential for generation of artifacts, WGA was carried out in triplicate, using 25 ng of primary DNA, and the products were pooled. DNA from ovarian cancer cell lines (CAOV3, SKOV3, ES-2, OVCAR-3, SWS-26, TOV-21G) was provided by Dr. John Carpten (Translational Genomics Research Institute).

Detection of FGFR2 mutations

Ovarian tumor samples were screened for FGFR2 mutations in exons 7, 8, 9, 10, 13, and 15, as previously described [20]. All sequencing was performed at the Translational Genomics Research Institute Sequencing Core.

BaF/3 Proliferation Assay

The S252W and Y376C mutations were introduced to the pEF1 α.FGFR2b.IRES.neo plasmid (NM_022970.3) using the Quikchange XL Site Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Mutagenesis primers were designed to introduce a missense mutation to encode for the desired mutation in FGFR2 (indicated in bold) and a silent mutation for diagnostic restriction digestion screening of clones (indicated by underline). Site-directed mutagenesis primer sequences were as follows: FGFR2 S252W Forward: 5'-GTTGTGGAGCGCT**G**GCCTCACCGGCC-3'; FGFR2 S252W Reverse: 5'-GGCCGGTGAGGC**C**AGCGCTCCACAAC-3'; FGFR2 Y376C Forward: 5'–GATTACAGCTTCCCCAGACT**G**CCTCGAGATAGCCAT-3'; FGFR2 Y376C Reverse: 5'–ATGGCTATCTCGAGG**C**AGTCTGGGGAAGCTGTAATC-3'. After restriction enzyme screening, the entire coding sequence of FGFR2 was sequenced for each clone to confirm the

presence of the intended mutation and to ensure that no other mutations were introduced during the mutagenesis process.

The IL-3 dependent murine pro B BaF3 cell line was transduced with empty vector, wildtype FGFR2b and mutant FGFR2b using Amaxa nucleofection, according to the manufacturer's instructions. Transduced cells were stably selected with 1.2 mg/mL Geneticin in the presence of 5 ng/ml IL-3 for 14 days, and then maintained under selection in RPMI supplemented with 10% FBS, 50 nM beta-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 1.2 mg/mL Geneticin and 5 ng/mL murine IL-3 (R&D Systems). For the proliferation assays, cells were washed in PBS to remove IL-3, and plated at 1 x 10^4 cells per well in triplicate in a 96 well plate in IL-3 free media containing 1nM FGF7 and 5 µg/mL heparin. Cells had a 50% volume media change on day 3 to provide fresh FGF ligand. On day five, bioluminescent measurement of ATP was assessed as an indicator of cell number using the ViaLight Plus Cell Proliferation/Cytotoxity Kit (Lonza Rockland, Inc.), according to the manufacturer's instructions. Experiments were performed twice in each of two independent sets of stable cell lines, with triplicate wells measured for each assay. Representative results are presented.

RESULTS AND DISCUSSION

To characterize the spectrum and frequency of FGFR2 mutations across the histological subtypes of ovarian cancer, 120 ovarian tumors, including 46 endometrioid, 41 serous, 14 mucinous, 12 clear cell, and 7 mixed histology ovarian tumors, were screened for mutations in FGFR2. Sequencing was performed for exons 7, 8, 9, 10, 13, and 15 of FGFR2, as 95% of the activating mutations we identified in endometrial cancer occurred within these exons. In

addition, the majority of germline mutations in the FGFR gene family associated with skeletal and craniosynostosis syndromes occur within these exons. Alternative splicing of exon 8 and exon 9 occurs in a tissue specific fashion, where cells of an epithelial lineage usually only express the 'b' isoform encoded by exon 8 (FGFR2b) and mesenchymally derived cells only express the 'c' isoform utilizing exon 9 (FGFR2c). These isoforms possess distinct ligand specificities and, with tissue specific control of ligand expression, mediate paracrine epithelial-mesenchymal signaling. Although no activating mutations in exon 8 have been identified in endometrial cancer to date, a large number of activating mutations in exon 9 of FGFR2 in craniosynostosis and skeletal dysplasia syndromes have been identified. Given that normal ovarian surface epithelium express FGFR2c (utilizing exon 9) and a majority of ovarian carcinomas express FGFR2b (utilizing exon 8) [11-13], we screened both exon 8 and exon 9 for mutations in these ovarian tumors.

Mutations in FGFR2 were identified in 1/46 (2.2%) endometrioid and 1/41 (2.4%) serous ovarian tumors (Table 1). Sequencing revealed the normal DNA was wildtype confirming the mutation were somatic in origin. No mutations were seen in mucinous, clear cell, or mixed histology ovarian tumors. In addition, no mutations were seen in the six ovarian cancer cell lines tested.

Both of the mutations identified in ovarian tumor samples have been previously reported in endometrial cancer [20]. S252W, the most common mutation observed in endometrioid endometrial cancer [20], was identified here in an endometrioid ovarian tumor and the Y376C mutation was identified in a serous ovarian tumor (Table 1). Extensive structural and biological studies evaluating the causative role of the S252W mutation in the craniosynostosis and limb pathologies of Apert syndrome have shown that this mutation results in activation of the

FGFR2b and FGFR2c receptors by two mechanisms, (1) increasing the binding affinity of the receptor isoforms for their cognate ligands and (2) by violation of FGFR2b and FGFR2c ligand binding specificities, eg the "c" isoform can now bind "b" isoform specific ligands, resulting in autocrine receptor activation [22-24]. Normal ovarian surface epithelial cells are unique in that they are the only epithelial tissue identified to date that express FGFR2c and FGF7, members of the FGF signaling family that are typically expressed in mesenchymally derived tissues [25]. Interestingly, a majority of ovarian carcinomas have been shown to express FGFR2b, and FGF7 has been shown to stimulate DNA synthesis in ovarian cancer cell lines expressing FGFR2b suggesting the establishment of an autocrine loop [14]. Whether these cells have maintained expression of FGFR2c or have undergone an isoform switch from FGFR2c to FGFR2b is currently unknown. Functional studies have shown that a neutralizing antibody to FGF7 can partially inhibit DNA synthesis in the FGFR2b-expressing ovarian carcinoma cell line, 41 M [14], suggesting that this isoform switching may play a role in ovarian tumorigenesis rather than just being a "passenger" event that accompanies the epithelial differentiation of this tumor type during malignant transformation. Although it is unknown whether the ovarian tumor with the S252W mutation in FGFR2 identified in this study predominantly expresses the "b" or the "c" isoform of FGFR2, it is tempting to speculate that this tumor expresses the "c" isoform only, and that the S252W mutation phenotypically mimics the previously identified isoform switching, thereby allowing the autocrine activation of FGFR2c by FGF7 in OSE cells. This finding is significant in that the identification of the pathogenic S252W mutation in this single tumor would in turn suggest that the isoform switching previously observed in ovarian tumors plays a role in the pathogenesis of these tumors. Similarly the low rate of activating mutations in FGFR2

identified to date may reflect that in this tissue type FGFR2 is already activated in a ligand dependent manner by isoform switching, and may not require additional mutational activation.

Given that the S252W mutation results in ligand-dependent receptor activation, we were interested to evaluate the ligand-dependency of the Y376C mutation. The homologous Y372C mutation in FGFR1 results in ligand independent activation of an osteocalcin FGF response element promoter-luciferase reporter in the osteogenic MC3T3 cell line [26], presumably by the formation of intermolecular disulfide bonds [27]. The homologous Y373C mutation in FGFR3c has been shown to result in receptor dimerization, but predominantly ligand dependent activation of the MAPK pathway in HEK293 cells and L8 myoblasts [28]. To determine if the Y376C mutation in FGFR2 resulted in ligand independent or dependent receptor activation, we employed mitogenic assays in IL-3-dependent BaF3 cells. These cells do not express endogenous FGF ligands or FGFRs and introduction and activation of FGFRs has been shown to substitute for IL-3 to promote cell proliferation [29]. As shown in Figure 1A, the Y376C mutation results in ligand independent receptor activation, as evidenced by BaF3 proliferation in the absence of FGF ligand. Stimulation with FGF7 resulted in increased proliferation in the Y376C FGFR2b BaF3 cells compared to wildtype FGFR2b (Figure 1B), demonstrating that this mutant receptor can be further activated by the addition of ligand. In comparison, the S252W mutation does not lead to BaF3 proliferation in the absence of ligand, similar to wildtype FGFR2b (Figure 1A) but does lead to increased BaF3 proliferation in response to its cognate ligand (Figure 1B), consistent with published literature [22]. We should note that these functional studies were carried out using the “b” isoform of FGFR2 as the effect of the Y376C mutation on receptor dimerization is independent of the “b” or “c” isoform on which it arises.

In conclusion, we identified an S252W mutation, the most predominant mutation identified in endometrioid endometrial cancers, in a single endometroid ovarian carcinoma and a Y376C mutation in a single serous ovarian tumor. This S252W mutation violates the ligand binding specificity of FGFR2b and FGFR2c and phenotypically mimics the occurrence of FGFR2 isoform switching previously observed in ovarian cancer. Together this data raises the possibility that inhibition of FGFR2 by either small molecule kinase inhibitors or extracellular blocking antibodies may be beneficial in those rare tumors with activating mutations, in addition to the larger number of tumors that undergo FGFR2 isoform switching.

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