Proliferation in the Normal FTE Is a Hallmark of the Follicular Phase, Not BRCA Mutation Status

Sophia H.L. George, Anca Milea, and Patricia A. Shaw

Abstract

Purpose: Women who have inherited germline mutations of BRCA1/BRCA2 are at increased risk of developing high-grade serous carcinoma, and many of these cancers arise in the distal fimbriated end of the fallopian tube. We have previously shown that the fallopian tube epithelia of BRCA1 mutation carriers (FTE-BRCA) have altered signaling pathways compared to nonmutation carriers. In this study, we sought to determine whether these differences result in a proliferative advantage to the epithelia in this high-risk patient population and to investigate whether the postovulation environment of the FTE-BRCA compared to FTE from nonmutation carriers experiences a differential abundance of immune cells.

Method: Immunohistochemistry for Ki67, CD3, CD8, CD20, and CD68 was performed on histologically normal tubal epithelium (ampulla, n = 83), fimbria (n = 18) with known ovarian cycle status and germline mutation status and for Ki67 on fimbrial epithelium from women (n = 144) with and without BRCA1 or BRCA2 mutations who underwent risk-reducing salpingo-oophorectomy (RRSO). Serous tubal intraepithelial carcinomas (STIC) with concomitant cancer (n = 15) were also analyzed for presence of immune infiltrates. All slides were digitized and analyzed using automated image analysis software.

Results: There was no significant difference in the proliferative index in histologically normal FTE between BRCA1/BRCA2 and non-BRCA, in 144 fimbriae and 83 ampullae. The FTE-BRCA1 epithelia did not exhibit a differential presence of lymphocytes or macrophages, however more macrophages were present in the luteal phase compared to the follicular phase epithelia. In STICs macrophages were more abundant than lymphocytes with an incremental increase noted with disease progression.

Conclusions: BRCA1/2 mutation carriers exhibited no significant increase in proliferation in the fallopian tube epithelial cells either in the ampulla or fimbriated ends of the tube. Rather, a significant proliferative increase was defined in the cases determined to be in the follicular, or proliferative, preovulatory phase of the ovarian cycle. Finally, we also show an incremental increase in leukocytes invading the STICs and HGSC, implicating a possible role of the leukocytes early in the progression or inhibition of tumor formation, which is independent of ovarian cycle status.

Introduction

High-grade serous carcinoma (HGSC) is the predominant histotype associated with germline mutations of BRCA1 and BRCA2, which can give rise to a 10-fold increased risk of developing HGSC. Detailed histo-pathologic examination of tubal epithelia (FTE) in this genetically high-risk population has led to the discovery of putative cancer precursor lesions in the fallopian tube, some of which, i.e., the p53 signature, are found with a similar frequency in BRCA mutation carriers and noncarriers. Occult invasive and serous tubal intraepithelial carcinomas (STIC) have been identified in the fallopian tubes of mutation carriers undergoing risk-reducing surgery; they are more frequent in mutation carriers (5–8). We have previously described molecular signatures of FTE from BRCA1/BRCA2 heterozygotes, providing some insight into the molecular events preceding the malignant transformation resulting in histologic abnormalities and defined precursors (9, 10). Some of these altered pathways include the MAP kinase pathway, the adipokine signaling pathway,
Translational Relevance
High-grade serous carcinoma is the most common type of ovarian cancer and despite recent advances in therapy a high mortality rate persists. Women who have BRCA1/2 mutations are at 40% to 60% increased lifetime risk for developing the disease. In recent years, with the identification of cancer precursors in the distal end of the fallopian tube, it has been recognized that the fallopian tube epithelium, not the ovarian surface epithelium, is the likely site of tumor initiation for this disease. Little is understood about how a mutation in 1 allele of the BRCA1 or BRCA2 gene leads to malignant transformation of the fallopian tube epithelium. This study interrogates the normal FTE microenvironment and shows that there is no inherent difference in proliferation in normal FTE of BRCA1/2 mutation carriers to the normal population, nor is there a difference in some immune cell populations within the histologic normal tissue. An increase in proliferation occurs later in tumor progression when the FTE have already lost cell-cycle progression barriers and there is histologic evidence of a precursor lesion.

epithelial signaling in helicobacter pylori infection and the p53 signaling pathway (9). We also identified pathways that are differentially represented in the pre (follicular) and post (luteal) ovulatory phases of premenopausal women. As shown by gene expression profiling, the FTE-BRCA cases have a higher transcriptional immune signature particularly in the luteal phase (MHC class II P = 0.0055, MHC protein complex P = 0.021 and chemokine receptor binding P = 0.014) than FTE–non-BRCA in either phase of the ovarian cycle (Fig. 1A). By ranking genes based on their GO terms biologic process we observed that the differentially expressed genes were highly represented in the cell-cycle regulation and immune defense categories (Fig. 1B).

We believe that the postovulatory microenvironment of the fallopian tube may contribute to key early events in serous carcinogenesis. The use of oral contraceptives (11), nonsteroidal anti-inflammatory drugs (12), and multiparity are all epidemiologic factors that reduce risk in of HGSC, presumably by reducing lifetime ovulation events and modulating effects of the hormonal and inflammatory milieu of the postovulatory environment on FTE. Inflammation has long been associated with ovulation, which is an epidemiologic risk factor in the development of high-grade serous ovarian cancer, seen in both sporadic and hereditary ovarian cancers (13, 14). Gene expression analyses have revealed that there is a significant elevation of inflammation or immune related mRNA in the normal fallopian tube of BRCA1 mutation carriers (9). Therefore, the chronic inflammatory state related to ovulation, gonadotropin, and hormonal stimulation (15, 16) as it relates to the presence of immune cells in the context of BRCA1/2 mutation carriers has not yet been described in human FTE. To further interrogate the effects of the observed transcriptional changes in histologic normal FTE (both in FTE-BRCA and FTE–non-BRCA), we analyzed more than 200 patient specimens: (a) to determine whether there is a proliferative advantage of FTE-BRCA cells over nonmutation carriers, (b) to identify any correlative relationship with cells of the immune system and the FTE, and (c) to investigate the relationship between ovarian cycle status, proliferation and immune infiltrates in histologically normal FTE.

Materials and Methods

Patient samples
The University Health Network Research Ethics Board approved this study protocol. Ovarian cycle status was determined after review of histologic sections of ovaries and endometrium by a gynecologic pathologist (PS). Two fallopian tube tissue microarrays (TMA) previously described were used comprising of 83 cases with known BRCA1/2 and ovarian cycle status (9, 10). In addition, 144 cases of fimbrial tissues using whole sections including FTE-BRCA and FTE–non-BRCA were analyzed (Table 1; ref. 5). Two fimbrial tissue sections were assayed for Ki-67 expression if available (n = 23/94). The mean age of the patients included in this study was 46.7 years with a range from 30 to 71 years. Whole sections of fallopian tubes from HGSC (n = 15) patients with lesions diagnosed as STIC (17) were analyzed.

Immunohistochemistry
The following antibodies were used at these dilutions: MIB1/Ki67 (Labvision) 1/1,000, p53 (Novocastra) 1/300, CD3 (Dako) 1/300, CD8 (Vector) 1/50, CD20 (Dako) 1/100, CD68 (Dako) 1/50, anti-Mouse-Cy3, and anti-Rabbit-FITC (Jackson). Appropriate negative and positive controls were performed to determine specificity of antibodies. Slides were imaged at 40× magnification using the ScanScope XT slide scanner (Aperio Technologies, Inc) or the Olympus BX50.

Two independent observers, blinded to case designations (ovarian cycle, age, and/or BRCA1/2 germline mutation status) scored digitized slides. For Ki-67+ , positive epithelial cells were scored as 0 (none), 1 (1% positive cells), 2 (2% to 4% positive cells), 3 (5% to 15% positive cells), or 4 (>15% positive cells) as previously described by Norquist and colleagues (ref. 18; Supplementary Fig. 1). Independently, automated image analysis was performed as a comparator of overall proliferation in the epithelial mucosa of the fallopian tube epithelium. Digitized slides were quantified for intensity and percentage of cells staining using automated image analysis algorithms for nuclear staining (Spectrum Plus, Image Analysis Toolbox, TMALab II, Aperio, Inc.; ref. 19). Images were annotated to include only the mucosal components of the tubes (Supplementary Fig. 3). For cases with STIC the same anatomic regions for normal FTE, STIC and HGSC were annotated to compare CD3, CD68, and Ki67 infiltrates in normal, cancer precursor lesion and cancer.
Figure 1. A, differentially expressed genes between FTE-BRCA and FTE-non-BRCA highly represented GO BP terms. B, representative Ki-67\(^+\) cells in the FTE cases. Distribution of Ki-67\(^+\) cells in the luteal phase indicate that nonepithelial, immune cells are also Ki-67\(^+\) (×20 magnification).
Microarray analysis

Profiling data for all samples deposited in the National Center for Biotechnology Information Gene Expression Omnibus8 and accessible through GEO Series accession number GSE28044 was used. 440 probes related to BRCA1 mutational status, which had a P-value of ≤0.05 and at least a 2-fold change in FTE-BRCA (n = 12) versus FTE–non-BRCA (n = 12), were deduced using GeneSpring v12. The database for visualization and integrated discovery (DAVID) v6.7 was used to generate the list of Gene Ontology biologic process terms (GO BP) that the ovarian cycle status has a greater effect on the proliferative index of FTE, we placed samples into 2 groups: 50 years ≤ and >50 years (with ranges from 45 to 55 years old). Menopausal status at the time of surgery was not available in most cases. We found that the younger age group had an overall higher proliferation than the older age group although this was not statistically significant (P = 0.2494) for any range analyzed (for Supplementary Fig. 2 and Fig. 2E).

We noted there were Ki-67+ cells within the submucosal stromal compartment of the fallopian tubes, and that some of the intraepithelial cells expressing Ki67 were lymphocytes (Figs. 1C and 3B). There was a statistically significant increase (P = 0.0017) in leukocytes measured by Ki-67+ cells in the luteal phase (n = 31) compared to the follicular phase (n = 22; Fig. 2F).

Inflammatory cell phenotype

To determine whether BRCA mutation status influences the infiltration of inflammatory cells within the microenvironment of the histologic normal FTE, we performed immunohistochemistry on CD3+ -T-lymphocyte cells, CD8+ -T-helper cells, CD20+ -B cells and CD68+ macrophages. In the fallopian tube cases (n = 111), CD3+ or CD8+ lymphocytes were not significantly differentially distributed when analyzed by either ovarian cycle status or BRCA1/2 mutation status (Table 2). There were minimal to no CD20+ cells present in the distal end of the fallopian tubes or the ampulla, therefore, we did not pursue further analyses (data not shown). Finally, as

Table 1. Patient characteristic of cases analyzed in this study

<table>
<thead>
<tr>
<th>Age range (years)</th>
<th>No. of cases</th>
<th>BRCA1</th>
<th>p53 signature</th>
</tr>
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<tbody>
<tr>
<td>34–71</td>
<td>46</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>30–64</td>
<td>7</td>
<td></td>
<td></td>
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<tr>
<td>44–77</td>
<td>0</td>
<td>N/A</td>
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<tr>
<td>37–47</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36–50</td>
<td>37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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has been previously shown in smaller studies (22, 23), there were a greater number of CD68+ cells in the luteal phase (4.58 ± 2.25%) compared to the follicular phase (2.97 ± 1.87%) in all histologic normal fallopian tube samples tested (P = 0.002). However, there were no differences in number of CD68+ cells observed between BRCA (3.63 ± 2.26%) and non-BRCA (3.64 ± 1.97%) cases (Fig. 3).

Sequential slides containing normal FTE, STIC, and HGSC were analyzed for the presence of macrophages (CD68+ cells) and T lymphocytes (CD3+ cells; Fig. 4). Identical regions of normal FTE, STIC, and HGSC were annotated and analyzed using Spectrum Plus to determine per cent positive macrophages and T lymphocytes in 15 individuals, analyzing the same region for each case. CD3+ cells did not display a significantly similar increase in infiltration with an increase in the degree of neoplasia within each case where, normal FTE to STIC (P = 0.5363), STIC to HGSC (P = 0.4107) and normal FTE to HGSC (P = 0.3061; Fig. 4B). An incremental increase in CD68+ invading cells was observed with disease progression: between normal FTE (benign) to STIC (P = 0.0035), STIC to HGSC (P = 0.0046) and normal FTE to HGSC (P = 0.003; Fig. 4C). Furthermore, the mean ratio of CD68+/CD3+ increased from the benign normal FTE 2.826 ± 0.7582 (range 1.064 to 12.13) to STIC 4.629 ± 1.104 (range 0.9642 to 14.7) to HGSC 6.532 ± 1.584 (range 0.9955 to 20.72) with a correlative increase in Ki67+ cells from benign to disease development (Fig. 4D).
Germline mutations in BRCA1 and BRCA2 are present in about 15% of ovarian cancer patients with HGSC (24). We have previously shown that cell-cycle genes and genes enriched for immune cell regulation were altered in BRCA1 heterozygote FTE, and it is possible that FTE cells with loss of 1 allele respond differentially to the microenvironment stimuli (refs. 9, 10, 25; Supplementary Table 1). In this study, we have investigated whether histologically normal FTE from BRCA1/2 heterozygotes had an increased proliferation compared to control FTE or an increased inflammatory infiltrate as suggested by previous gene expression analyses. A previous study by Norquist and colleagues, identified Ki-67+ cells in fallopian tubes to be significantly higher in BRCA mutation carriers than in normal control cases (18). These authors however did not control for age or ovarian cycle status in their study, an important omission as FTE cells are responsive to reproductive hormone stimulation. Controlling for these parameters, we found no significant difference in proliferation between mutation carriers and nonmutation carriers either in the high anatomic risk zone, the fimbria, or in the tubal ampulla. Analyses of the proliferative index of women at ages 45 to 55 < P < 55 years and >45 to 55 years used as a surrogate for menopausal status, and as a possible indicator of proliferative advantage revealed no significant differences between these 2 groups. Most importantly, the ovarian cycle status alone, independent of BRCA mutation status, resulted in a significant difference (P < 0.001) between

![Figure 3. A, distribution of CD3+, CD8+, and CD68+ cells in fallopian tube epithelium cases. B, immunofluorescence of double positive Ki67 immune cells in normal FTE (>40 magnification). C, representative IHC of immune infiltrates CD3+, CD8+, and CD68+ cells in histologic normal FTE, in BRCA1, follicular, luteal, and controls cases (>40 magnification). * P < 0.01.](image-url)
the proliferation index of the FTE in the follicular preovulatory phase and the postovulatory luteal phase. In addition, when analyzing the tissues, particular care was taken to only count Ki67+ tubal epithelium, and not Ki67+ intraepithelial lymphocytes or stromal cells (Fig. 3B). Our results indicate that increased tubal proliferative activity is linked to the estrogen dominant preovulatory (follicular/proliferative) phase in premenopausal women, and not to BRCA germline mutation status. In tubal epithelium from patients with a diagnosis of HGSC and concomitant STIC, background histologically normal FTE showed no increased proliferative activity as also recently proposed by Vang and colleagues and Khun and colleagues (17, 26). These results together indicate that the cellular events resulting in increased proliferative activity occur relatively late in the development of carcinoma.

We observed an increased presence ($P = 0.0017$) of Ki67+ cells in the stroma of FTE in the luteal phase. Our previous publication also highlighted pathways involved in cytokine–cytokine receptor interaction and antigen processing and presentation (9), indicating that the FTE-BRCA epithelial cells potentially have an increased number of inflammatory cells present in the microenvironment or have an inflammatory secretory signature, higher levels of NAMPT, IL8, and C/EBP-δ for example, which could potentially activate immune cells in the case of FTE-BRCA tissue (9). CD68+ cells were equally distributed between low and high-risk fallopian tube epithelium, but were more prevalent in the postovulatory phase of the ovarian cycle. There are no significant differences in the presence of CD3+, CD8+ and CD68+ between BRCA and non-BRCA FTE. Although studies have shown the presence of T cells to be important prognostic factors in HGSC, the presence of the cells themselves within the histologically normal fallopian tube epithelium in high-risk individuals, is not an indicator of an increased or decreased risk in developing the disease.

Immune-regulated pathways influence multiple aspects of tumor and fallopian tube biology and possibly tumor development. We are the first to report that immune infiltrates are altered early in malignant transformation of FTE, as noted by the statistically incremental increase of macrophages associated with the FTE, STIC and cancers of individual cases analyzed with the concomitant increase in proliferation. Macrophages were also the predominant inflammatory cell type within the normal FTE, STIC, and cancers. It seems that infiltration of CD3+ T lymphocytes comes late in the progression of the disease compared to CD68+ macrophages. We also noted that macrophages and lymphocytes were equally distributed between STIC and HGSC cases that stained positive or negative for p53.

Finally, these data suggest that a significant increase in proliferation is a late event in the transformation of FTE even within the BRCA mutation population. The postovulatory (the luteal phase) microenvironment, has effects on FTE and as discussed previously, cytokines can contribute to reactive oxygen species and can inflict DNA damage to the epithelium, which in a haploinsufficiency BRCA1 background may lead to lesions in the fallopian tube epithelium. The autocrine (secretion of cytokines and chemokines) and paracrine relationship with inflammatory cells within the FTE needs to be further understood as there are many more events taking place in the FTE which will influence the progression of histologic normal epithelium to develop p53 signatures (and possibly other types of “signatures”) leading to formation of STICS.

<table>
<thead>
<tr>
<th>Mutation status/ ovarian cycle status–FTE</th>
<th>Mean% positive</th>
<th>Mutation status/ ovarian cycle status–FTE</th>
<th>Mean% positive</th>
<th>T-test – $P$ value</th>
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<tr>
<td>CD3</td>
<td></td>
<td>CD8</td>
<td></td>
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<tr>
<td>Non-BRCA</td>
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<td>BRCA</td>
<td>3.423</td>
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<td>BRCA-Luteal</td>
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<td>0.779</td>
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</table>

Table 2. Per cent positive immune infiltrates in normal fallopian tube epithelium.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: S.H.L. George, P. Shaw
Development of methodology: S.H.L. George, P. Shaw
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.H.L. George, P. Shaw
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.H.L. George, A. Milea, P. Shaw
Writing, review, and/or revision of the manuscript: S.H.L. George, A. Milea, P. Shaw
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.H.L. George, P. Shaw
Study supervision: S.H.L. George, P. Shaw

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Figure 4. A, immune infiltrates are also observed in STICs in both p53⁺ and p53⁻ cases as observed in the corresponding HGSC (>20 magnification). B, percent CD3⁺. C, percent CD68⁺. D, percent Ki67⁺ cells in normal FTE, STIC, and concomitant cancer. *, P < 0.01; **, P < 0.006; ***, P < 0.0001.
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