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Using a Panel of Immunohistochemical Stains to Determine Risk of Lymph Node Metastases in Women with Endometrioid Adenocarcinoma of the Endometrium

Karina Zapiecki, Greg A. Miller, Zhen Zhou, Kelly J. Manahan, John P. Geisler*

Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University of Toledo Medical Center, Toledo, USA.
Email: *john.geisler@utoledo.edu

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ABSTRACT

Objectives**:** *The purpose of this study was to determine whether a correlation exists between a panel of immunohistochemical stains (consisting of estrogen receptor (ER), progesterone receptor (PR) and wild type p53 (p53)) and nodal status in women with endometrioid endometrial cancer.* ***Methods*****:** *Three hundred forty-three women underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy, bilateral pelvic and para-aortic lymph node dissection performed. All tumors were stained for ER, PR and p53. This panel was compared to the patient's nodal status and other clinic-pathologic factors. All data was collected from the patients' charts.* ***Results*****:** *One hundred eight patients had grade 1 tumors (83.3% node negative), one hundred forty three had grade 2 (86.6% node negative), and seventy had grade 3 (74.3% node negative). One hundred thirty six patients (39.6%) had tumors that were positive for ER, PR and negative for p53. Twenty eight patients (8.1%) had tumors that were negative for ER, PR and positive for p53. One hundred seventy nine patients (52.1%) had tumors that had mixed staining. Only 6 (4.4%) patients with ER+, PR+, p53– tumors had positive node nodes (P = 0.005). None of the sixty patients with grade 1 tumors that stained ER+, PR+, p53– had positive nodes found. Conclusion: In women with grade 1 disease, no positive nodes were found if the tumors stained positively for ER and PR and negatively for p53. Further studies will look at staining in diagnostic biopsies specimens and their correlation with nodal status.*

Keywords**:** *Correlation, Estrogen Receptor, Progesterone Receptor, p53, Endometrial Cancer, Nodes*

1. Introduction

Endometrial cancer is the most common gynecological cancer in the western world. It is estimated that 2.5% of women born today in the United States will be diagnosed with cancer of the endometrium [1], this means that 1 out of 40 women will be affected. It was predicted that in the United States in 2009 there were 42,160 new cases and 7780 resulting deaths [2]. Type 1 occurs in 70% - 80% of cases being preceded by hyperplastic endometrium, occurring at younger ages. The appearance of estrogen and progesterone receptors is correlated with a favorable prognosis [3]. Type 2 which takes place at advanced ages and accompanied usually with estrogen and progesterone receptor negativity is typically linked to more aggressive types and poorer prognosis.

Accurate surgical staging is the foundation in planning treatment. The staging procedure includes total abdominal hysterectomy, bilateral salpingo-oophorectomy and also pelvic and para-aortic lymphadenectomy. Systematic pelvic and para-aortic lymphadenectomy has been an accepted as part of the staging system since 1988. There are supporting studies for the necessity of the lymphadenectomy for all patients regardless of clinical stage. [4-6] In contrast, some authors believe that some women who present with clinical stage I disease would not benefit from lymphadenectomy [4,7-9]. The ASTEC study supported those that believe lymphadenectomy is not necessary in clinically early disease [10].

As more knowledge is gained on the molecular nature of tumors, more tailored treatments are possible. The immunohistologic staining of estrogen and progesterone receptors is a central feature in determining treatment

and prognosis in tumors such as breast cancer. Studies have shown a relationship between receptor positivity, lower tumor grade and more positive overall survival. [11-14] The estrogen receptor (ER) status is described as the most powerful predictive marker in survival and response in treatment for breast cancer [15]. In ovarian cancer, the data on hormone receptors is not as clear. While Arias-Pulido and others have demonstrated that ER and PR present prognostic information in epithelial ovarian cancer [16], Tangjitgamol has not found any significant impact on survival with either expression of ER or any other receptor combination [17].

The influence of these receptors on the endometrium was and is a major research subject. The loss of steroid hormone receptors is linked with a more aggressive tumor type and presence of recurrent tumors [18-21]. The ability to search for these receptors and modify medical treatment is significant in hormone dependent carcinomas. One of the products of research was tamoxifen. Its development had a considerable impact on the treatment of breast cancer. It prolongs overall survival, enhances disease-free survival and has become the gold standard in the treatment of ER receptor positive breast carcinoma. However, it may increase the possibility of endometrial cancer and thromboembolic complications [22].

The p53 gene plays a significant role in tumor supression. It is located on chromosome 17. Initiating a cascade of reaction, p53 is responsible for stopping uncontrolled cell division and tumor growth. Altered p53 can not follow the normal pathway which includes stimulation of p21 production. p21 and the cell division-stimulating protein prevent mutant cells from proceeding in the cell cycle. With the missing "stop signal", the tumor can grow [23]. There are various studies which support the critical part of p53 in diverse cancers including endometrial cancer. p53 expression is related to much higher tumor grade and stage [24]. Positive p53 staining (*i.e.*, mutant p53) is commonly found in type 2 endometrial cancer. Positive staining p53 is described as an independent prognostic factor in endometrial cancer in many studies [24-26]. Also there is a described relation between p53 expression and positive lymph node involvement [27].

This study's purpose was to develop a new approach to combining the existing staging procedures with a new way of locating low risk patients in order to tailor their surgical procedure. Undergoing the entire staging for patients with low-risk endometrial cancer could be a disadvantage for the patients as well as a waste of hospital and economic resources.

2. Materials and Methods

This study was performed in accordance with the standards of the institutional review board. All patients underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy, bilateral pelvic and para-aortic lymphadenectomy and washings.

Specimens of endometrial carcinoma were snap-frozen and stored at –80˚C. Five-micrometer sections of frozen tissue were used for immunohistochemical staining with the p53 monoclonal antibody (pAb 1801; Novocastra Laboratories, Newcastle-upon-Tyne, UK). Sections were fixed in 10% neutral buffered formalin for 15 minutes and rinsed in phosphate-buffered saline (PBS) 3 times. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 10 minutes. Slides were stained with the pAb 1801 (1:100 dilution) using the Fisher Code-On Immuno/DNA slide stainer (Instrumentation Laboratories, Lexington, MA). Slides then were incubated in a moist chamber for 2 hours at room temperature with a 1:100 dilution of pAb 1801. After rinsing in PBS, biotinylated anti-mouse immunoglobulin (IgG) and avidin-biotin-complex (ABC kit PK6102; Vector Lab, Burlingame, CA) were used for immunohistochemical staining. The sections were developed with 3'-3'-diaminobenzidine tetrahydrochloride (Polyscience Inc., Warrington, PA) and counterstained with 1% methylgreen in 0.1 M sodium acetate buffer, pH 4.0. Sections were dehydrated with 100% isopropanol and mounted with permount. Internal controls of known positive and known negative endometrial and colon carcinomas were used with each batch stained. Quantification of mutant p53 immunostaining was accomplished using the CAS 200 image analysis system, Quantitative Proliferation Index Program (Bacus Laboratories, Inc., Lombard, IL). Mutant p53 expression was reported as percentage of positive nuclear area staining and as positive or negative. p53 expression was also semiquantitatively called positive or negative based on the absolute presence or absence of staining as observed by the CAS 200 image analyzer. All p53 stained by these standard immunohistochemical methods was considered to be mutant p53.

The fresh frozen endometrial carcinoma tissue was used for immunohistochemical detection of ER and PR protein. Five-micrometer sections were cut with Cryostat (Reichert-Jung Cryocut 1800; Leica Microsystems, Buffalo, NY) and immediately fixed in neutral buffered formalin for 48 hours. The monoclonal antibody used for ER staining was NCL-ER-6F11 (Novocastra Laboratories, Newcastle-upon-Tyne, UK) and PR staining was NCL-L-PGR-312 (Novocastra Laboratories, Newcastle-upon-Tyne, UK). After fixation, the slides were rinsed with distilled water and endogenous peroxidases were blocked with 1% hydrogen peroxide in methanol for 10 minutes. A plastic pressure cooker that contained 1000

mL of 0.01 M citrate acid buffer (CAB), pH 6.0, was used for antigen retrieval. The pressure cooker was placed in a microwave oven, and the buffer was boiled at full power for 13 minutes. The slides were put in a stainless rack, immersed into boiling CAB, and heated in the microwave oven for another 5 minutes. After removal from the pressure cooker, the slide rack immediately was placed in the water bath and then in the PBS buffer. The slides were incubated with the respective antibody at 1:30 dilution (overnight at 40˚C in a moist chamber). After rinsing in PBS, biotinylated anti-mouse IgG and avidin-biotin complex (ABC kit PK 6102; Vector Laboratories) was used for immunohistochemical staining. The slides were developed with 3, 3 diaminobenzidine tetrahydrochloride (Polyscience Inc, Warington, PA) and counterstained with 0.5% methyl green in 0.1 M sodium acetate buffer (pH 4.0) for 1 minute. Sections were dehydrated with 100% isopropanol and mounted with Permount. Normal endometrial tissue was used as positive control with each batch staining. Quantification of expression was accomplished using the CAS 200 image analysis. ER and PR expression was reported as both percentage of positive nuclear area stained and as positive or negative. At least five areas were measured and more than 200 malignant cancer nuclei were examined for each tumor.

Before data collection, the instrument was calibrated using a control slide of rat hepatocytes that was stained with each batch of slides. For each sample, the total nuclear optical density of at least 200 structurally well preserved and separate neoplastic nuclei was obtained at a wavelength of 546 μm. The DNA content was derived from the total nuclear optical density and was expressed as picograms of DNA. The internal diploid controls consisted of a mixture of nuclei from normal stromal cells and lymphocytes. For each sample, a DNA histogram then was generated, and the DNA index (DI) of all main peaks was determined. DI was calculated from the mean DNA content of a neoplastic G_0/G_1 peak divided by the mean DNA content of the normal diploid G_0/G_1 peak. A sample was considered diploid (DI = 1.0 ± 0.1) when a single G_0/G_1 peak occupied the same histogram position as the diploid control G_0/G_1 peak and no other G_0/G_1 peak with greater than 10% of the total number of nuclei was present. An aneuploid population (DI ≠ 1.0) was defined as the presence of one or more G_0/G_1 peaks that were outside the diploid range and contained greater than 10% of the total number of nuclei. The aneuploid samples were subclassified according to the value of DI (hyperdiploid, 1.1 < DI < 1.8; tetraploid, 1.8 ≤ DI ≤ 2.2; hypertetraploid, DI > 2.2; and multiploid, 2 DI -; 1.0). In addition to having a DI between 1.8 and 2.2, tetraploid samples had to contain greater than 20% of the total nuclei in the 2n G_2/M peak region on the histogram and nuclei in the 8n position (2n G_2/M peak for a tetraploid population). The DNA histograms that contained two closely overlapping peaks were classified as having questionable DNA ploidy. Statistics were performed utilizing SPSS for Windows version 9.0 (Chicago, IL), namely, Student *t* test, one-way analysis of variance, or Cox regression analysis.

3. Results

Three hundred forty three women were diagnosed with endometrial adenocarcinoma. One hundred eight had grade 1 tumors (83.3% node negative), one hundred forty three grade 2 (86.6% node negative), seventy grade 3 (74.3% node negative, **Figure 1**). One hundred thirty six patients (39.6%) had tumors that were positive for ER, PR and negative for p53. Twenty eight patients (8.1%) had tumors that were negative for ER, PR and positive for p53. One hundred seventy nine patients (52.1%) had tumors that had mixed staining (**Figure 2**). Only 6 (4.4%) patients with ER+, PR+, p53– tumors had positive node nodes (P = 0.005). All of these patients had grade 2 or 3 disease. None of the sixty patients with grade 1 tumors that stained ER+, PR+, p53– had positive nodes found.

A correlation was found between positive tumor stain-

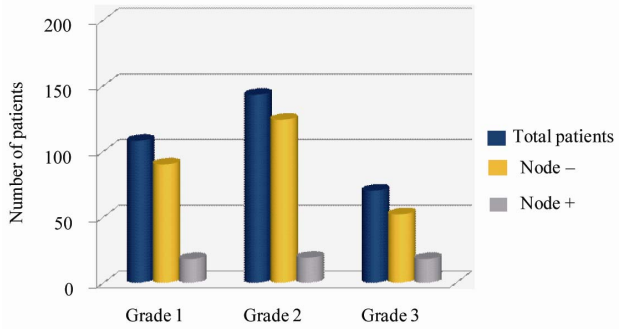

Figure 1. Histologic grade and nodal status.

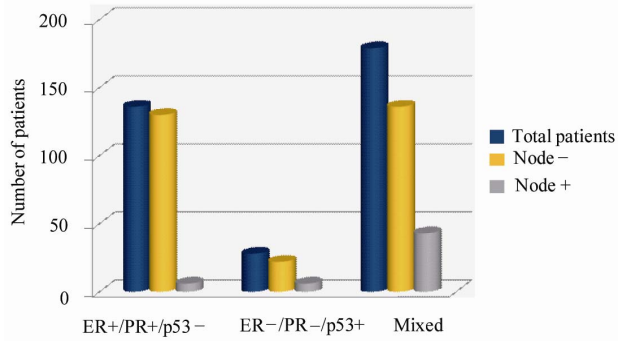

Figure 2. Staining and nodal status.

ing for ER, PR, negative for p53 and low tumor grade ($p = 6.48$ times 10^{-9}, **Figure 3**), as well as low tumor stage ($p = 0.0005$). Patients with tumor staining positive for ER, PR and negative for p53 had a much better chance of node negativity in comparison with patients with tumor staining negative for ER, PR and positive for p53 ($p = 0.0065$) and also had a much lower stage than patients with negative staining for ER, PR and positive for p53 ($p = 0.0041$). **Figure 4** depicts the correlation between FIGO stage and the status of the three stains As can be seen by the bar graph, as stage goes up, hormone receptors are less likely positive.

4. Discussion

The standardizing of the surgical therapy in patients with endometrial adenocarcinoma through FIGO was an extraordinary step in directing patients to receive the appropriate operation. The goal was to help patients get appropriate care and to be able to compare data among institutions. However, recent discoveries including advanced diagnostic tools, pre- and postoperative screening methods and molecular research, are leading to more personalization of care for every patient.

The importance of the role of steroid hormone receptors in providing predictive information can not be overlooked in the treatment of endometrial cancer patients. The expression of both ER and PR was correlated with low-grade and early stage tumor, non-recurrent tumor and good survival [10,19]. Certain patterns of ER and PR expression showed prognostic information [16]. A positive correspondence was found between presence of ER and PR and degree of tumor differention [21]. With our study we could show the positive correlation between positive tumor staining for ER, PR, negative for p53 and low tumor stage ($p = 0.0005$) and also for low tumor grade ($p = 6.4 \times 10^{-9}$).

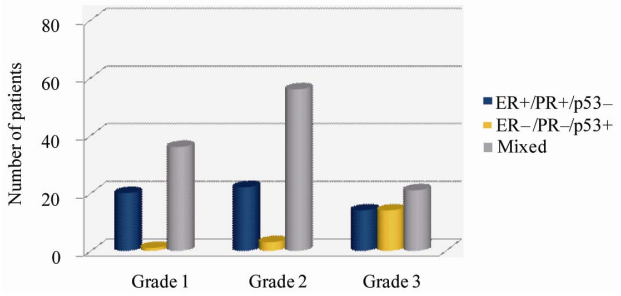

Figure 3. Correlation of staining with histologic grade.

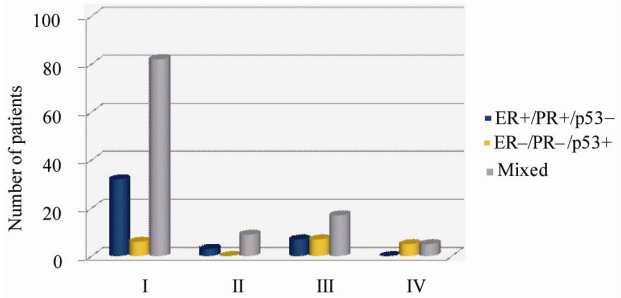

Figure 4. Correlation of staining with FIGO stage.

The expression of the tumor suppressor gene p53 is connected with unfavorable clinic pathologic factors such as advanced stage and grade, non-endometrial type, advanced age and lymph node involvement [19,28]. Appel and others also described a direct correlation of p53 with nodal positivity and risk of death [27]. The resulting p-value = 0.0041 in this study supports the assertion of p53 involvement in more advanced stage and grade. p53 is further described as an independent prognostic indicator in endometrioid endometrial cancer and as an indicator of disease recurrence [24,26,29].

The ability to gain more information about the molecular side of endometrial cancer should lead to a more tailored and advances in treatment. Patients with a low risk tumor and positive tumor staining for ER and PR, negative tumor staining for p53 could be treated according to modifications of current standards. The supporting studies have shown that these patients experience less frequently high tumor stage or grade, lymph node involvement or recurrent disease. Following this conclusion these patients would benefit from a procedure without lymphadenectomy. The MRC ASTEC trial included 1408 patients with proven endometrial cancer. For women with early endometrial cancer there was no shown benefit in overall or recurrence-free survival [10].

In the current study, low risk patients displayed no lymph node involvement. The accurate staging is the critical first step [30]. Advanced tumor stage and grade, high-risk histology are indications for lymphadenectomy with more than eleven nodes which have shown to be a important prognostic variable [30-33]. In this patient population, if a tumor if a tumor stained positively for ER and PR but negative for p53, there was less than a 5% chance of finding positive nodes (6 of 136). Thus, we propose the following: if a patient undergoes a dilation and curettage, and the tumor stains positively for ER and PR and negatively for p53, there may be only a very small chance of positive lymph nodes being found. In fact, in this study, no patients with histologic grade 1 disease and a tumor that stains positively for ER and PR and negatively for p53 were found to have positive lymph nodes after a thorough lymphadenectomy. If this data can be proven through a prospective study, this could help refine referral patterns and surgical management.

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