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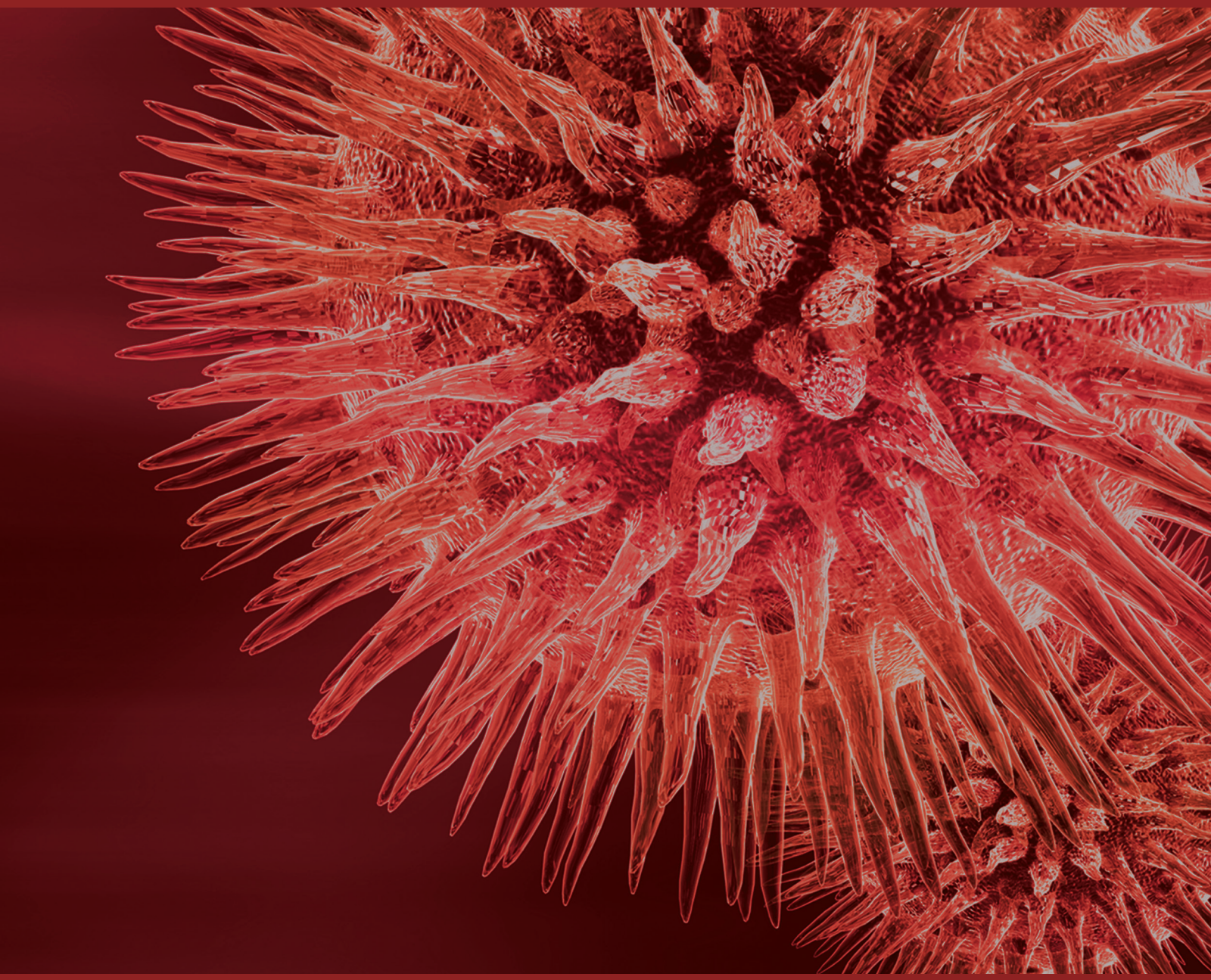
Challenges in Gynecological Cancer: Biology, Diagnosis, Surgical, and Medical Treatment

Guest Editors: Ignacio Zapardiel, Shalini Rajaram, Elisa Piovano, and Marco Petrillo



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Contents

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Editorial

Challenges in Gynecological Cancer: Biology, Diagnosis, Surgical, and Medical Treatment

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The last few years have witnessed a better understanding of molecular events that cause gynecological cancers and new insights are changing the practice of gynecologic oncology. The implementation of new concepts into daily clinical practice may improve treatment received by patients, and, more importantly, it could influence their survival and quality of life, which should be our final endpoint. The aim of this special issue was to synthetize several advances in gynecological cancer, both molecular aspects and their clinical application. We have included six research papers, half of which focused on basic research aspects and the other half focused on clinical management and usefulness.

U. Andergassen et al. report in 235 breast cancer samples that glycosylation patterns identified by immunohistochemistry could be used as a marker of early tumorigenesis since glycosyltransferases are related to smaller breast cancer tumors as well as well-differentiated ones. The Chinese group of X. Zhao et al. studied levonorgestrel effects on endometrial cancer, which is a topical subject, considering the increasing social demand for fertility preservation management for gynecological cancers. They found a time-dependent inhibition of cell proliferation with an increase of apoptosis in both human endometrial stromal cells and glandular cells by means of the upregulation of Cx43 expression in the gap junctional intercellular communication. Another basic research article focuses on the study of microvessel density and its survival implications among women with uterine leiomyosarcoma. M. Bobiński et al. did not find among their 50 patients studied a significant correlation between the microvessels density and patients' overall and 2-year survival. Their conclusions suggest that additional mechanisms apart from angiogenesis should be considered and studied in future research on uterine leiomyosarcoma.

A. Wong and J. Ngeow reviewed the influence of pathological features in the risk assessment of hereditary endometrial cancer syndromes. Specifically they studied the microscopic features as well as immunohistochemical and polymerase chain reaction based tests for DNA mismatch repair and PTEN gene mutations to establish a more targeted and cost-effective approach to those patients with hereditary syndromes. With the aim of solving the unanswered question of the early diagnosis of ovarian cancer, A. Barua et al. examined the feasibility of using interleukin-16 targeted ultrasound imaging for the detection of ovarian tumors. They found significant enhanced ultrasound signal intensity among ovarian cancers compared to other ovarian pathologies, both in early and in late stages of the disease. These findings could improve the early diagnosis of ovarian cancer by ultrasonographic scan. Another article includes novel information on the clinical usefulness of immunochemistry in the differential diagnosis of an uncommon disease, specifically the complete mole. S. Sasaki et al. studied the staining of $p57^{kip2}$ in 14 cases of gestational trophoblastic disease. They demonstrated its usefulness for the differential diagnosis

between complete and partial mole which is much cheaper than the current gold standard DNA analysis.

To conclude, we consider the current special issue a good opportunity to update our knowledge on the latest research of two very common cancers such as breast and endometrial cancer. Interesting articles on uterine sarcoma, ovarian cancer, and gestational trophoblastic disease may aid the practicing gynecologic oncology in decision making.

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Research Article

Microvessels Density in Uterine Leiomyosarcoma

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Uterine leiomyosarcomas (LMS) are rare tumors typically presenting rapid growth and unfavorable outcome. Nowadays the results of uterine LMS treatment do not meet expectations. Angiogenesis is one of processes investigated to be target for future treatment. The aim of the research was to assess microvessels density (MVD) in tumor samples collected from 50 patients with histological confirmed uterine leiomyosarcoma and to investigate statistical relations between MVD, patients survival, and FIGO stage of tumor. The assessment was carried out using immunohistochemistry methods with anti-CD34 antibody. No significant difference in MVD between FIGO stages was observed. Furthermore, contrary to many other malignancies, we found no significant relation between MVD and patients overall and 2-year survival. Results obtained in the study suggest that processes on vascular mimicry and mesenchymal to epithelial transition (MET) may play important role in development of LMS. No statistical relation between MVD and survival leads to conclusion that not only angiogenesis but other mechanisms as well should be taken into consideration in planning future research.

1. Introduction

Angiogenesis is one of crucial processes in the development of various types of tumor. The correlation between this process and patients outcome has been investigated in many gynecological and nongynaecological malignancies [1, 2]. Recently many studies investigating potential role of angiogenesis in tumors diagnostics, prognostication, and therapy were released. During last few years many antiangiogenic agents were developed; therefore better understanding of angiogenesis and its role in tumors' biology seems to be necessary for the introduction of new therapeutic strategies.

Uterine sarcomas are very rare mesenchymal tumors; thus the number of reports concerning the biology is limited. The most common histological type of uterine sarcoma is leiomyosarcoma (LMS). These tumors usually present rapid growth and poor clinical outcome [3].

The activity of tumor's angiogenesis can be assessed by measuring microvessels density in its tissue. The CD34 glycoprotein is widely used as a marker of blood vessels' endothelial cells. Besides endothelium CD34 is also expressed in a membrane of steam cells, hemopoietic cells, and osteoclasts. Furthermore its expression was found in a few malignancies, that is, gastrointestinal stromal tumors, Kaposi sarcoma, and lymphoblastic leukemia [4].

The aim of this study was to assess microvessels density (MVD) in tumors tissues of uterine leiomyosarcomas using immunohistochemical staining with anti-CD34 antibody. Furthermore we aimed to indentify statistical relations of tumors MVD with overall survival (OS) and FIGO stage of the disease.

2. Materials and Methods

2.1. Patients and Tumor Samples. The retrospective study was performed using clinical data, follow-up, and paraffin samples of uterine leiomyosarcomas diagnosed among patients operated on in the 1st Department of Gynaecological

Table 1: FIGO stages among patients.

FIGO stage	Number of patients
IA	10
IB	23
II	6
III	5
IV	6

Oncology and Gynaecology, Medical University in Lublin, Poland, from 2000 to 2013. Fifty patients were included to the study group. Mean age was 52,84 years (median: 51,50 y., SD: 12,36 y., min: 29 y., and max: 76 y.). FIGO stages among the patients are shown in Table 1. Histological diagnosis was than confirmed by two independent, experienced pathologists based on WHO criteria.

Forty-six patients (92%) underwent total hysterectomy and bilateral salpingo-oophorectomy (as first line or "second look" therapy); in two cases (4%) no surgical treatment was available and procedures were limited to collecting excisions, one patient (2%) had total hysterectomy without adnexectomy, and in one case (2%) retroperitoneal tumor was resected. Adjuvant treatment was provided to 9 patients, chemotherapy in 2 cases (4%), radiotherapy in 6 (12%), and radiochemotherapy in 1 case (2%).

2.2. Immunohistochemical Procedure. For immunohistochemical staining the most representative samples of tumors were chosen. Samples with extensive necrosis were excluded to avoid misinterpretation of vessel density, since areas close to necrosis are usually highly vascularized.

Tissue specimens have been cut into 3 μm slides and fixed on silanized glass slides. Specimens were cleaned from paraffin and antigens were unmasked (using DAKO EnVision FLEX Retrieval Solution Low pH (50x) in DAKO PT Link, Pretreatment Module for Tissue Specimens (DAKO, Denmark)). Afterwards endogenous peroxidase was blocked by washing with hydrogen peroxide for 5 min. CD34 glycoprotein was marked by using specific antibodies (primary antibody: Monoclonal Mouse Anti Human CD34 Class II Clone QBEnd 10, secondary antibody: Dako EnVision+ System/HRP Labeled Polymer Anti Mouse). To gain colorful reaction, specimens were washed with DAB (diaminobenzidine) (DAKO, Denmark); afterwards cellular nuclei were stained with Meyer's hematoxylin.

2.3. MVD Assessment. MVD was estimated by counting vessels containing CD34 positive cells in 10 HPF (high power field, magnification: 200x). MVD was expressed in absolute values as a number of CD34 positive cells in a field of 15,7 mm^2. Figure 1 presents microscopic view of LMS tissue stained with antibody against CD34.

2.4. Statistical Analysis. The distribution of results was tested with Kolmogorow-Smirnow with Lilliefors' modification and Shapiro-Wilk tests and assessed as nonnormal. To assess the correlation between MVD, FIGO, and overall survival Mann-Whitney test was used. To investigate relations between MVD and OS the group was divided into two subgroups, with high and low MVD, using median as the cut-off point. Differences in survival functions were analyzed with log-rank test (Mantel-Cox).

Table 2: MVD_{CD34} depending FIGO stage.

	N	Mean value ± SD	Median	Min–max	P
FIGO IA	10	849,40 ± 342,75	771,50	379–1594	
FIGO IB	23	1059,74 ± 751,89	742,00	139–2917	
FIGO II	6	721,17 ± 411,44	729,00	67–1226	0,923
FIGO III	5	733,40 ± 361,70	776,00	139–1044	
FIGO IV	6	933,50 ± 529,71	1024,00	126–1454	

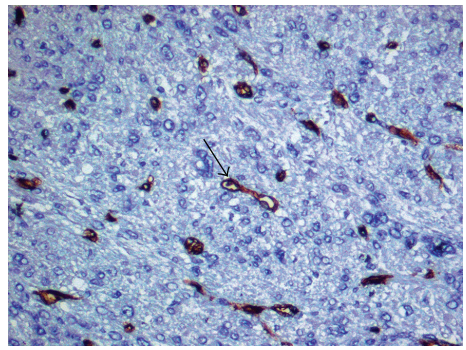

Figure 1: Colorful reaction with antibody against CD34 glycoprotein. Cellular nuclei stained with hematoxilin. The arrow shows CD34 positive microvessel. Magnification: 200x.

3. Results

CD34 positive vessels were observed in all the cases. MVD_{CD34} was ranged between 67 and 2917. Mean MVD_{CD34} was 929,26, median 766, and SD 592,03.

Microvessels density in several FIGO stages is presented in Table 2 and Figure 2.

No significant difference in MVD_{CD34} between FIGO stages was observed (P = 0,923).

The MVD_{CD34} values in groups of patients with OS longer and shorter than 2 years are presented in Table 3 and Figure 3.

No significant differences in MVD_{CD34} in groups of patients with OS longer and shorter than 2 years were detected.

No significant differences in OS depending on MVD_{CD34} among patients with uterine leiomyosarcoma were detected (P = 0,814) (see Figure 4).

4. Discussion

4.1. MVD and Treatment Outcome in Uterine Sarcoma. Microvessels density is considered to be a marker of angiogenesis. The process of angiogenesis is crucial for tumors development. To asses MVD in tumors tissues many markers were used, that is, CD34, CD31, CD105, and von Willebrand

Table 3: MVD_{CD34} values in a groups of patients with OS longer and shorter than 2 years.

	N	Mean ± SD	Median	Min–max	P
Survival ≤ 2 years	14	818,21 ± 491,71	861,00	67–1454	0,779
Survival > 2 years	36	972,44 ± 627,73	754,50	139–2917	

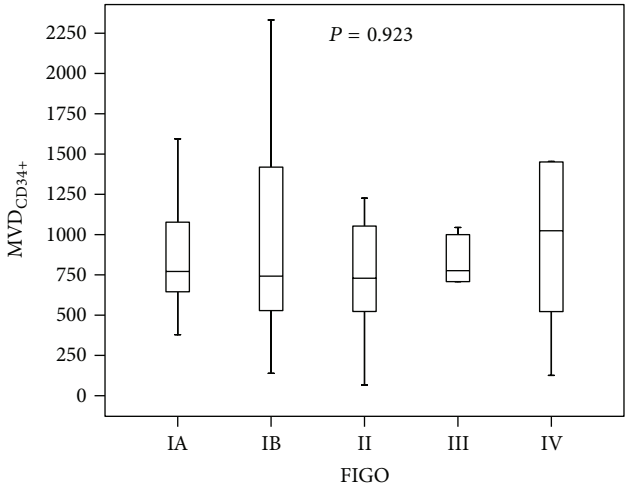

Figure 2: MVD_{CD34} depending FIGO stage.

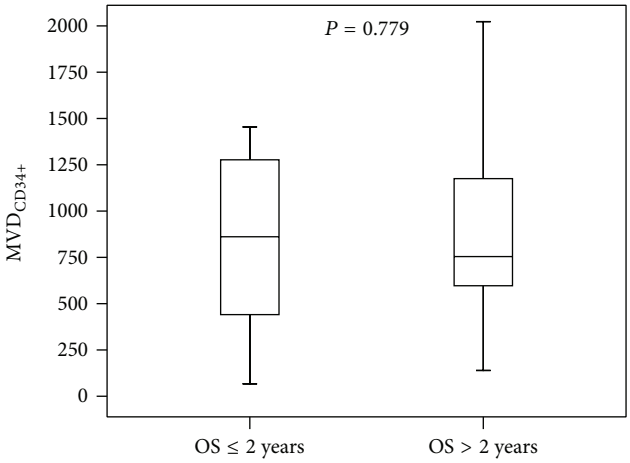

Figure 3: The MVD_{CD34} values in a groups of patients with OS longer and shorter than 2 years.

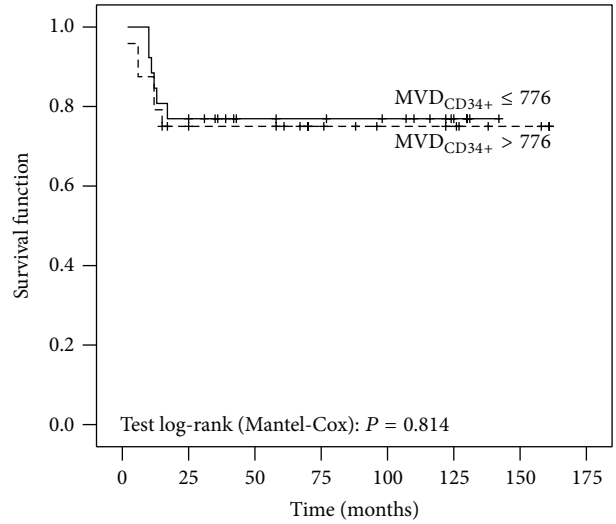

Figure 4: The Kaplan-Meyer survival functions of patients with uterine leiomyosarcoma by MVD_{CD34}.

factor [5–7]. However, it is still not proven which marker is the most suitable for such research. The undisputed advantage of glycoprotein CD34 is its high sensitivity and specificity, especially in endothelial cells staining.

In many malignancies MVD was found to be correlated with both overall and disease-free survival. In the studies carried on patients suffering from endometrial cancer, colorectal cancer, and lung cancer it was considered to be independent prognostic factor, where higher MVD was correlated with poorer outcome [8–10].

Taking into consideration typical limitations of sarcoma research such as small study groups, lack of prospective study, and not-standardized therapeutic strategies, only a few studies treating vascularity in uterine sarcoma have been published so far.

Poncelet et al. [6] investigated MVD in uterine leiomyosarcoma using antibodies against von Willebrand factor, CD34, and CD31 and concluded that MVD assessed only with antibodies against von Willebrand factor has prognostic value in this type of malignancy. Interestingly, lower MVD was correlated with poorer outcome. Interestingly there was no such relation with MVD assessed with antibodies against CD34 and CD31. Importantly, it occurs that MVD in sarcomas tissues was lower than in healthy myometrium. However, it is worth underlining that in cited research only 12 cases of LMS were analyzed.

Another research focused on MVD in leiomyosarcoma was conducted on a group of 66 patients by Avdalyan et al. [5]. They used antibodies against CD31 and concluded that MVD in tumor tissue does not affect survival but it does when it is assessed in peritumoral area.

In our research no statistical relation between MVD_{CD34+} and survival parameters was found. This fact combined with positive correlation with poorer prognosis and MVD in peritumoral area reported by Avdalyan et al. [5] may lead to the conclusion that there is a need to investigate other processes that may play an important role in the development of LMS. The role of vascularity in LMS tissue seems to be difficult to define. Recently, in a few centers sarcomas are investigated using xenografts and this method seems to be promising and may allow clarifying their role in angiogenesis in these rare tumors [11].

4.2. Angiogenesis as the Therapeutic Target in Uterine Sarcoma. Nowadays most of drugs used in chemotherapy express their activity by blocking cells division or inducing apoptosis in dividing cells. These therapies are efficient against cells that are dividing quickly, but it is widely known that many tumors (e.g., uterine sarcomas) have heterogenic histology and include, for example, steam like cells (cancer cells that express some features of steam cells, i.e., ability for self-renewal) with quite stable cell cycle that divide very seldom (tumorigenic cells) [12]. The presence of such cell populations in tumors' tissues is considered to be responsible for presence of recurrence.

Tumor development depends on oxygen and other substances' supply. This fact leads to concept of using inhibition of angiogenesis as antitumor therapy.

Interestingly many old, well-known drugs were found to have antiangiogenic activity (i.e., acetylsalicylic acid, thalidomide, and gold based antirheumatic drugs) [13].

During last few years many interesting ideas of antiangiogenic therapy were introduced. A few trials were conducted with uterine LMS as well.

In the phase II trial investigated activity of thalidomide in LMS it was concluded that it has no antitumor activity in this type of malignancies [14].

Acetylsalicylic acid (aspirin) widely using cyclooxygenase (COX) inhibitor is another agent that is tested to assess its antiangiogenic activity. The mechanism of COX inhibitors influence on angiogenesis is based on decreasing expression of proangiogenic factors such as VEGF-A and VEGF-C in tumor cells. Promising results were achieved using high dose of aspirin to inhibit growth of sarcoma cells cultures among mice [15]. In the research cited above except from inhibition of tumors growth lower MVD was noted as well.

Among novel antiangiogenic agents sunitinib was considered to be one of the most promising agents. Sunitinib is multitarget tyrosine kinase inhibitor that expresses activity against, for example, VEGF receptors, PDGF receptors, and stem cell factor receptor (KIT). Molecules mentioned above play an important role in tumors angiogenesis and development. Unfortunately, in latest research sunitinib was considered to be inactive in uterine LMS [16].

Trials aimed to assess activity of antiangiogenic drugs in LMS mostly have not met the expectations. The extrapolation of these conclusions may support results obtained in the presenting study.

The fact that MVD does not affect OS in uterine sarcoma may lead to deduction that LMS present special features that allow them to develop in a different way than other malignancies where relation between MVD and OS was observed.

This observation may be explained by phenomenon of "vascular mimicry" and "mesenchymal to epithelial transition" (MET).

4.3. Vascular Mimicry in LMS. Vascular mimicry is the formation of vascular-like structures but unprovided with endothelial cells. Walls of these structures are built of tumor cells that are suspected to present some antigens typical for endothelial cells (i.e., CD31). Interestingly, red blood cells have the ability to pass though mimicry structure similarly to passing through microvessels [17]. The presence of mimicry was observed in a few types of mesenchymal tumors, for example, rhabdomyosarcomas, esophageal stromal tumors, and it was linked with poor prognosis [18].

The process of vascular mimicry is still not fully understood and its role in development of LMS remains unclear. However, the lack of difference in MVD between tumors with various prognosis and clinical stages demands explanation and investigating presence of mimicry in this tumor seems to be promising way of further research.

4.4. MET in Sarcomas. Another recently described process that may be suspected to play a role in development of LMS' vascularity is "mesenchymal to epithelial transition." Simplifying, it is the process of acquiring by mesenchymal tumor cells characteristics typical for epithelial cells. The presence of this process in LMS was already noted [19].

Possible ability of LMS cells to present endothelial-like phenotype could be important in understanding biology of these tumors. Glycoprotein CD34 is the one of epithelial markers that were observed among mesenchymal cells that undergone MET [20].

Assuming that at least part of tumors cells is able to differentiate into epithelial-like cells may explain its resistance for antiangiogenic therapy. This assumption allows supposing that even if its vascularity is affected by therapy, tumor has ability to build new endogenous vascular system to supply itself with necessary substances.

5. Conclusions

The fact that no relevance between MVD, OS, and FIGO stage exists in uterine sarcoma leads to conclusion that process of angiogenesis in these rare tumors demands further research. The efficiency of experimental antiangiogenic therapy remains unsatisfactory. Vascular mimicry and MET may play important role in these tumors and may occur promising prognostic factors and therapeutic targets. Future research on LMS should take into consideration possibility of presence of multipotential, steam-like tumors.

Disclosure

Preliminary analysis of the results has been presented during 5th meeting of European Network of Individualized Treatment in Endometrial Cancer (ENITEC) held on June 26-27, 2014, in Leiden, Netherlands.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Marcin Bobiński was responsible for preparation of the paper, data collection, design of the study, Grzegorz Polak was responsible for preparation of the paper, Wiesława Bednarek

and Jan Kotarski were responsible for design of the study and correction of the paper, Justyna Szumiło was responsible for immunohistochemical analysis, and Marek Cybulski was responsible for statistical analysis. All authors approved the final version of the paper.

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References

[1] N. J. Nadkarni, K. D. Geest, T. Neff et al., "Microvessel density and p53 mutations in advanced-stage epithelial ovarian cancer," *Cancer Letters*, vol. 331, no. 1, pp. 99–104, 2013.

[2] Y. Liu, Z. Ye, H. Sun, and R. Bai, "Grading of uterine cervical cancer by using the ADC difference value and its correlation with microvascular density and vascular endothelial growth factor," *European Radiology*, vol. 23, no. 3, pp. 757–765, 2013.

[3] E. D'Angelo and J. Prat, "Uterine sarcomas: a review," *Gynecologic Oncology*, vol. 116, no. 1, pp. 131–139, 2010.

[4] T. L. Holyoake, M. J. Alcorn, and I. M. Franklin, "The CD34 antigen: potential clinical advantages of CD34 selection," *Clinical Oncology*, vol. 8, no. 4, pp. 214–221, 1996.

[5] A. Avdalyan, I. Bobrov, V. Klimachev, and A. Lazarev, "Prognostic value of microvessel density in tumor and peritumoral area as evaluated by CD31 protein expression and argyrophilic nucleolar organizer region count in endothelial cells in uterine leiomyosarcoma," *Sarcoma*, vol. 2012, Article ID 594512, 11 pages, 2012.

[6] C. Poncelet, R. Fauvet, G. Feldmann, F. Walker, P. Madelenat, and E. Darai, "Prognostic value of von Willebrand factor, CD34, CD31, and vascular endothelial growth factor expression in women with uterine leiomyosarcomas," *Journal of Surgical Oncology*, vol. 86, no. 2, pp. 84–90, 2004.

[7] O. Erdem, C. Taskiran, M. A. Onan, M. Erdem, H. Guner, and O. Ataoglu, "CD105 expression is an independent predictor of survival in patients with endometrial cancer," *Gynecologic Oncology*, vol. 103, no. 3, pp. 1007–1011, 2006.

[8] Y. Li, S. Li, D. Sun, L. Song, and X. Liu, "Expression of 15-hydroxyprostaglandin dehydrogenase and cyclooxygenase-2 in non-small cell lung cancer: correlations with angiogenesis and prognosis," *Oncology Letters*, vol. 8, no. 4, pp. 1589–1594, 2014.

[9] Y. Wang, X. Yao, J. Ge, F. Hu, and Y. Zhao, "Can vascular endothelial growth factor and microvessel density be used as prognostic biomarkers for colorectal cancer? A systematic review and meta-analysis," *The Scientific World Journal*, vol. 2014, Article ID 102736, 13 pages, 2014.

[10] A. Czekierdowski, S. Czekierdowska, B. Czuba et al., "Microvessel density assessment in benign and malignant endometrial changes," *Journal of Physiology and Pharmacology*, vol. 59, supplement 4, pp. 45–51, 2008.

[11] J. Stebbing, K. Paz, G. K. Schwartz et al., "Patient-derived xenografts for individualized care in advanced sarcoma," *Cancer*, vol. 120, no. 13, pp. 2006–2015, 2014.

[12] B. Choijamts, S. Jimi, T. Kondo et al., "$CD133^{+}$ cancer stem cell-like cells derived from uterine carcinosarcoma (malignant mixed Müllerian tumor)," *Stem Cells*, vol. 29, no. 10, pp. 1485–1495, 2011.

[13] M. F. He, X. P. Gao, S. C. Li et al., "Anti-angiogenic effect of auranofin on HUVECs in vitro and zebrafish in vivo," *European Journal of Pharmacology*, vol. 740, pp. 240–247, 2014.

[14] D. S. McMeekin, M. W. Sill, K. M. Darcy et al., "A phase II trial of thalidomide in patients with refractory leiomyosarcoma of the uterus and correlation with biomarkers of angiogenesis: a gynecologic oncology group study," *Gynecologic Oncology*, vol. 106, no. 3, pp. 596–603, 2007.

[15] X. Zhang, Z. Wang, Z. Wang et al., "Impact of acetylsalicylic acid on tumor angiogenesis and lymphangiogenesis through inhibition of VEGF signaling in a murine sarcoma model," *Oncology Reports*, vol. 29, no. 5, pp. 1907–1913, 2013.

[16] M. L. Hensley, M. W. Sill, D. R. Scribner Jr. et al., "Sunitinib malate in the treatment of recurrent or persistent uterine leiomyosarcoma: a Gynecologic Oncology Group phase II study," *Gynecologic Oncology*, vol. 115, no. 3, pp. 460–465, 2009.

[17] H. Zhao and X.-M. Gu, "Study on vasculogenic mimicry in malignant esophageal stromal tumors," *World Journal of Gastroenterology*, vol. 14, no. 15, pp. 2430–2433, 2008.

[18] B. Sun, S. Zhang, X. Zhao, W. Zhang, and X. Hao, "Vasculogenic mimicry is associated with poor survival in patients with mesothelial sarcomas and alveolar rhabdomyosarcomas," *International Journal of Oncology*, vol. 25, no. 6, pp. 1609–1614, 2004.

[19] J. Yang, J. A. Eddy, Y. Pan et al., "Integrated proteomics and genomics analysis reveals a novel mesenchymal to epithelial reverting transition in leiomyosarcoma through regulation of slug," *Molecular and Cellular Proteomics*, vol. 9, no. 11, pp. 2405–2413, 2010.

[20] J. Yang, X. Du, G. Wang et al., "Mesenchymal to epithelial transition in sarcomas," *European Journal of Cancer*, vol. 50, no. 3, pp. 593–601, 2014.

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Research Article

Glycosyltransferases as Markers for Early Tumorigenesis

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Background. Glycosylation is the most frequent posttranslational modification of proteins and lipids influencing inter- and intracellular communication and cell adhesion. Altered glycosylation patterns are characteristically observed in tumour cells. Normal and altered carbohydrate chains are transferred to their acceptor structures via glycosyltransferases. Here, we present the correlation between the presence of three different glycosyltransferases and tumour characteristics. *Methods*. 235 breast cancer tissue samples were stained immunohistochemically for the glycosyltransferases N-acetylgalactosaminyltransferase 6 (GALNT6), β-1,6-N-acetylglucosaminyltransferase 2 (GCNT2), and ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosamine α-2,6-sialyltransferase 1 (ST6GALNac1). Staining was evaluated by light microscopy and was correlated to different tumour characteristics by statistical analysis. *Results*. We found a statistically significant correlation for the presence of glycosyltransferases and tumour size and grading. Specifically smaller tumours with low grading revealed the highest incidences of glycosyltransferases. Additionally, Her4-expression but not pHer4-expression is correlated with the presence of glycosyltransferases. All other investigated parameters could not uncover any statistically significant reciprocity. *Conclusion*. Here we show, that glycosyltransferases can identify small tumours with well-differentiated cells; hence, glycosylation patterns could be used as a marker for early tumourigenesis. This assumption is supported by the fact that Her4 is also correlated to glycosylation, whereas the activated form of Her4 does not show such a connection with glycosylation.

1. Introduction

Glycosylation is the most common posttranslational modification of proteins and lipids, creating structural diversity [1]. The addition of carbohydrate chains influences diverse mechanisms such as cell-cell adhesion [2, 3], communication of cells with their environment [4], or antigen recognition by the immune system [5]. There are two major forms of glycosylation: first, the so-called N-glycosylation. During this process, oligosaccharide precursor chains are covalently linked to Asparagine-residues of proteins. The second form is called O-glycosylation. Here, in a first step, GalNAc residues are attached to Serine- or Threonine-residues under the control of ppGalNac-transferases [6–8]. These carbohydrate residues are later modified in a tissue-specific manner [9, 10].

Cancer cells and tissues are often characterized by an altered glycosylation pattern [11, 12]. As early as 1985, it was shown that cancer tissues stain positive with antibodies against abnormal carbohydrate chains [13]. Many of these tumour associated carbohydrate antigens (TACAs) are well known and described, for example, T-, Tn-, sTn-, and the Lewis-antigens [14, 15], and have been studied extensively in cell culture models [16, 17]. TACAs seem to regulate cellular functions such as signal transduction, antigenicity, interaction with immune effector cells, and cell-cell adhesion [18–22]. Taken together, altered glycosylation seems to contribute to tumourigenesis and tumour progression [23] and hence might offer new targets for diagnosis, prognosis, and therapeutic strategies [24–26].

In breast cancer, altered glycosylation has been linked to a worse prognosis and a shortened overall survival before

[27]. The most frequent alterations of glycosylation in breast cancer are shortened O-glycans [28] and increased sialylation [29]. Moreover, altered glycosylation effects the morphological transformation of tumour cells which can ultimately lead to metastasis formation [6].

The main reason for the occurrence of altered glycosylation is a change in the expression of glycosyltransferases—the responsible enzymes for glycosylation [30, 31]. These enzymes are located in the membranes of the endoplasmic reticulum and the Golgi apparatus and transfer carbohydrate chains to acceptor molecules. They are categorized in different subfamilies with regard to the respective transferred carbohydrate. Although the families share almost no sequence homologies and do not have comparable exon-intron-structures, protein domain structures are rather similar: they mostly have an N-terminal cytoplasmatic tail, a signal anchor domain of 16–20 amino acids, an extended stem region, and a catalytic C-terminal domain [32, 33]. Expression profiling of glycosyltransferases has been studied extensively [34–38] and it was reported that oncogenic transformation is regulated at the transcriptional level [39]. Therefore, the expression of glycosyltransferases is an important marker for tumour prognosis and therapeutic outcome [40, 41].

In the present study, we sought to determine correlations between the incidence of glycosyltransferases and other tumour characteristics, such as histology, grading, tumour size, expression of Her2 and Her4, or hormone receptor status. For that purpose, three different glycosyltransferases, namely, N-acetylgalactosaminyltransferase 6 (GALNT6), β-1,6-N-acetylglucosaminyltransferase 2 (GCNT2), and ST6(α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosamine α-2,6-sialyltransferase 1 (ST6GALNac1), were immunohistochemically investigated in paraffin-embedded tumour tissue sections. GALNT6 is involved in the first steps of O-glycosylation [42]. It is known that breast cancer expresses GALNT6 mRNA and this phenomenon is mainly associated with smaller tumours (T1) [43]. GCNT2 is related to metastasis formation and influences cell proliferation, migration, and invasion of endothelial cells [44]. ST6GALNac1 synthesizes the sTn-antigen, which is known to be overexpressed in epithelial cancers like breast cancer. Moreover, the expression of ST6GALNac1 results in increased cell migration and reduced cell adhesion [16].

It could be shown that GALNT6 especially is correlated to a small tumour size and low grading, meaning that small, still good differentiated tumours are glycosylated, and thus glycosylation is a marker for a good prognosis for therapy and outcome. Furthermore a correlation of all three glycosyltransferases with Her4, but not with the activated form of Her4, pHer4, could be seen. Her4 is also known to be mostly expressed in tumour tissues which are still more differentiated [45], supporting our hypothesis.

2. Materials and Methods

2.1. Tumour Tissue Samples. Tumour tissue samples of 235 breast cancer patients undergoing breast cancer surgery between July 1998 and May 2000 were collected (ethical vote 048-08 and 148-12, Ludwig-Maximilians University of Munich, compliant to the Declaration of Helsinki), subsequently embedded in paraffin, and archived. Patient samples in this study were not preselected for certain criteria and therefore show different tumour characteristics with respect to age at time of surgery, histology, grading, tumour size, nodal state, formation of remote metastasis, and hormone receptor state.

Tissue samples can be assigned to characteristics shown in Figure 1.

2.2. Immunohistochemistry. The paraffin-embedded samples were cut into 2-3 μm thick sections with a sliding microtome, subsequently placed on specially covered microscope slides (SuperFrost Plus, Menzel GmbH, Braunschweig), and air-dried over night at 56–58°C. For immunohistochemical staining, samples were deparaffinized with xylol (Merck, Darmstadt, Germany) for 20 min and successively washed with different dilutions of ethanol (100%, 90%, and 75%). To prevent unspecific staining of tissue samples, endogenous peroxidase activity was reduced by incubation of the samples in 3% H_2O_2 (VWR International, Radnor, USA) for 20 min. Afterwards samples underwent further washes in ethanol and water. Next, antigen retrieval was carried out in boiling Na-citrate buffer (pH 6.00) for 5 min. (Merck, Darmstadt, Germany). After cooling down, tissue samples were washed again in water and PBS (Biochrom, Berlin, Germany).

The prepared slides were first blocked in 10% normal goat serum for 20 min (Vector Laboratories, Burlingame, USA) to prevent unspecific binding of the primary antibody. After removing the blocking solution, primary antibodies were added in optimized concentrations (see Table 1).

Primary antibodies were incubated for 18 h at 4°C. Slides were then washed twice with PBS and subsequently incubated with biotinylated secondary antibodies for 30 min. at room temperature. After removing the secondary antibody, slides were treated with ABC-reagent (Vector Laboratories, Burlingame, USA) according to the manufacturers' instructions for 30 min. Next, DAB-reagent (Dako, Carpinteria, USA) diluted in H_2O_2 was added to the slides for 1 min. for ideal staining. Enzyme reaction was stopped by washing the slides in water. Nuclei were counterstained with Hemalaun (AppliChem, Darmstadt, Germany) for 5 min. Last, samples were dehydrated with ethanol and xylol and embedded in Eukitt (Medite, Burgdorf, Germany). The stained samples were then analysed and archived for further evaluations.

Before starting the staining procedure on tumour tissue samples positive and isotype control have to be carried out (Figure 2). For positive control a sample from a tissue certainly expressing the antigen of interest is stained to test antibody function and to determine an appropriate dilution of the antibody for staining (Table 1). The isotype control reveals background staining due to primary antibody. Therefore the same tissue used for positive control (GALNT6: Placenta, GCNT2: Colon, ST6GALNac1: Uterus) is stained, but primary antibody is replaced by a control serum.

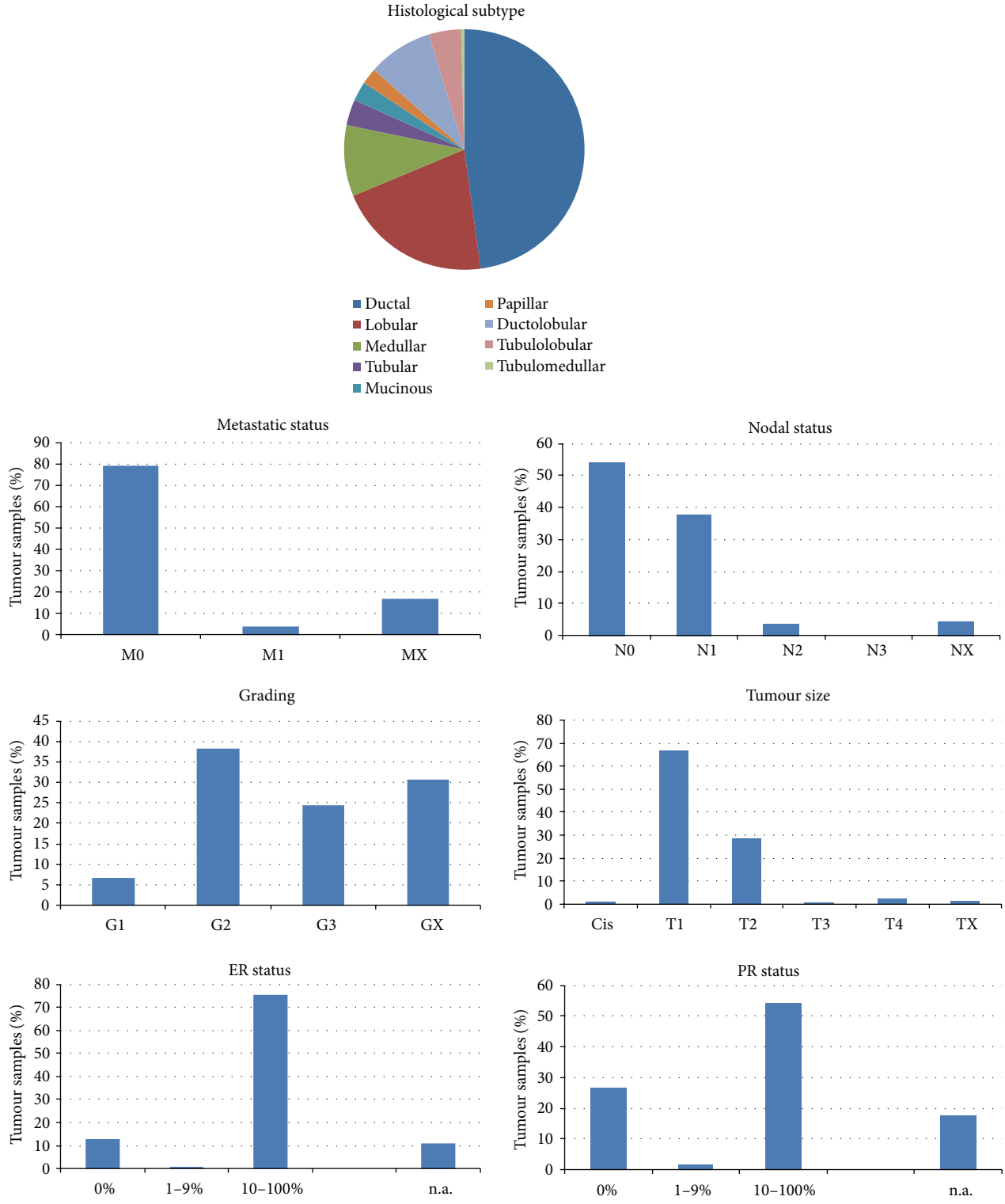


Figure 1: Characteristics of tumour samples used for staining of glycosyltransferases.

2.3. Microscopy and Evaluation of Staining. Samples were analysed and evaluated with a Leitz Diaplan light microscope (Ernst Leitz GmbH, Wetzlar, Germany). Four objectives with different magnifications (6,3x, 10x, 25x, and 40x) were used (Figure 3; Figure 2: positive and isotype controls).

Stainings were evaluated following the immune-reactive-score (IRS) described by Remmele and Stegner in 1987 [46]. The IRS is obtained by multiplication of staining intensity by the number of stained cells. Staining intensity can be classified into groups from 0 to 3, with 0 being "no staining

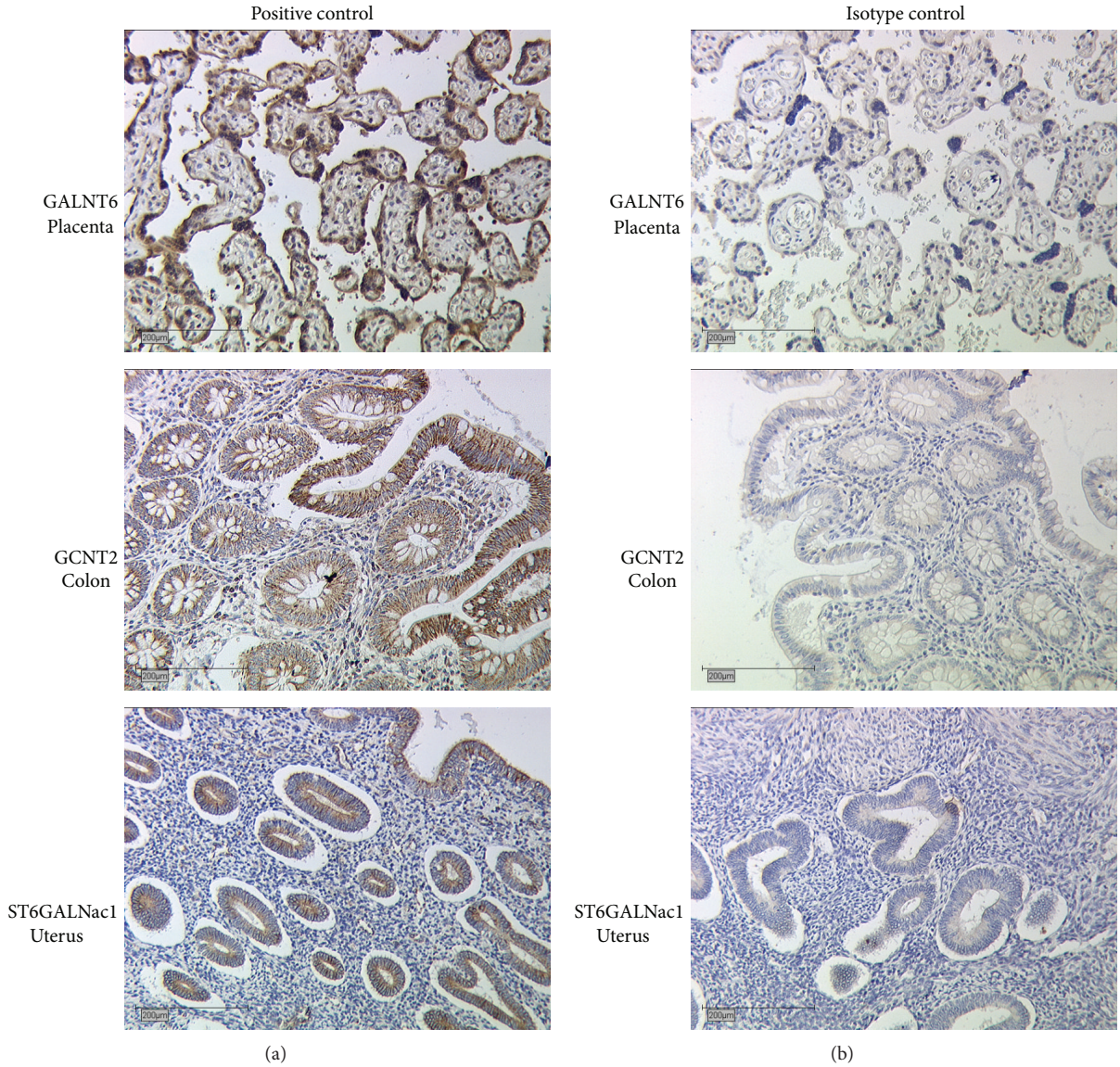


Figure 2: Positive control (a) and isotype control (b). For positive control tissues certainly expressing the questionable antigens were stained. Placental tissue was used for GALNT6 staining, Colon tissue was used for GCNT2 and Uterus was chosen for ST6GALNac1 staining. Furthermore appropriate antibody concentrations were determined in the positive controls. For isotype control same tissues as for positive control are used, but primary antibody is replaced by a control serum, thus excluding unspecific binding signals of the primary antibody.

Table 1: Antibodies used for immunohistochemical staining of breast cancer tissue samples.

Antigen	Host	Isotype	Manufacturer	Positive control tissue	Dilution (in PBS)
GALNT6	Rabbit	Polyclonal IgG	GeneTex	Placenta	1 : 1000
GCNT2	Rabbit	Polyclonal IgG	Novus Biologicals	Colon	1 : 400
ST6GALNac1	Rabbit	Polyclonal IgG	Novus Biologicals	Uterus	1 : 500

reaction" and 3 being "strong colour reaction"; numbers of stained cells are classified in a similar manner from 0, "0% stained cells," to 4 "81–100% stained cells." Thus, the IRS is in a range from 0 to 12. The IRS of different tumour characteristics were compared (Figure 4).

2.4. Statistical Evaluation. Statistical analysis was done by SPSS (SPSS Inc. Headquarters, Chicago, USA) version 20.0. As patient samples are not normally distributed, nonparametric Mann-Whitney U test was applied comparing two variables; for more variables Kruskal-Wallis test was applied.

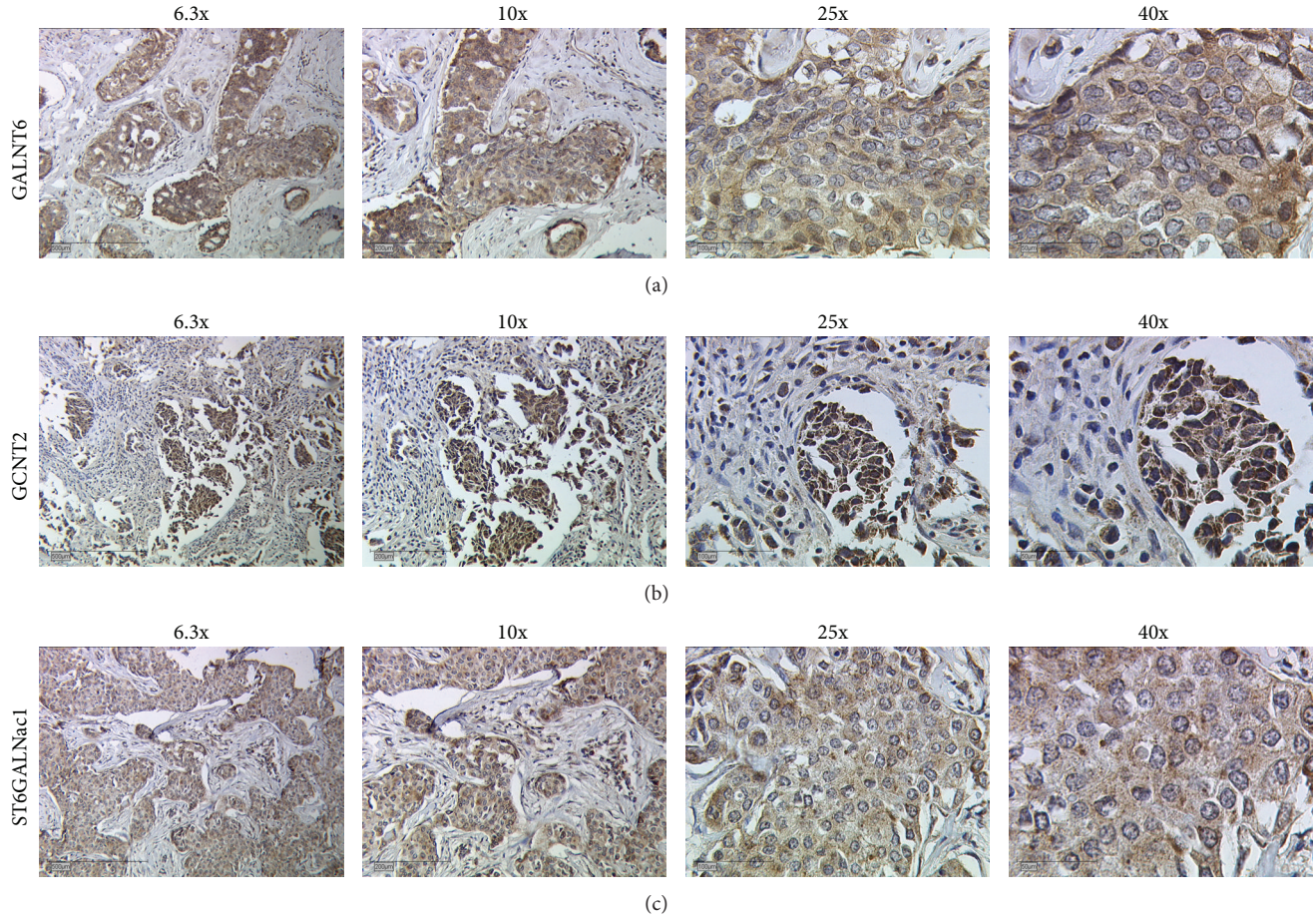


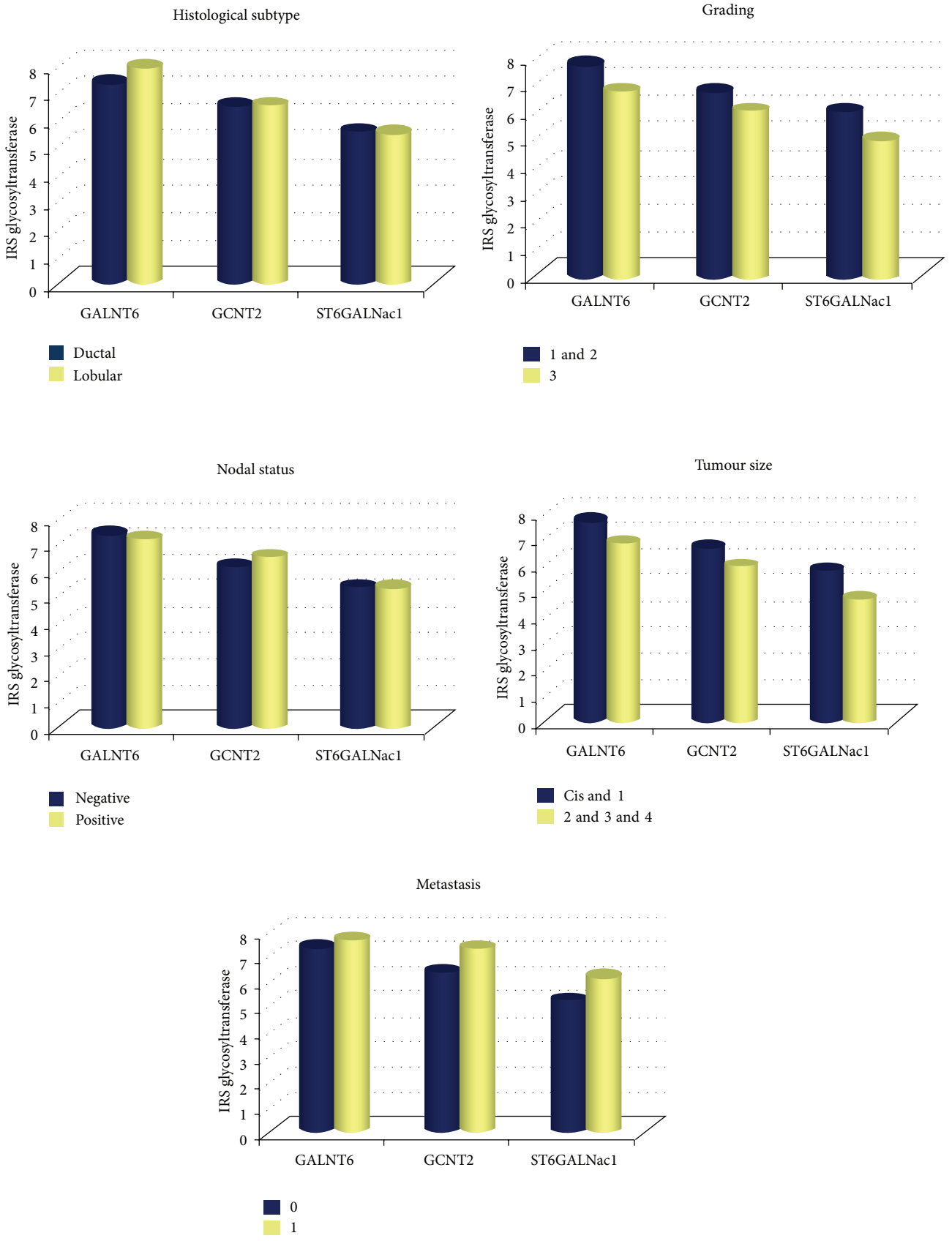
FIGURE 3: Staining of malignant breast tissue with antibodies against the three glycosyltransferases GALNT6 (a), GCNT2 (b), and ST6GALNac1 (c). Pictures were taken with different objectives (6,3x, 10x, 25x, and 40x; from left to right column) resulting in different magnifications of tissue structures.

A P value of ≤0,05 was regarded as statistically significant (Table 2). Correlations were calculated with the Spearman-Rho test. Survival curves were drawn using Kaplan-Meier analysis (Figure 5).

3. Results and Discussion

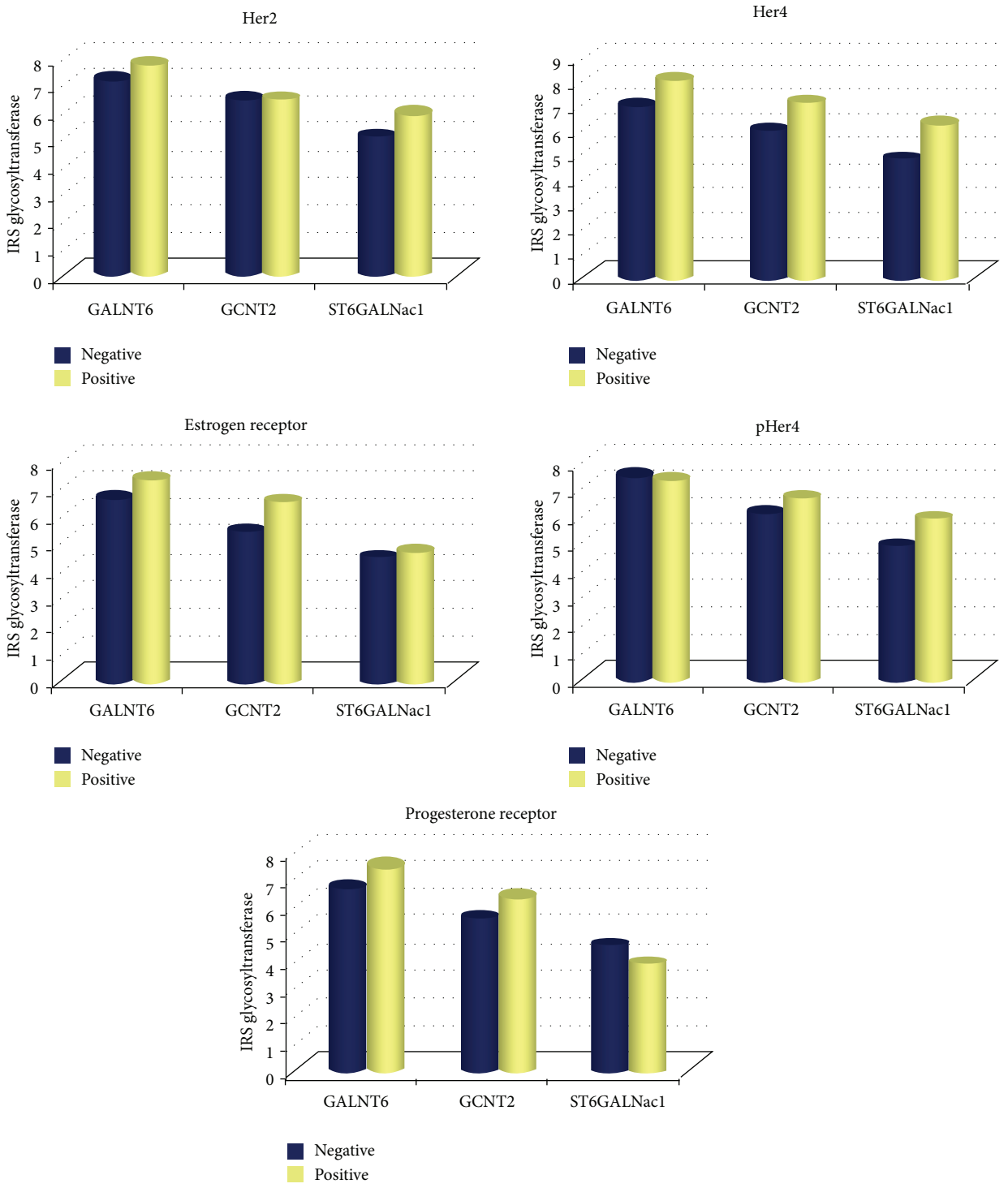
235 tumour tissue samples were stained for all three different glycosyltransferases (GALNT6, GCNT2, and ST6GALNac1) (Figures 3 and 2 for controls) and their IRS was determined by light microscopic and statistical evaluation. The resulting IRS was then compared to multiple different tumour characteristics in order to detect potential correlations.

First of all, we could not detect any association between the histological subtype and the presence of glycosyltransferases (Figure 4(a)). Lobular and ductal breast cancer revealed similar IRS for all three examined glycosyltransferases with no statistical significant difference (P = 0,203, P = 0,984, and P = 0,904 for GALNT6, GCNT2, and ST6GALNac1, resp.; Table 2). Similarly, we could not detect any significant differences of glycosyltransferase expression in comparison to the nodal status or metastatic setting (Figure 4(a), Table 2). When correlating the tumour grading to the presence of glycosyltransferases, GALNT6 was significantly higher expressed in low grade tumours (grades 1 and 2) compared to high grade tumours (grade 3) (Figure 4(a), Table 2). GCNT2 and ST6GALNac1 were seemingly higher expressed in the same group, however, at a level of borderline significance (P = 0,104 for GCNT2 and P = 0,094 for ST6GALNac1). With reference to tumour size, we were able to find a similar tendency (Figure 4(a), Table 2). Here, IR-scores for all three enzymes were higher for smaller tumours (Cis and T1) compared to bigger tumours ($T \geq 2$), with a significant difference for GALNT6 (P = 0,012) and borderline significant difference for the other two investigated glycosyltransferases (GCNT2: P = 0,066, ST6GALNac1: P = 0,059) (Figure 4(a), Table 2). However there seem to be coherences between tumour grading and tumour size and glycosyltransferases. The results suggest that tumours of low grading (grades 1 and 2) are seemingly more dependent on glycosyltransferases than tumours of high grading (grade 3). The latter group contains tumours that are much more dedifferentiated and underwent major changes in their cellular structure making them possibly



(a)

Figure 4: Continued.



(b)

Figure 4: (a) Comparison of IRS-values of glycosyltransferases with histological subtype, nodal status, grading, tumour size, and metastasis. (b) Comparison of IRS-values of glycosyltransferases with Her2, ER, and PR status and Her4 and pHer4.

more independent from glycosyltransferase enzymes. With reference to tumour size, we were able to find a similar tendency (Figure 4(a), Table 2). Furthermore IR-scores for all three enzymes were higher for smaller tumours (Cis and T1) compared to bigger tumours ($T \geq 2$) leading to the thought that glycosyltransferases are important in early phases of breast tumorigenesis. GALNT6 especially seems to play a role in early tumour formation, a finding that is in consistency with the results of Berois et al. [43]. GALNT6 seems to be characteristic of small, low grade tumours while GCNT2 and

Table 2: Statistical analysis of the investigated features. Statistically significant P values are seen for Her4 (all glycosyltransferases) and for tumour size (GALNT6; GCNT2 and ST6GALNac1 show borderline significance).

	GALNT6 (P value)	GCNT2 (P value)	ST6GALNac1 (P value)
Histological subtype, ductal versus lobular	0,203	0,948	0,904
Nodal status, negative versus positive	0,532	0,331	0,891
Metastatic status, 0 versus 1	0,957	0,383	0,497
Grading, 1 and 2 versus 3	0,029	0,104	0,094
Tumour size, CIS and 1 versus 2, 3, and 4	0,012	0,066	0,059
Her4, negative versus positive	0,003	0,005	0,001
pHer4, negative versus positive	0,622	0,113	0,039
Her2 status, negative versus positive	0,142	0,925	0,077
Estrogenreceptor status, negative versus positive	0,378	0,125	0,672
Progesterone receptor status, negative versus positive	0,324	0,266	0,575

ST6GALNac1 are obviously markers of a little more advanced tumour stage, with higher IRS-values in metastatic patient tissue samples and also with a little higher correlation to OAS, which is again in line with former findings [44]. Another correlation was seen between the glycosyltransferases and Her4/pHer4 (Figure 4(b), Table 2), as we detected that the nonphosphorylated form of Her4 seems to correlate strongly with the presence of all three glycosyltransferases (P = 0,003, P = 0,005, and P = 0,001 for GALNT6, GCNT2, and ST6GALNac1, resp.), while the phosphorylated form, pHer4, did not. Only IR-scores of ST6GALNac1 correlated at a statistically significant level with the presence of pHer4 (P = 0,039). Her4 is another member of the family of epidermal growth factor receptors and is, hence, a receptor tyrosine kinase. Epidermal growth factor binds to one of the type I transmembrane receptors which leads to a homo- or heterodimerization and subsequently activates the intrinsic kinase domain by autophosphorylation. The phosphorylated domain then serves as starting point for many intracellular signalling cascades [47]. In our observations, we detected that the "inactive," nonphosphorylated form of Her4 seems to correlate strongly with the presence of all three glycosyltransferases, while the "activated," phosphorylated form, pHer4, did not correlate at such a strong level. Only IR-scores of ST6GALNac1 correlated at a statistically significant level with the presence of pHer4.

Her2 or the hormone receptors for Estrogen and Progesterone did not reveal any significant correlations to the presence of glycosyltransferases (Figure 4(b), Table 2). Only the presence of Her2 showed a borderline significance with IR-scores for ST6GALNac1 (P = 0,077); however, GALNT6 and GCNT2 did not appear to be correlated with Her2.

Last, we tried to evaluate whether expression of glycosyltransferases correlated with the overall survival of patients included into this study. The Kaplan-Meier curves indicate that survival is not dependent on glycosylation (Figure 5), since high or low IR-scores did not deviate significantly (P values: GALNT6: 0,802, GCNT2: 0,406, and ST6GALNac1: 0,422).

All these findings are showing and underline a strong coherence between early tumorigenesis and the increased presence of glycosyltransferases.

4. Conclusion

From the results presented and discussed in the present study, we conclude that glycosylation of tumour cells does not correlate with tumour histology, formation of metastasis, nodal status, or hormone receptors. On the other hand, glycosyltransferases seem to be abundantly expressed in small and well-differentiated tumours. It is known that altered glycosylation can protect malignant cells from recognition by the immune system [48]. Specifically small and nascent tumours are prone to escape immunosurveillance in order to establish tumour growth and supportive cancer microenvironment. Hence, the overexpression of such enzymes might indicate a useful early step in breast tumorigenesis.

Of the three investigated glycosyltransferases, GALNT6 seems to offer the best correlations with regard to tumour size and grading. Since GALNT6 catalyses early steps in O-glycosylation [43] and therefore plays a central role in the process of glycosylation, it stands out as a potential marker.

A further interesting focus for future research could be to clarify the interrelation between Her4/pHer4 and glycosylation patterns. It is well known that Her4 is heavily glycosylated [45] and could serve in combination with GALNT6 to detect starting and endpoint of the glycosylation cascade. Once Her4 becomes activated by phosphorylation, intracellular signalling pathways are activated, leading to further dedifferentiation of tumour cells. This might eventually lead to a reduction of glycosylation. On the other hand, Her4 is regarded as a marker of favourable prognosis [45], since it is inversely correlated with the histological grading of a tumour [49] and is elevated in sera of early breast cancer patients [50]. In this regard, our data confer the correlation of Her4 to the presence of glycosyltransferases, especially its presence in small tumours and good differentiation.

The above-described hypothesis requires further insights and research. Additionally, the role of ST6GALNac1 should be investigated in more detail, since this glycosyltransferase in particular seems to be correlated with pHer4 and Her2.

It is the task of future research to analyse a wider array of glycosyltransferases for their role in tumour formation and progression, depicting a more detailed scheme of the roles of different glycosyltransferases in early and later tumorigenesis. Additionally the role of glycosyltransferases should be investigated in other gynaecological

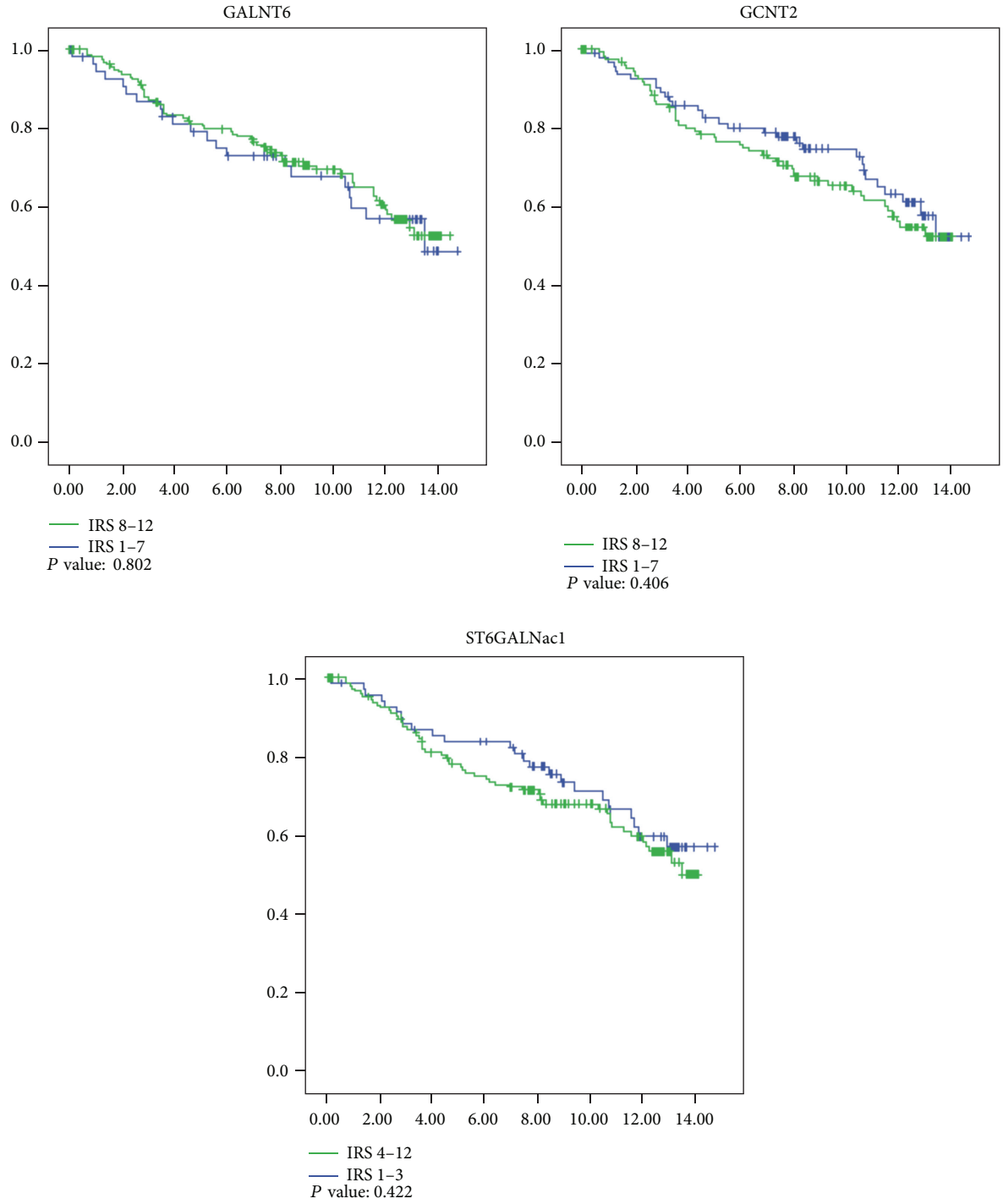


Figure 5: Kaplan-Meier analysis of overall survival with respect to glycosyltransferase expression. *P* values indicate that no differences in overall survival were found in respect to glycosyltransferase expression.

tumour entities, like ovarian or endometrial adenocarcinomas to gain more detailed insight into the onset of cancer formation.

The results of the presented experiments furthermore give a hint towards the utility of the methodology and the usefulness of glycosyltransferases in terms of tumour characterization. The method is fast and cost-efficient and glycosyltransferases play an important role in tumour development and are independent of processes like epithelial mesenchymal transformation (EMT), so that they could be useful biomarkers in the analysis of tumour tissue samples. This could in turn help to individualize tumour treatment, reducing side effects of any applied therapy while simultaneously increasing the efficiency of a therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] T. P. Skelton, C. Zeng, A. Nocks, and I. Stamenkovic, "Glycosylation provides both stimulatory and inhibitory effects on cell surface and soluble CD44 binding to hyaluronan," *The Journal of Cell Biology*, vol. 140, no. 2, pp. 431–446, 1998.

[2] M. S. Diamond, D. E. Staunton, S. D. Marlin, and T. A. Springer, "Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation," *Cell*, vol. 65, no. 6, pp. 961–971, 1991.

[3] L. M. McEvoy, H. Sun, J. G. Frelinger, and E. C. Butcher, "Anti-CD43 inhibition of T cell homing," *The Journal of Experimental Medicine*, vol. 185, no. 8, pp. 1493–1498, 1997.

[4] J. W. Dennis, M. Granovsky, and C. E. Warren, "Glycoprotein glycosylation and cancer progression," *Biochimica et Biophysica Acta: General Subjects*, vol. 1473, no. 1, pp. 21–34, 1999.

[5] P. M. Rudd, T. Elliott, P. Cresswell, I. A. Wilson, and R. A. Dwek, "Glycosylation and the immune system," *Science*, vol. 291, no. 5512, pp. 2370–2376, 2001.

[6] H. Clausen and E. P. Bennett, "A family of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyl-transferases control the initiation of mucin-type O-linked glycosylation," *Glycobiology*, vol. 6, no. 6, pp. 635–646, 1996.

[7] F. L. Homa, T. Hollander, D. J. Lehman, D. R. Thomsen, and A. P. Elhammer, "Isolation and expression of a cDNA clone encoding a bovine UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase," *The Journal of Biological Chemistry*, vol. 268, no. 17, pp. 12609–12616, 1993.

[8] K. G. Ten Hagen, T. A. Fritz, and L. A. Tabak, "All in the family: the UDP-GalNAc:polypeptide N-acetylgalactosaminyl-transferases," *Glycobiology*, vol. 13, no. 1, pp. 1R–16R, 2003.

[9] J. Gomes, N. T. Marcos, N. Berois et al., "Expression of UDP-*N*-acetyl-D-galactosamine: polypeptide *N*-acetylgalactosaminyl-transferase-6 in gastric mucosa, intestinal metaplasia, and gastric carcinoma," *Journal of Histochemistry and Cytochemistry*, vol. 57, no. 1, pp. 79–86, 2009.

[10] U. Mandel, H. Hassan, M. H. Therkildsen et al., "Expression of polypeptide GalNAc-transferases in stratified epithelia and squamous cell carcinomas: immunohistological evaluation using monoclonal antibodies to three members of the GalNAc-transferase family," *Glycobiology*, vol. 9, no. 1, pp. 43–52, 1999.

[11] S.-I. Hakomori, "Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives," *Cancer Research*, vol. 45, no. 6, pp. 2405–2414, 1985.

[12] S.-I. Hakomori, "Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism," *Cancer Research*, vol. 56, no. 23, pp. 5309–5318, 1996.

[13] T. Feizi, "Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are oncodevelopmental antigens," *Nature*, vol. 314, no. 6006, pp. 53–57, 1985.

[14] I. Brockhausen, "Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions," *EMBO Reports*, vol. 7, no. 6, pp. 599–604, 2006.

[15] S. Hakomori, "Carbohydrate-to-carbohydrate interaction in basic cell biology: a brief overview," *Archives of Biochemistry and Biophysics*, vol. 426, no. 2, pp. 173–181, 2004.

[16] S. Julien, E. Adriaenssens, K. Ottenberg et al., "ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity," *Glycobiology*, vol. 16, no. 1, pp. 54–64, 2006.

[17] S. Julien, C. Lagadec, M.-A. Krzewinski-Recchi, G. Courtand, X. Le Bourhis, and P. Delannoy, "Stable expression of sialyl-Tn antigen in T47-D cells induces a decrease of cell adhesion and an increase of cell migration," *Breast Cancer Research and Treatment*, vol. 90, no. 1, pp. 77–84, 2005.

[18] S. E. Baldus, K. Engelmann, and F.-G. Hanisch, "MUC1 and the MUCs: a family of human mucins with impact in cancer biology," *Critical Reviews in Clinical Laboratory Sciences*, vol. 41, no. 2, pp. 189–231, 2004.

[19] P. R. Crocker, "Siglecs in innate immunity," *Current Opinion in Pharmacology*, vol. 5, no. 4, pp. 431–437, 2005.

[20] M. Gerloni, P. Castiglioni, and M. Zanetti, "The cooperation between two CD4 T cells induces tumor protective immunity in MUC.1 transgenic mice," *The Journal of Immunology*, vol. 175, no. 10, pp. 6551–6559, 2005.

[21] T. M. Onami, L. E. Harrington, M. A. Williams et al., "Dynamic regulation of T cell immunity by CD43," *Journal of Immunology*, vol. 168, no. 12, pp. 6022–6031, 2002.

[22] A. Varki and T. Angata, "Siglecs—the major subfamily of I-type lectins," *Glycobiology*, vol. 16, no. 1, pp. 1R–27R, 2006.

[23] A. Cazet, S. Julien, M. Bobowski, J. Burchell, and P. Delannoy, "Tumour-associated carbohydrate antigens in breast cancer," *Breast Cancer Research*, vol. 12, article 204, 2010.

[24] E. Dabelsteen, "Cell surface carbohydrates as prognostic markers in human carcinomas," *The Journal of Pathology*, vol. 179, no. 4, pp. 358–369, 1996.

[25] M. A. Hollingsworth and B. J. Swanson, "Mucins in cancer: protection and control of the cell surface," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 45–60, 2004.

[26] Y. J. Kim and A. Varki, "Perspectives on the significance of altered glycosylation of glycoproteins in cancer," *Glycoconjugate Journal*, vol. 14, no. 5, pp. 569–576, 1997.

[27] D. W. Miles, L. C. Happerfield, P. Smith et al., "Expression of sialyl-Tn predicts the effect of adjuvant chemotherapy in node-positive breast cancer," *British Journal of Cancer*, vol. 70, no. 6, pp. 1272–1275, 1994.

[28] J. M. Burchell, A. Mungul, and J. Taylor-Papadimitriou, "O-linked glycosylation in the mammary gland: changes that occur during malignancy," *Journal of Mammary Gland Biology and Neoplasia*, vol. 6, no. 3, pp. 355–364, 2001.

[29] K. O. Lloyd, J. Burchell, V. Kudryashov, B. W. T. Yin, and J. Taylor-Papadimitriou, "Comparison of *O*-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines: demonstration of simpler and fewer glycan chains in tumor cells," *The Journal of Biological Chemistry*, vol. 271, no. 52, pp. 33325–33334, 1996.

[30] P. Buckhaults, L. Chen, N. Fregien, and M. Pierce, "Transcriptional regulation of *N*-acetylglucosaminyltransferase V by the src oncogene," *The Journal of Biological Chemistry*, vol. 272, no. 31, pp. 19575–19581, 1997.

[31] N. Le Marer, V. Laudet, E. C. Svensson et al., "The c-Ha-ras oncogene induces increased expression of β-galactoside α-2,6-sialyltransferase in rat fibroblast (FR3T3) cells," *Glycobiology*, vol. 2, no. 1, pp. 49–56, 1992.

[32] D. H. Joziasse, "Mammalian glycosyltransferases: genomic organization and protein structure," *Glycobiology*, vol. 2, no. 4, pp. 271–277, 1992.

[33] J. C. Paulson, J. Weinstein, E. L. Ujita, K. J. Riggs, and P. H. Lai, "The membrane-binding domain of a rat liver Golgi sialyltransferase," *Biochemical Society Transactions*, vol. 15, no. 4, pp. 618–620, 1987.

[34] E. P. Bennett, H. Hassan, U. Mandel et al., "Cloning and characterization of a close homologue of human UDP-N-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-T3, designated GalNAc-T6. Evidence for genetic but not functional redundancy," *The Journal of Biological Chemistry*, vol. 274, no. 36, pp. 25362–25370, 1999.

[35] S. A. Brooks, T. M. Carter, E. P. Bennett, H. Clausen, and U. Mandel, "Immunolocalisation of members of the polypeptide *N*-acetylgalactosaminyl transferase (ppGalNAc-T) family is consistent with biologically relevant altered cell surface glycosylation in breast cancer," *Acta Histochemica*, vol. 109, no. 4, pp. 273–284, 2007.

[36] T. Freire, N. Berois, C. Sóñora, M. Varangot, E. Barrios, and E. Osinaga, "UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 6 (ppGalNAc-T6) mRNA as a potential new marker for detection of bone marrow-disseminated breast cancer cells," *International Journal of Cancer*, vol. 119, no. 6, pp. 1383–1388, 2006.

[37] M. Nomoto, H. Izumi, T. Ise et al., "Structural basis for the regulation of UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyl transferase-3 gene expression in adenocarcinoma cells," *Cancer Research*, vol. 59, no. 24, pp. 6214–6222, 1999.

[38] H. H. Wandall, S. Dabelsteen, J. A. Sørensen, A. Krogdahl, U. Mandel, and E. Dabelsteen, "Molecular basis for the presence of glycosylated onco-foetal fibronectin in oral carcinomas: the production of glycosylated onco-foetal fibronectin by carcinoma cells," *Oral Oncology*, vol. 43, no. 3, pp. 301–309, 2007.

[39] J. C. Paulson and K. J. Colley, "Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation," *The Journal of Biological Chemistry*, vol. 264, no. 30, pp. 17615–17618, 1989.

[40] M. Hebbar, M.-A. Krzewinski-Recchi, L. Hornez et al., "Prognostic value of tumoral sialyltransferase expression and circulating E-selectin concentrations in node-negative breast cancer patients," *International Journal of Biological Markers*, vol. 18, no. 2, pp. 116–122, 2003.

[41] M.-A. Recchi, M. Hebbar, L. Hornez, A. Harduin-Lepers, J.-P. Peyrat, and P. Delannoy, "Multiplex reverse transcription polymerase chain reaction assessment of sialyltransferase expression in human breast cancer," *Cancer Research*, vol. 58, no. 18, pp. 4066–4070, 1998.

[42] J.-H. Park, T. Nishidate, K. Kijima et al., "Critical roles of mucin 1 glycosylation by transactivated polypeptide *N*-acetylgalactosaminyltransferase 6 in mammary carcinogenesis," *Cancer Research*, vol. 70, no. 7, pp. 2759–2769, 2010.

[43] N. Berois, D. Mazal, L. Ubillos et al., "UDP-*N*-acetyl-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-6 as a new immunohistochemical breast cancer marker," *Journal of Histochemistry & Cytochemistry*, vol. 54, no. 3, pp. 317–328, 2006.

[44] H. Zhang, F. Meng, S. Wu et al., "Engagement of I-branching β-1, 6-*N*-acetylglucosaminyltransferase 2 in breast cancer metastasis and TGF-β signaling," *Cancer Research*, vol. 71, no. 14, pp. 4846–4856, 2011.

[45] C.-P. Chuu, R.-Y. Chen, J. L. Barkinge, M. F. Ciaccio, and R. B. Jones, "Systems-level analysis of ErbB4 signaling in breast cancer: a laboratory to clinical perspective," *Molecular Cancer Research*, vol. 6, no. 6, pp. 885–891, 2008.

[46] W. Remmele and H. E. Stegner, "Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue," *Pathologe*, vol. 8, no. 3, pp. 138–140, 1987.

[47] T. Holbro and N. E. Hynes, "ErbB receptors: directing key signaling networks throughout life," *Annual Review of Pharmacology and Toxicology*, vol. 44, pp. 195–217, 2004.

[48] C. B. Madsen, C. Petersen, K. Lavrsen et al., "Cancer associated aberrant protein O-glycosylation can modify antigen processing and immune response," *PLoS ONE*, vol. 7, no. 11, Article ID e50139, 2012.

[49] T. Y. Kew, J. A. Bell, S. E. Pinder et al., "c-*erb*B-4 protein expression in human breast cancer," *British Journal of Cancer*, vol. 82, no. 6, pp. 1163–1170, 2000.

[50] M. Hollmén, P. Liu, K. Kurppa et al., "Proteolytic processing of ErbB4 in breast cancer," *PLoS ONE*, vol. 7, no. 6, Article ID e39413, 2012.

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Review Article

Hereditary Syndromes Manifesting as Endometrial Carcinoma: How Can Pathological Features Aid Risk Assessment?

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Endometrial carcinoma is the most common gynecological tumor worldwide. It can be the presenting malignancy, acting as the harbinger, of an undiagnosed hereditary syndrome. Up to 50% of females with Lynch syndrome present in this manner. Differentiation between Lynch, Muir-Torre, and Cowden syndromes can at times be challenging due to the overlapping features. Our review emphasizes on the strengths, pitfalls, and limitations of microscopic features as well as immunohistochemical and polymerase chain reaction- (PCR-) based tests used by laboratories to screen for DNA mismatch repair (MMR) and *PTEN* gene mutations in patients to enable a more targeted and cost effective approach in the use of confirmatory gene mutational analysis tests. This is crucial towards initiating timely and appropriate surveillance measures for the patient and affected family members. We also review the evidence postulating on the possible inclusion of uterine serous carcinoma as part of the spectrum of malignancies seen in hereditary breast and ovarian carcinoma syndrome, driven by mutations in *BRCA1/2*.

1. Introduction

Many genetic mutations have been elucidated in the past half century leading to either the discovery or better understanding of hereditary syndromes associated with malignancies in the female genital tract. The discovery of the *BRCA1/2* gene in the early 1990s [1] and subsequent work on gene linkage analysis identified it as the main causative gene in hereditary breast and ovarian carcinoma (HBOC) syndrome [2]. Since then, other mutations in molecular pathways such as DNA mismatch repair (MMR) [3] and *PTEN* [4] have shown to result in syndromes causing endometrial carcinomas, the most common gynecological carcinoma to afflict women worldwide [5].

Gene mutations inherited in a Mendelian fashion have been associated with up to 10% of all malignancies occurring in humans [6]. As such, it is imperative that syndromes are identified in probands who present with malignancies to enable prompt initiation of appropriate counseling and testing for the individual and family to reduce morbidity and mortality amongst these individuals [7]. As some of these syndromes may have overlapping clinical features, clinicians or geneticists can be faced with a few possible differential diagnoses [8] as summarized in Table 1. In this aspect, close collaboration between oncologists, pathologists, and geneticists is necessary to ensure confirmatory genetic testing proceeds in a cost effective and timely manner for the patient and family members [9].

With the advent of immunohistochemical (IHC) markers and molecular testing for specific gene mutations, anatomic pathologists now play a bigger role than ever aiding oncologists and geneticists towards a more directed approach towards confirmatory genetic testing. This is particularly so for proband patients with sentinel tumors as the initial manifestation for any given family. Although risk assessment and predictive tools for various hereditary syndromes exist

Table 1: A summary of the epidemiological, mutational, clinical, and pathological characteristics and features encountered in hereditary syndromes manifesting as endometrial carcinoma. The indicators noted by pathologists to augur a need to notify clinicians on the possible need for referral to a geneticist for further clinical assessment and confirmatory gene mutational testing. Highlighted in the extreme right column are the histological features seen on microscopy and ancillary tests including immunohistochemistry and polymerase chain reaction- (PCR-) based tests such as microsatellite instability analysis and MLH-1 methylation study.

Syndrome	Incidence in general population	Lifetime risk of developing endometrial carcinoma	Most common sentinel tumor in women (%)	Germline gene mutation	Associated malignancies	Indicators to prompt pathologist to alert clinician on the possible need for referral to a geneticist
Lynch syndrome or hereditary nonpolyposis syndrome (HNPCC)	1 in 300 to 1 in 500	40%–60%	Endometrial carcinoma (50%)	*MLH1*, *MSH2*, *MSH6*, *PSM2*, *EPCAM*	Endometrial, ovarian, gastric, breast, intestinal, pancreatic, urinary tract, renal, and bile duct carcinoma	**Microscopic findings**: Mixed carcinoma. Undifferentiated or differentiated carcinomas. Significant peritumoral and/or intratumoral lymphocytic infiltrate. Tumors arising from lower uterine segment. Synchronous endometrial and ovarian carcinomas. **Ancillary investigations**: Immunohistochemistry: Loss of MMR protein staining for MLH1, MSH2, MSH6 or PSM2. Methylation study: Absence of promoter methylation of MLH1 in cases with loss of IHC staining. Microsatellite instability analysis: Instability of >1 (MSI-H) or instability in 1 locus (MSI-L)
Muir-Torre syndrome	Variant of Lynch syndrome with an incidence of 9% among Lynch syndrome patients	20%–60%	Colorectal carcinoma (47%) #if benign lesions also taken into account, the most common sentinel tumor is sebaceous neoplasms (50%)	Similar to Lynch syndrome	Similar to Lynch syndrome	**Microscopic findings**: Sebaceous neoplasm: sebaceous adenoma, sebaceoma, sebaceous carcinoma. (sebaceous hyperplasia not shown to be associated with Muir-Torre syndrome). **Ancillary investigations**: (1) Immunohistochemistry: loss of MIHC staining for MLH1, MSH2, MSH6 or PSM2. (Poor positive predictive value without proper clinical assessment for syndrome).
Cowden syndrome	Estimated at 1 in 200,000 (likely underestimated due to difficulty in identifying such patients)	~28%	Breast carcinoma (48%) #if benign lesions are also taken into account, the most common sentinel tumor are mucocuteneous lesions (~85%)	*PTEN*	Breast, thyroid, and endometrial carcinoma	**Microscopic findings**: Trichilemmommas, particularly if multiple Breast hamartoma, especially if prominent hyalinised stroma. Breast carcinoma with dense hyalinised collagenous stroma. Multiple hamartomatous gastrointestinal polyp. Oesophageal glycogenic acanthosis. Histologic confirmation of radiological suspicion of Lhermitte-Duclos. **Ancillary investigation**: Immunohistochemistry: PTEN IHC loss in trichilemmommas has not been shown to be particularly helpful in identifying cases as it is also commonly lost in sporadic cases as well.

MSI-L, microsatellite low; MSI, microsatellite high; and MMR, mismatch repair genes (i.e., *MLH1*, *MSH2*, *MSH6*, and *PMS2*).

to aid clinician in identifying such patients, some patients fail to fulfill the criteria and are only picked up by pathologists during examination of the tumor tissue specimens.

In this review, we discuss these hereditary endometrial carcinoma syndromes and the important role gynecologists play in identifying at-risk patients as well as in the surveillance of such patients. We further place special emphasis on the role the pathologist plays in terms of appreciating the histological nuances present in tumor tissue using traditional light microscopy as well as the interpretation of newer ancillary investigations performed in the laboratory that may assist clinicians assessing potential patients with an underlying syndrome. We have included HBOC syndrome in this discussion as we wish to highlight the possible association of uterine serous carcinoma with this syndrome. The less common syndromes such as Muir-Torre syndrome and Cowden syndrome are emphasized as these may be missed if clinicians do not actively consider them when assessing patients.

2. DNA Mismatch Repair (MMR)

2.1. Lynch Syndrome

2.1.1. Background. Lynch syndrome (LS), also known as hereditary nonpolyposis colorectal cancer (HPNCC), is an autosomal dominant syndrome [10, 11]. The incidence in the general population is estimated to be between 1 in 300 and 1 in 500 [12]. In LS, mutations in the DNA mismatch repair (MMR) gene result in widely dispersed replication errors or instability in highly repetitive error prone areas found primarily in intronic sequences of the genome, known as microsatellites [13]. Microsatellite instability (MSI) can be seen in patients harboring either germline or somatic DNA MMR gene mutations. LS is a result of germline mutations in the DNA MMR genes *MLH1, MSH2, MSH6, and PSM2* [14]. Nonhereditary somatic mutation is due to promoter hypermethylation of the *MLH1* gene resulting in silencing of the gene causing similar MSI levels in the genome seen in 10% to 25% of sporadic tumors, especially colorectal and endometrial carcinomas [15]. Unlike colorectal carcinomas, somatic mutations in the *BRAF* gene resulting in sporadic cases are far rarer in endometrial carcinomas [16, 17]. Germline deletions in a non DNA MMR gene, *EPCAM*, can result in inactivation of *MSH2* in approximately 1% of LS patients [18]. LS patients are at risk of developing colorectal cancer (80%), endometrial cancer (60%), ovarian cancer (12%), and other malignancies in the stomach, pancreas, upper urinary tract, biliary tract, and small intestines [19].

2.1.2. Endometrial Carcinomas in Lynch Syndrome. Approximately 2% to 6% of all endometrial carcinomas can be attributed to germline mutations in the DNA MMR genes [20, 21]. Up to 50% of female patients with LS will present with endometrial carcinoma as their sentinel tumor [19, 22]. Germline mutation in the *MSH6* gene is associated with the highest risk for developing endometrial carcinomas [23, 24]. Mutations in *MLH1* and *MSH6* genes result in a higher risk of developing colorectal carcinoma [25]. Individuals with germline mutations in *PMS2* have the lowest overall risk of developing LS-associated tumors [26]. The median time for LS patients with endometrial carcinoma to develop a second tumor is estimated to be 11 years [27]. Therefore, identification of proband LS patients with endometrial carcinomas can result in timely and appropriate management to help reduce the potential of a second tumor in the patient or, in the case of her relatives, preventing tumors all together.

2.1.3. Identification of Lynch Syndrome amongst Proband Patients with Endometrial Carcinoma

(1) Clinical Evaluation. LS patients have traditionally been identified by clinical assessment using validated criteria followed by confirmatory gene testing as described in Table 2. Sensitivity and specificity were increased in the 2004 revised Bethesda guidelines but still fell short due to the failure to specify gynecological tumors requiring further testing [28]. Among women with LS presenting with endometrial carcinomas, between 50% and 70% do not meet the Amsterdam or Bethesda guidelines due to the absence of a personal or family history suggestive of LS [20, 29–31]. Mutations in *MSH6* and *PSM2* are more likely to result in failure to meet either of the guidelines [29]. Clinical predictive tools relying on personal and family history such as $\text{PREMM}_{1,2,6}$, MMRpredict, and MMRpro have been developed to quantify the risk of harboring germline DNA MMR mutations in colorectal patients [32]. Risk is determined by calculating the area under the receiver curve (AUC) with a ≥5% cutoff [33]. A large study involving 563 patients with endometrial carcinomas showed the three predictive tools to be having inferior sensitivity and specificity compared with IHC and polymerase chain reaction- (PCR-) based MSI analysis in identifying patients requiring confirmatory germline DNA MMR gene testing [33].

The deficiencies of the Amsterdam and Bethesda guidelines have resulted in the implementation of utilization of IHC and/or MSI analysis on tumor tissue to boost the ability to identify patients with LS. The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) working group recommends IHC and MSA testing to be offered to all newly diagnosed colorectal carcinoma patients as part of the workup to identify all possible LS individuals [34, 35]. Currently, this proposal does not extend to include endometrial carcinoma patients. The Society of Gynecologic Oncologists (SGO) and National Comprehensive Cancer Network (NCCN) have only proposed blanket IHC and/or MSI analysis of women with endometrial carcinoma under the age of 50 years [36]. However, the suggestion to offer universal testing has been advocated as many proband LS patients present with endometrial carcinoma as their sentinel tumor without appropriate family history to trigger IHC and/or MSI analysis testing. Furthermore, the majority of LS patients will present with endometrial carcinoma at 50 years and above [20, 21, 37]. The SGO and NCCN guidelines very likely fail to optimally identify patients with LS and subsequent initiation of appropriate cancer surveillance.

(2) Histological Evaluation. Endometrial carcinomas associated with LS have been shown to exhibit a tendency to occur

Table 2: Amsterdam I and II criteria as well as the Revised Bethesda Guidelines for diagnosis of Lynch syndrome. The Revised Bethesda Guidelines was developed with the intention of identifying individuals who should undergo investigation for Lynch syndrome by evaluation of MSI analysis and/or immunohistochemistry (IHC) testing of their tumors. (Adapted from [43]).

Amsterdam I criteria
(i) Three or more relatives with histologically verified colorectal cancer, one of which is a first-degree relative of the other two. Familial adenomatous polyposis should be excluded.
(ii) Two or more generations with colorectal cancer.
(iii) One or more colorectal cancer cases diagnosed before the age of 50 years.
Amsterdam II criteria
(i) Three or more relatives with histologically verified Lynch syndrome-associated cancer (colorectal cancer, cancer of the endometrium, small bowel, ureter, or renal pelvis), one of which is a first-degree relative of the other two. Familial adenomatous polyposis should be excluded.
(ii) Cancer involving at least two generations.
(iii) One or more cancer cases diagnosed before the age of 50 years.
Revised Bethesda Guidelines
(i) Colorectal carcinoma diagnosed at younger than 50 years.
(ii) Presence of synchronous or metachronous colorectal carcinoma or other Lynch Syndrome-associated tumors[#].
(iii) Colorectal carcinoma with MSI-high pathologic-associated features (Crohn-like lymphocytic reaction, mucinous/signet cell differentiation, or medullary growth pattern) diagnosed in an individual younger than 60 years old.
(iv) Patient with colorectal carcinoma and colorectal carcinoma or Lynch syndrome-associated tumor diagnosed in at least 1 first-degree relative younger than 50 years old.
(v) Patient with colorectal carcinoma and colorectal carcinoma or Lynch syndrome-associated tumor[#] at any age in two first-degree or second-degree relatives.

[#]Lynch syndrome-associated tumors include tumor of the colorectum, endometrium, stomach, ovary, pancreas, ureter, renal pelvis, biliary tract, brain, and small bowel.
Caveat: Muir Torre syndrome is considered a subset of Lynch syndrome with patients also having sebaceous neoplasms and/or keratoacanthomas.

in the lower uterine segment (LUS) with up to third of such tumors attributed to this syndrome [38]. Histologically, LS-associated tumors have a diverse morphological appearance. The most common subtype is endometrioid carcinoma but serous carcinoma, clear cell carcinoma, and carcinosarcoma are also well accounted for [39]. Nonendometrioid carcinomas such as clear cell carcinoma, serous cell carcinoma, and carcinosarcoma are known to occur in LS patients at a younger age than is commonly seen in non-LS patients [40]. There is also a well-documented predisposition for LS-associated tumors to exhibit high grade features with a mixed histology which can at times represent a huge challenge to anatomic pathologists attempting to subtype the tumor components into the various neat categorical variants [41, 42]. Difficulty in separating the various tumor components comprising endometrioid, serous, and/or clear cell carcinomas is not uncommon in such situations [42]. Interestingly, tumors seen arising in the LUS have been shown to occasionally disclose histological and immunohistochemical features which are difficult to ascertain if the tumor is an endometrial or endocervical primary adenocarcinoma [38].

Among tumors with an endometrioid appearance, a few histological features have been shown to suggest the possibility of an underlying MSI. Undifferentiated and dedifferentiated endometrioid carcinomas have been associated with MSI, in particular *MLH1/PMS2* gene mutation, due to either promoter methylation or germline mutation [40, 46, 47]. Undifferentiated endometrioid carcinomas consist of solid sheets of round to polygonal cells with vesicular nuclei and prominent nucleoli without any evidence of gland formation [48]. A tumor is deemed to be dedifferentiated when areas of moderately or even well differentiated endometrioid carcinoma are discernible [48]. Another histological feature often associated with MSI, both in germline mutated or sporadic promoter methylated tumors, is a heavy lymphocytic infiltrate within and around the endometrial carcinomas [46, 49].

Synchronous ovarian and endometrial tumors have also been connected to MSI. The most common pattern is that of endometrioid carcinomas in both the endometrium and ovary [50–52]. However, some patients may exhibit synchronous clear cell or undifferentiated carcinomas [50]. Serous carcinomas are uncommon. Gynecologists practicing in centers conducting universal screening for LS using IHC should consider requesting for the test to be performed when encountering young patients with ovarian cell carcinomas. This is due to a strong association with LS in patients in this age group [50]. This should also be extended to patients with synchronous uterine endometrioid carcinoma and ovarian clear cell carcinoma as rare reports have been documented in MSI patients [47, 50].

(3) Ancillary Laboratory Tests. The current gold standard confirmatory test for LS is the expensive gene mutational analysis of DNA MMR genes [21]. Cost effective and readily available screening tools available in most laboratories are (1) IHC to look for abnormal loss of DNA MMR proteins, (2) MSI analysis by PCR to detect for increased microsatellite

Table 3: Endometrial carcinoma testing result using MSI analysis and/or immunohistochemistry with additional testing strategies for Lynch Syndrome. Additional suggested testing strategies for patients who have been tested using either MSI analysis and/or immunohistochemistry with a four-panel marker (*MHL1, MSH2, MSH6, and PMS2*) or a [#]two-panel marker (MSH6 and PMS2) (adapted from [43]).

MSI analysis	Immunohistochemistry protein expression				Possible causes	Further action
	MLH1	MSH2	MSH6[#]	PMS2[#]		
MSS/MSI-L	+	+	+	+	Sporadic carcinoma	None.
MSI-H	+	+	+	+	Germline mutation in MMR or EPCAM genes	MLH1, MSH2, then MSH6, PMS2, and EPCAM genetic testing
MSI-H	NA	NA	NA	NA	Sporadic or germline mutation in the MMR or EPCAM genes	Consider IHC to guide germline testing if IHC is not done germline testing of MLH1, MSH2, MSH6, PMS2, and EPCAM genes
MSI-H or NA	−	+	+	−	Sporadic cancer or germline mutation of MLH1	MLH1 promoter methylation testing. MLH1 genetic testing if absent hypermethylation or if testing not done
MSI-H or NA	−	+	+	+	Germline mutation MLH1	MLH1 genetic testing
MSI-H or NA	+	+	+	−	Germline mutation of PMS2, rarely MLH1	PMS2 genetic testing if negative MLH1 testing
MSI-H or NA	+	−	−	+	Germline mutation of MSH2 or EPCAM, rarely of MSH6	MSH2 genetic testing, if negative EPCAM, if negative MSH6
MSI-H or NA	+	−	+	+	Germline mutation of MSH2	MSH2 genetic testing if negative EPCAM testing
MSI-H, MSI-L or MSS	+	+	−	+	Germline mutation of MSH6, less likely MSH2	MSH2 genetic testing if negative MSH6 testing

MSI-L, microsatellite low; MSI, microsatellite high; MMR, mismatch repair genes (i.e., *MLH1*, *MSH2*, *MSH6*, and *PMS2*); NA, not available; +, protein expression present in tissue; and −, protein expression not present in tissue.

repeats in specific loci and when required, and (3) *MLH1* promoter methylation also utilizing PCR [21]. The usual initial workflow to identify DNA MMR genes is by IHC for the 4 DNA MMR proteins (*MLH1, PMS2, MSH2, and MSH6*) and/or MSI analysis [53]. Some centers perform both tests on every tumor specimen to maximize detection [20, 22, 54] as described in Table 3. Tumor testing by IHC and/or MSI analysis has been reported to generally detect abnormal DNA MMR protein expression in 15–25% of unselected patients with endometrial carcinomas [20, 33]. Subsequent to this, all MSI patients exhibiting loss of DNA MMR protein *MLH1* expression can be further segregated using *MLH1* gene methylation testing to identify those with as somatic promoter methylation and those most likely to benefit from confirmatory germline mutational analysis [53, 54]. In contrast to patients with colorectal cancers [16], patients with sporadic MSI *MLH1* methylated endometrial carcinomas do not benefit from additional testing for V600E mutation of the *BRAF* gene as less than 1% display this mutation [17, 55].

(a) Immunohistochemistry (IHC). IHC is performed on paraffin embedded tumor tissue. The sensitivity and specificity when using the four DNA MMR protein markers are 91% and 83%, respectively [56]. However, it is important to recognize that IHC will not detect germline mutations where the DNA MMR protein is produced but nonfunctioning as in the case of missense mutations [57].

Normal DNA MMR proteins function as heterodimer complexes by forming pairs of dimers with *MLH1* partnering *PMS2* and *MHS2* pairing up with *MSH6*, with *MLH1* and *MSH2* acting as obligatory partners [58, 59]. Mutation in one of the protein in the pairing results in concurrent loss of staining of its partner protein. Somatic mutation via epigenetic methylation silencing of the *MLH1* promoter gene results in loss of *PMS2* IHC staining. Epigenetic silencing can also occur in the *MSH2* gene following deletions in the *EPCAM* gene leading to loss of IHC staining in the partner protein, *MSH6* [18]. Germline mutations in *MSH6* or *PMS2* genes do not result in loss of IHC staining in its obligatory partner [57, 60]. Individual loss of *PMS2* or *MSH6* IHC staining indicates the possible germline mutations in the respective genes [57, 60]. As such, a two IHC panel utilizing *PMS2* and *MSH6* is feasible [61].

An abnormal IHC staining pattern is where there is a total loss of nuclear staining in tumor cells [10]. Normal staining is seen in lymphocytes, stroma, and normal endometrium and these act as internal positive controls. A common problem arising from normal staining of intratumoral lymphocytes

is mistaking these for tumor cells, resulting in false positive result of normal retention of MSI in the tumor [62–64]. Another common challenge is the difficulty in assessing the heterogeneous staining nature of the *MSH6* IHC marker [63]. Only small areas may exhibit normal retention of nuclear staining. As such, tumor tissue of an adequate size should be selected for IHC staining. When accurate interpretation on a tissue block remains problematic despite repeated testing, an equivocal or inconclusive report will be rendered with the recommendation to consider an alternative test such as MSI analysis.

(b) Polymerase Chain Reaction (PCR). MSI analysis is a PCR test which measures errors in DNA replication caused by loss or abnormal function of the DNA MMR protein. It is performed on paraffin-embedded tumor tissue using either mononucleotides only or a combination of nucleotides and dinucleotides to amplify common sites of instability in the genome [35, 65, 66]. The Bethesda MSI panel consists of two mononucleotides and three dinucleotides (BAT25, BAT26, D2S123, D5S346, and D17S250) [67] and is still widely used despite two well-known pitfalls caused by the dinucleotides [66]. The National Cancer Institute (NCI) panel of five mononucleotide markers (BAT25, BAT26, NR21, NR24, and NR27) in contrast has been shown to be more effective and reproducible [62, 66, 68]. MS-stable (MSS) phenotype tumors will show normal microsatellite repeats as normal tissues [65]. MSI-high (MSI-H) tumors show microsatellite instability in two or more of the tested loci while MSI-low (MSI-L) tumors show instability at one locus [65]. Although MSI-H is seen when germline mutation occurs in any of the four DNA MMR genes, mutations in *MSH6* more frequently results in MSI-L or even MSS status [14, 20, 69, 70]. MSS *MSH6* mutated cases are more commonly seen with the use of the Bethesda panel [69, 70]. One study previously showed 11.8% (12/102) of MSI-H tumors retained normal IHC staining, of which 2/12 of the discordant cases were patients with endometrial carcinomas and a family history of LS [54]. Possible reduced detection rates can occur in centers only relying on IHC. As such, MSI analysis is concurrently used in conjunction with IHC for testing in some centers [54]. MSI analysis is not capable of differentiation between tumors with *MLH1* promoter methylation and germline mutation [65]. Tumors with loss of *MLH1* IHC staining will need to undergo an additional PCR-based test to detect for *MLH1* gene promoter methylation [15, 71]. A patient with a tumor which tests negative with the *MLH1* gene promoter methylation test should be encouraged to undergo germline mutation testing for LS [71].

(c) Confirmatory Gene Mutational Analysis for Lynch Syndrome. Direct gene sequencing using the traditional Sanger sequencing method to uncover mutations in DNA MMR genes is carried out in conjunction with multiplex ligation-dependent probe amplification (MLPA) [20, 21]. MLPA is utilized to detect large genomic rearrangements. MLPA is also used to detect deletions in the *EPCAM* gene, which results in somatic methylation of the *MSH2* gene [72].

Some gene mutational analysis results of the four DNA MMR genes will indicate missense mutations [73], the significance of which is often unknown and are classified under the "variants of uncertain significance" (VUS) category [74]. A certain proportion of patients with IHC and/or MSI analysis results suggestive of LS will have no mutations in *DNA MMR* genes [73]. Current sequencing protocols may not be sufficiently sensitive to identify such mutations which reside deep within the introns or promoter regions of the genes. Unknown novel epigenetic effects have also been postulated as a cause.

2.1.4. Recommended Surveillance. One of the main aims of identifying proband patients with LS is to unearth unsuspecting relatives who are carriers of the deleterious DNA MMR genes. The general expert consensus is that surveillance for the patient and family members with LS is required [54, 75] as highlighted in Table 4.

We concentrate on endometrial surveillance required for the proband's female family members. Female relatives of LS proband patients who are suspected or confirmed to be DNA MMR mutation carriers should be offered the option to undergo surveillance in the effort to prevent endometrial carcinomas. There is some support for the use of transvaginal ultrasound and endometrial biopsy either annually or every 2 years from the age of 30 to 35 [76]. In a recent retrospective study over a 10-year period, gynecologists were the designated physicians to perform surveillance colonoscopy and endometrial curettage under sedation to reduce discomfort, at the same outpatient visit scheduled every 1 to 2 years apart [77]. Endometrial curettage usually provides a large amount of tissue compared to other biopsy methods and is, thus, an added advantage during histological examination. The 55 LS mutation carriers in this study had a combined 111 surveillance visits with 4.5% (5/111) of these visits resulting in abnormal biopsy findings. Four patients had complex hyperplasia and one patient was diagnosed with endometrioid carcinoma, FIGO grade 1 stage 1a. The patient with endometrioid carcinoma and three others with complex hyperplasia did not have thickened endometrium on transvaginal ultrasound to warrant a biopsy. With the findings by Nebgen et al. [77] in mind, it is prudent that if it is decided that no active surveillance is to be carried out, female carriers of the DNA MMR gene mutations must be educated on the need to seek immediate medical attention for further investigation if they have any abnormal uterine bleeding.

2.2. Muir-Torre Syndrome, a Variant of Lynch Syndrome

2.2.1. Background. Muir-Torre syndrome (MTS) is now considered a subtype of LS [78, 79] with an estimated overall frequency of 9.2% among individuals with LS [80]. MTS is mostly due to germline mutations in *MSH2* and *MLH1* [81]. It is characterized by sebaceous gland neoplasms (except sebaceous hyperplasia) and keratoacanthoma with 57% of patients presenting with diagnostic skin lesions as their sentinel pathology [81, 82]. Recognition of MTS is a problem and may stem from patients considering these lesions as insignificant and not disclosing in their medical histories

Table 4: Guidelines for screening at-risk or affected persons with Lynch syndrome. Recommendations are based on the strength of confidence and Grades of Recommendation, Assessment, Development, and Evaluation (GRADE). GRADE is a well-accepted rating of evidence relying on expert consensus about whether new research is likely to change the confidence level (CL) of recommendations (adapted from [43]).

Intervention	Recommendation	Strength of recommendation
Colonoscopy	Every 1 to 2 years beginning at age 20 to 25 or 2 to 5 years younger than youngest age at diagnosis of colorectal carcinoma in family if diagnosis before age 25. Considerations: start at age 30 in MSH6 and age 35 in PMS2 families Annual colonoscopy in MMR mutation carriers	Strong recommendation: Level of evidence (III): well-designed and conducted cohort or case-controlled studies from more than 1 group with cancer #GRADE rating: moderate
Pelvic examination with endometrial sampling	Annually beginning at age 30 to 35	Offer to patient: Level of evidence (V): expert consensus #GRADE rating: low
Transvaginal ultrasound	Annually beginning at age 30 to 35	Offer to patient: Level of evidence (V): expert consensus #GRADE rating: low
Esophagogastroduodenoscopy with biopsy of the gastric antrum	Beginning at age 30 to 35 and subsequent surveillance every 2 to 3 years can be considered based on patient risk factors	Offer to patient: Level of evidence (V): expert consensus #GRADE rating: low
Urinalysis	Annually beginning at age 30 to 35	Consideration: Level of evidence (V): expert consensus #GRADE rating: low

unless specifically asked [83]. It is important to be vigilant for new onset skin lesions in patients with a previous history of endometrial carcinoma. For patients who have never been tested for LS, these skin lesions may indicate the need to testing. In such instances, the patient's original endometrial tumor tissue blocks can be used for IHC or MSI analysis testing.

Endometrial carcinoma is not the most common visceral tumor to be associated with MTS. However, there have been early case reports in the literature clearly documenting endometrial carcinomas presenting as the sentinel tumor in a few patients with MTS [84, 85]. An old meta-analysis study uncovered 120 patients reported in the literature to have had internal malignancies, of whom seven patients were noted to have been diagnosed with endometrial carcinoma [86]. Colorectal carcinoma was shown to afflict almost half of all MTS patients with internal malignancies and a quarter was reported to have genitourinary tract malignancies [86].

In a similar vein to colorectal and endometrial carcinomas, IHC markers have been proposed as part of the work-up to identify patients presenting with sebaceous neoplasms requiring confirmatory DNA MMR gene mutational analysis [83, 87–89]. More recent studies have shown IHC to be less reliable when performed on sebaceous neoplasms that are on colon or endometrial carcinomas [89, 90]. IHC has been shown to have an unacceptably high false-positive rate of 52% with a positive predictive value (PPV) of 22% and negative predictive value (NPV) of 95% [90]. Variable results within individual patients with multiple sebaceous neoplasms have also been demonstrated [90]. Despite the pitfalls, some center may still opt to perform IHC on sebaceous neoplasms [89]. Individuals with germline *MSH6* mutations are associated with higher risk of developing endometrial carcinomas [23, 24] and generally do not conform to the classic LS presentation with personal and family histories of young-onset colorectal cancer [37]. *MSH6* germline mutations are not uncommon in MTS patients [89, 90]. Selected IHC testing prompted by clinical history may potentially result in the missed the opportunity of identifying MTS patients harboring mutations in the *MSH6* gene.

3. PTEN

3.1. Cowden Syndrome

3.1.1. Background. Cowden syndrome (CS) is an autosomal dominant syndrome with incomplete penetrance and variable expressivity characterized by multiple hamartomas, skin lesions, abnormal CNS lesions, and an increased risk of developing carcinomas in the breast, endometrium, thyroid, and genitourinary tract [4, 91]. CS is due to germline mutations in the phosphatase and tensin homologue (*PTEN*) gene located on chromosome 10q23.3 [92]. The incidence of CS in the general population is difficult to ascertain due to the variable and often subtle expression resulting in difficulty in diagnosing proband patients. A clinical epidemiological study utilizing confirmatory *PTEN* gene mutation analysis suggests a prevalence of between 1 in 200,000 and 1 in 250,000 [93] but is likely an underestimation [94]. *PTEN* is a tumor suppressor gene containing 9 exons which encodes for a 403 amino acid protein [94]. The *PTEN* protein plays a role in the *PTEN* pathway by negatively regulating the PI3K/AKT/mTOR pathway by dephosphorylating phosphatidylinositol (3,4,5-)triphosphate (PIP3) to PIP2 [94]. This

results in a decrease in downstream PI3K kinase activity such as phosphoinositide dependent kinase 1 (PDK-1), Akt, mTOR, and ribosomal protein s6 kinase (S6K1) [95–97]. The loss or reduction of *PTEN* activity results in activation by phosphorylation of important cellular proteins in key signaling pathways involved in cell cycle progression, metabolism, translation, growth, migration, invasion, angiogenesis, and apoptosis [95–97].

3.1.2. Endometrial Carcinomas in Cowden Syndrome. Early tumorigenesis from benign endometrium to endometrial intraepithelial neoplasia has been shown to involve the loss of *PTEN* function [98, 99]. It is the most common gene mutation in endometrial carcinomas with approximately 40% reported to harbor *PTEN* gene somatic mutations [98, 99]. In two separate studies, between 16% and 17% of women with endometrial carcinomas who fulfill the clinical diagnostic criteria for CS have been shown to carry the deleterious germline *PTEN* mutations [100, 101].

Endometrial carcinoma is considered as one of the major criteria by NCCN for the diagnosis of CS. The lifetime risk of CS patients with *PTEN* germline mutation developing endometrial carcinomas is between 13% and 19%, which is a substantially higher lifetime risk of the general population estimated to be between 2% and 4% [102–105]. Among female individuals with CS, 12% will present to an oncology department with endometrial carcinoma as their sentinel malignancy [106]. Among female CS patients previously afflicted with a malignancy, 10% were shown represent at a later date with endometrial carcinoma as their second malignancy [106]. It must be stressing that, due to the inherent rarity of CS, the syndrome itself accounts for only a minor proportion of unselected endometrial carcinomas [107]. In a study of 240 unselected endometrial carcinoma patients, no germline mutation of *PTEN* was discovered [107]. In a recent study, CS and CS-like patients with endometrial carcinoma were shown to be linked to mutations other than the *PTEN* gene [108]. In women with CS, the majority of endometrial carcinomas have been noted to occur between the ages of 30 and 50 [101, 109]. However, there have been case reports of endometrial carcinomas occurring in CS patients under the age of 20, which is a highly unusual phenomenon in the general population [110–112]. These tumors have generally been endometrioid carcinomas [110–112] with one case preceded by the finding of an atypical polypoid adenomyoma in the uterine curetting [113].

The majority of reports indicate endometrioid carcinoma to be most common subtype to afflict women with CS [110–113]. However, in a large prospective multicenter study involving 371 CS and CS-like patients with endometrial carcinoma, only 42% were noted to have carcinoma of the endometrioid subtype [108]. The remaining 58% were reported to been diagnosed with a nonendometrioid carcinoma, of which 50% were labeled as endometrial carcinoma, NOS. Serous/clear cell carcinoma (5%), mucinous carcinoma (0.3%), and sarcoma (2.7%) were shown to account for the other malignancies in the nonendometrioid carcinoma group [108]. As all the patients were recruited based on an inclusion criterion of endometrial carcinoma, we assume that the sarcoma (2.7%) stated may in fact be carcinosarcomas [108]. Carcinosarcomas are recognized under the WHO tumor classification system to be of epithelial origin containing at least an area of malignant stromal transformation [48]. Unfortunately, the study was limited by the lack of central pathology review and we are therefore unable to use the reported histology subtypes to help guide clinical risk assessment for germline PTEN mutations. We hope that future studies will address this issue.

In addition to endometrial carcinoma, CS patients are also predisposed to uterine leiomyomata, benign ovarian cysts, and functional menstrual abnormalities [114, 115].

3.1.3. Identification of Individuals with Cowden Syndrome. Clinical criteria remain the bedrock of identifying and diagnosing CS. The latest diagnostic criteria incorporating pathognomonic features with major and minor indications have been outlined by the 2014 NCCN [116]. A suggested workup for suspected individuals can be found in Table 3. Validated pediatric and adult risk calculators to determine the risk of an individual harboring *PTEN* gene mutations have been made freely available online (http://www.lerner.ccf.org/gmi/ccscore/) [101].

3.1.4. Clinicopathological Features of Cowden Syndrome

(1) Other Malignant Manifestations Seen in Individuals with CS. The lifetime risk for breast carcinoma among patients with CS is between 25% and 50% [117]. Among women with CS presenting with breast carcinoma, 48% present with it as their first malignancy and of this group, and 22% are likely to represent with at a later date with a new primary breast carcinoma [106]. Histologically, there is a rare but highly distinctive and striking feature of CS-associated ductal adenocarcinomas seen on microscopy as dense hyalinized collagenous stroma. When present, this distinctive stroma can be seen enveloping the malignant cells [118]. The second most common malignancy among CS patients is thyroid carcinoma, with a reported lifetime risk of between 3 and 10% [119]. Thyroid carcinoma is the initial presenting tumor in 11% of CS patients [106] with an overrepresentation of follicular histology compared to the general populations [119]. Other malignancies such as renal cell carcinoma, melanoma, and colorectal and gastric carcinoma may occur [101, 103, 120, 121]. One study showed colorectal carcinoma to be present in 13% of CS patients, all of whom were <50 years at the time of diagnosis [120].

(2) Benign Entities Seen in Individuals with CS. The most characteristic benign lesion in CS is mucocutaneous hamartomas which can present at birth and, typically, by adulthood [115, 122–124]. Hamartomatous ganglioneuromatous, adenomatous, or hyperplastic polyps involving the gastrointestinal tract simply seen as polyps on endoscopy are another common finding affecting between 35% and 85% of CS patients [114, 120, 125–129]. Other benign lesions are gastritis and the stigmata of CS, esophageal glycogenic acanthosis [120, 128]. Benign thyroid lesions are also a common feature [119, 130, 131]. Meanwhile, CS patients often present with benign

breast lesions ranging from fibrocystic breast disease to a characteristic mammary hamartoma-like lesion with densely hyalinised collagen [118, 132]. These lesions have a propensity to be multiple and bilateral [118]. Rarely, the lesion may be so extensive as to diffusely replace most of the normal breast tissue [118]. Identification of such lesion on histology should prompt the pathologist to inform the breast surgeon to clinically assess the patient for CS. This is especially pertinent if the patient presents with the mammary hamartomatous lesion and has yet to develop breast carcinoma. The pathognomonic lesion of CS is adult Lhermitte-Duclos disease, a slow growing hamartomatous outgrowth of the cerebellum [133, 134].

3.1.5. Ancillary Laboratory Tests

(1) Immunohistochemistry (IHC). To date, only the *PTEN* IHC clone 6H2.1 by Dako has been shown to provide reproducible staining results with good kappa scores among pathologists assessing endometrial carcinomas to determine patients' eligibility for targeted chemotherapy [135–138]. Djordjevic et al. [137] showed *PTEN* protein IHC marker to be lost in 64% endometrial carcinomas, of which only 67% of these were subsequently proven to contain *PTEN* mutations. This study did not determine if the *PTEN* gene mutations identified were somatic or germline [138]. Hence, it can be inferred that *PTEN* protein IHC if used to identify patients requiring confirmatory gene mutational analysis for CS will result in a high level of false positives and, clinically, is not useful for the identification of patients at risk of CS.

(2) Confirmatory Gene Mutational Analysis. The majority of patients meeting the guidelines for the diagnosis of CS have a mutation of the *PTEN* gene (up to 80%) but the figure is lower when patients are referred from the community (approximately 25%) [101]. Germline mutations are seen throughout the 9 exons of the *PTEN* gene but most appear to cluster on exon 5 [139–144]. It has been shown that de novo *PTEN* mutations are found in approximately 40% of proband patients who fulfill the criteria of possibly harboring the *PTEN* gene mutation [145]. Mutations in genes such as *SDBH-D* and *KLLN* which can result in similar disruption to the *PTEN* gene are also seen in some CS patients [108, 119, 146]. These genetic and epigenetic factors can serve as phenotypic modifiers.

3.1.6. Recommended Surveillance and Management. Current recommendations are highlighted in Table 5.

The 2014 NCCN guidelines currently indicate that women with CS aged 30–35 should be considered for annual random endometrial biopsies and/or ultrasound on an individual basis. These women must be educated on the need to respond to promptly symptoms [116]. The statement on the consideration of enrollment in a clinical trial to determine effectiveness and necessity of screening has been omitted [116]. The 2014 NCCN guidelines also suggest that risk-reducing hysterectomy is discussed as an option on a case-by-case basis [116]. However, in view of reports of endometrial carcinoma presenting even in adolescence with CS, any abnormal uterine bleeding should be an indication for an endometrial biopsy in all CS patients.

4. BRCA

4.1. Is Uterine Serous Carcinoma Part of the Spectrum of Hereditary Breast and Ovarian Carcinoma Syndrome?

4.1.1. Background. Women with germline mutations in *BRCA1* and *BRCA2* genes have an increased lifetime risk of developing breast (40%–85%) and ovarian (10–39%) carcinomas [147]. Additionally, germline *BRCA1/2* mutations have also been associated with carcinomas of the fallopian tube, colon, melanoma, and pancreas [148–151].

BRCA1 or *BRCA2* genes play a crucial role in maintaining the integrity of the genome via repair of DNA double stranded breaks using the homologous recombination pathways [152]. *BRCA1* and *BRCA2* proteins link with *RAD51* at damaged DNA and recombination sites [153]. Cells with mutations in either *BRCA* gene result in hypersensitivity to crosslinkage agents such as cisplatin or poly (ADP-ribose) polymerase (PARP) inhibitors which produce double stranded breaks [154–156].

4.1.2. Evidence for Inclusion of Uterine Serous Carcinoma as Part of the Spectrum of Hereditary Breast and Ovarian Carcinoma Syndrome. Uterine serous carcinoma (USC) is a high grade variant of endometrial carcinoma [157, 158]. It accounts for 10% of all endometrial carcinomas [159]. USC is an aggressive disease usually seen in older women with low estrogen states and is usually associated with widespread peritoneal involvement, advanced stage at initial presentation, and, prior to the introduction of platinum therapy, dismal survival rates [159, 160]. It shares similar prognosis and identical histological features with high grade serous carcinomas of the ovary and primary peritoneum [161]. Further evidence suggesting a possible underlying difference in biology special to USC was the finding of a higher incidence of subsequent breast cancer in women with USC compared to endometrioid endometrial carcinoma (25% versus 3.2%, resp., $P < 0.001$) [162].

Although USC is not currently recognized as a feature of any hereditary cancer syndrome, there have been previous speculations on its possible association with HBOC syndrome. It has previously been demonstrated that not only do serous carcinomas of the fallopian tube, uterus, and ovary resemble one another in histology and clinical behavior, comparative genomic hybridization has been shown to have strikingly similar mutations [163]. The endometrial carcinoma genomic characteristics published by the Tumor Cancer Genome Atlas (TCGA) also provide data to suggest a link between USC and serous carcinomas of the ovary [164]. Interestingly, recent evidence suggests most serous cell carcinoma of the ovarian carcinomas very likely arise from the epithelium fallopian tube which has undergone malignant transformation [165, 166]. Further support for this notion is the observation that USC responds to therapeutic agents used for ovarian and peritoneal serous carcinomas [160].

Table 5: Recommendations for diagnostic workup and cancer surveillance in patients with PTEN mutations. (Adapted from [44]).

	Paediatric (<18 years)	Adult female	Adult male
Baseline workup	(i) Targeted history and physical examination (ii) Baseline thyroid ultrasound (iii) Dermatologic examination (iv) Formal neurologic and psychological testing	(i) Targeted history and physical examination (ii) Baseline thyroid ultrasound (iii) Dermatologic examination	(i) Targeted history and physical examination (ii) Baseline thyroid ultrasound (iii) Dermatologic examination
Cancer surveillance			
From diagnosis	(i) Annual thyroid ultrasound (ii) Skin examination	(i) Annual thyroid ultrasound (ii) Skin examination	(i) Annual thyroid ultrasound (ii) Skin examination
From age 30*	As per adult recommendations	(i) Annual mammogram (for consideration of breast MRI instead of mammography if dense breasts) (ii) Annual endometrial sampling or transvaginal ultrasound (or from 5 years before age of earliest endometrial cancer)	
From age 40*	As per adult recommendations	(i) Biannual colonoscopy** (ii) Biannual renal ultrasound/MRI	(i) Biannual colonoscopy** (ii) Biannual renal ultrasound/MRI
Prophylactic surgery	Nil.		

*Surveillance may begin 5 years before the earliest onset of a specific cancer in the family but not later than the recommended age cutoff.
**The presence of multiple nonmalignant polyps in patients with PTEN mutations may complicate noninvasive methods for colon evaluation. More frequent colonoscopy should be considered for patients with a heavy polyp burden.

A case report in 1999 documented two Ashkenazi Jewish sisters, where one sister presented with postmenopausal USC followed by the other sister with ovarian serous carcinoma [167]. Both were later found to harbor one of the *BRCA1* founder mutations. This resulted in the postulation of a possible connection between USC and HBOC syndrome [167]. Following on to this, other studies have gone on to investigate this link with varying results [168–174] with all but one [174] concentrating either mainly or solely on sequencing for the founder mutations found in Ashkenazi Jews. A large Israeli study of 199 endometrial carcinoma Ashkenazi Jewish patients, mostly with endometrioid carcinoma, was negative for *BRCA1/2* mutations [169]. Seventeen patients in this study had been diagnosed with USC. Subsequent to this, four other studies involving only Jewish patients with USC found an increased mutation rate in *BRCA1* between 14% and 27% [170–173] which is significantly higher than the 2.3% mutation rate in Israeli population [175]. Between 50% and 100% of these patients with *BRCA1/2* mutations either had a personal history of breast carcinoma or at least a first degree relative with breast carcinoma or ovarian carcinoma [170–173].

However, a Canadian study involving 56 non-Jewish women with USC failed to detect any of the four commonest *BRCA1* or *BRCA2* mutations, of which three are founder mutations in Ashkenazi Jews [168]. A recent study involving 151 non-Jewish patients with USC found the frequency of *BRCA1* germline mutations to be 2% compared to the general non-Ashkenazi American population of 0.06% [174, 176]. A comprehensive search for all classes of *BRCA1/2* mutations was performed using the BROCA panel. Interestingly, 36 other patients had nondeleterious or variants of unknown significant mutations, 12 of which are reported as benign on the Breast Cancer Information Core database (BIC). It would be important that as longitudinal studies of patients with BRCA mutations mature it will help clarify if USC is truly a *BRCA*-related tumor.

4.1.3. Recommended Surveillance. Current surveillance guidelines are described in Table 6.

Risk reducing surgeries have led to cancer specific survival benefit as well as a reduction in all causes of mortality [177] but remain unclear if patients electing for risk reducing salpingo-oophorectomy should have concurrent opportunistic hysterectomy. However, in view of the rarity of USC as a whole, the absolute risk of developing it is likely to be small. It does not seem warranted at this point to recommend for women to undergo hysterectomy as part of primary prevention of BRCA-associated USC. However, other more crucial issues as indicated by the recent findings in the GOG-0199 study suggest postmenopausal age, abnormal transvaginal ovarian ultrasound findings and elevated serum CA-125 are associated with elevated risk of harboring invasive

Table 6: Recommended surveillance and management of individuals with hereditary breast and ovarian carcinoma syndrome family members (from [45]).

Women
(i) Breast awareness (periodic and consistent breast self-exam) starting at age 18.
(ii) Clinical breast exam, every 6 to 12 months, starting at age 25.
(iii) Breast screening
(a) Age 25–29, annual MRI screening (preferred) or mammogram if MRI is unavailable based on earliest age of onset in family.
(b) Age >30 to 75, annual mammogram and breast MRI screening.
(c) Age >75, management should be considered on an individual basis.
(iv) Discuss the option of risk reducing mastectomy
(a) Counseling may include a discussion regarding degree of protection, reconstruction options, and risk.
(v) Recommend risk-reducing salpingo-oophorectomy, ideally between 35 and 40 years of age and upon completion of child bearing or individualized based on earliest age of onset of ovarian carcinoma in the family.
(a) Counseling includes a discussion of reproductive desires, extent of cancer risk, degree of protection for breast and ovarian cancer, management of menopausal symptoms, possible short term hormone replacement therapy (HRT) to recommend maximum age of natural menopause, and related medical issues.
(vi) Address psychological, social, and quality-of-life aspects of undergoing risk-reducing mastectomy and/or salpingo-oophorectomy.
(vii) For those patients who have not elected risk-reducing salpingo-oophorectomy, consider transvaginal ultrasound (preferably day 1 to day 10 of menstrual cycle in premenopausal women) and CA-125 (preferably after day 5 of menstrual cycle in premenopausal women), every 6 months starting at age 30 or 5 to 10 years before earliest age of first diagnosis of ovarian cancer in the family.
(viii) Consider chemoprevention options for breast and ovarian cancer, including risks and benefits.
(ix) Consider investigational imaging and screening studies, when available (e.g., novel imaging technologies and more frequent screening intervals) in the context of a clinical trial.
Men
(i) Breast self-exam training and education starting at age 35.
(ii) Clinical breast exam every 6 to 12 months, starting at age 25.
(iii) Consider baseline mammogram at age 40; annual mammogram if gynaecomastia or parenchyma/glandular breast density on baseline study.
(iv) Starting at age 40:
(a) Recommend prostate cancer screening for BRCA2 carriers.
(b) Consider prostate cancer screening for BRCA1 carriers.
Men and women
(i) Education regarding signs and symptoms of cancer(s), especially those associated with BRCA gene mutations.
Risk to relatives
(i) Advise about possible inherited cancer risk to relatives, options for risk assessment, and management.
(ii) Recommend genetic counseling and consideration of genetic testing for at-risk relatives.
Reproductive options
(i) For couples expressing the desire that their offspring not carry a familial BRCA mutation, advise about options for prenatal diagnosis and assisted reproduction, including preimplantation genetic diagnosis. Discussion should include known risks, limitations, and benefits of these technologies.
(ii) For BRCA2 mutation carriers, there is a risk of a rare (recessive) Fanconi anaemia/brain tumor phenotypes in offspring if both partners carry a BRCA2 mutation should be discussed.

serous carcinoma in fallopian tube, ovary, and peritoneum or serous tubal intraepithelial carcinoma (STIC) in the fallopian tube of asymptomatic patients with deleterious *BRCA1/2* gene mutations [177]. Not to be taken lightly is also the host of psychological issues faced by such patients [178]. These need to be taken into account when arriving at a clinical decision if such women should be advised to have a hysterectomy and possibly bilateral salpingo-oophorectomy.

5. Conclusion

Endometrial carcinomas can be the first presentation of an underlying hereditary cancer syndrome. Endometrial carcinoma can arise in patients with LS and in lesser known conditions such as MTS, CS, and possibly HBOC. Clinicians and pathologists alike play vital roles in identifying who may require genetic testing by better understanding the associated

malignant and nonmalignant features of these conditions and the pitfalls of existing diagnostic tests. To better understand how best to screen these high-risk patients for endometrial carcinomas, we will need further research.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] J. M. Hall, M. K. Lee, B. Newman et al., "Linkage of early-onset familial breast cancer to chromosome 17q21," *Science*, vol. 250, no. 4988, pp. 1684–1689, 1990.

[2] L. S. Friedman, E. A. Ostermeyer, C. I. Szabo et al., "Confirmation of *BRCA1* by analysis of germline mutations linked to breast and ovarian cancer in ten families," *Nature Genetics*, vol. 8, no. 4, pp. 399–404, 1994.

[3] Y. Ionov, M. A. Peinado, S. Malkhosyan, D. Shibata, and M. Perucho, "Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis," *Nature*, vol. 363, no. 6429, pp. 558–561, 1993.

[4] M. R. Nelen, G. W. Padberg, E. A. J. Peeters et al., "Localization of the gene for Cowden disease to chromosome 10q22-23," *Nature Genetics*, vol. 13, no. 1, pp. 114–116, 1996.

[5] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA: A Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[6] R. Nagy, K. Sweet, and C. Eng, "Highly penetrant hereditary cancer syndromes," *Oncogene*, vol. 23, no. 38, pp. 6445–6470, 2004.

[7] Z. Ketabi, A.-M. Gerdes, B. Mosgaard, S. Ladelund, and I. Bernstein, "The results of gynecologic surveillance in families with hereditary nonpolyposis colorectal cancer," *Gynecologic Oncology*, vol. 133, no. 3, pp. 526–530, 2014.

[8] J. L. Mester, R. A. Moore, and C. Eng, "PTEN germline mutations in patients initially tested for other hereditary cancer syndromes: would use of risk assessment tools reduce genetic testing?" *Oncologist*, vol. 18, no. 10, pp. 1083–1090, 2013.

[9] J. M. Lancaster, C. B. Powell, L. M. Chen, and D. L. Richardson, "Statement on risk assessment for inherited gynecologic cancer predispositions," *Gynecologic Oncology*, 2014.

[10] W. Kohlmann and S. B. Gruber, "Lynch syndrome," in *GeneReviews(R)*, R. A. Pagon, M. P. Adam, H. H. Ardinger et al., Eds., University of Washington, Seattle University of Washington, Seattle, Wash, USA, 1993.

[11] H. T. Lynch and T. C. Smyrk, "Hereditary colorectal cancer," *Seminars in Oncology*, vol. 26, no. 5, pp. 478–484, 1999.

[12] C. R. Boland and M. Shike, "Report from the Jerusalem workshop on Lynch syndrome-hereditary nonpolyposis colorectal cancer," *Gastroenterology*, vol. 138, no. 7, pp. 2197.e1–2197.e7, 2010.

[13] L. A. Loeb, "Microsatellite instability: marker of a mutator phenotype in cancer," *Cancer Research*, vol. 54, no. 19, pp. 5059–5063, 1994.

[14] H. F. A. Vasen, Y. Hendriks, A. E. de Jong et al., "Identification of HNPCC by molecular analysis of colorectal and endometrial tumors," *Disease Markers*, vol. 20, no. 4-5, pp. 207–213, 2004.

[15] S. B. Simpkins, T. Bocker, E. M. Swisher et al., "MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers," *Human Molecular Genetics*, vol. 8, no. 4, pp. 661–666, 1999.

[16] M. B. Loughrey, P. M. Waring, A. Tan et al., "Incorporation of somatic BRAF mutation testing into an algorithm for the investigation of hereditary non-polyposis colorectal cancer," *Familial Cancer*, vol. 6, no. 3, pp. 301–310, 2007.

[17] L. M. Peterson, B. R. Kipp, K. C. Halling et al., "Molecular characterization of endometrial cancer: a correlative study assessing microsatellite instability, MLH1 hypermethylation, DNA mismatch repair protein expression, and PTEN, PIK3CA, KRAS, and BRAF mutation analysis," *International Journal of Gynecological Pathology*, vol. 31, no. 3, pp. 195–205, 2012.

[18] R. P. Kuiper, L. E. L. M. Vissers, R. Venkatachalam et al., "Recurrence and variability of germline EPCAM deletions in Lynch syndrome," *Human Mutation*, vol. 32, no. 4, pp. 407–414, 2011.

[19] M. Aarnio, R. Sankila, E. Pukkala et al., "Cancer risk in mutation carriers of DNA-mismatch-repair genes," *International Journal of Cancer*, vol. 81, no. 2, pp. 214–218, 1999.

[20] H. Hampel, W. Frankel, J. Panescu et al., "Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients," *Cancer Research*, vol. 66, no. 15, pp. 7810–7817, 2006.

[21] C. H. M. Leenen, M. G. F. Van Lier, H. C. Van Doorn et al., "Prospective evaluation of molecular screening for Lynch syndrome in patients with endometrial cancer ≤ 70 years," *Gynecologic Oncology*, vol. 125, no. 2, pp. 414–420, 2012.

[22] K. H. Lu, M. Dinh, W. Kohlmann et al., "Gynecologic cancer as a "sentinel cancer" for women with hereditary nonpolyposis colorectal cancer syndrome," *Obstetrics and Gynecology*, vol. 105, no. 3, pp. 569–574, 2005.

[23] M. Miyaki, M. Konishi, K. Tanaka et al., "Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer," *Nature Genetics*, vol. 17, no. 3, pp. 271–272, 1997.

[24] J. Wijnen, W. de Leeuw, H. Vasen et al., "Familial endometrial cancer in female carriers of MSH6 germline mutations," *Nature Genetics*, vol. 23, no. 2, pp. 142–144, 1999.

[25] J. G. Dowty, A. K. Win, D. D. Buchanan et al., "Cancer risks for MLH1 and MSH2 mutation carriers," *Human Mutation*, vol. 34, no. 3, pp. 490–497, 2013.

[26] L. Senter, M. Clendenning, K. Sotamaa et al., "The clinical phenotype of Lynch syndrome due to germ-line *PMS2* mutations," *Gastroenterology*, vol. 135, no. 2, pp. 419.e1–428.e1, 2008.

[27] A. K. Win, N. M. Lindor, I. Winship et al., "Risks of colorectal and other cancers after endometrial cancer for women with lynch syndrome," *Journal of the National Cancer Institute*, vol. 105, no. 4, pp. 274–279, 2013.

[28] A. Umar, C. R. Boland, J. P. Terdiman et al., "Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability," *Journal of the National Cancer Institute*, vol. 96, no. 4, pp. 261–268, 2004.

[29] W. Sjursen, B. I. Haukanes, E. M. Grindedal et al., "Current clinical criteria for Lynch syndrome are not sensitive enough to identify MSH6 mutation carriers," *Journal of Medical Genetics*, vol. 47, no. 9, pp. 579–585, 2010.

[30] J. C. Strafford, "Genetic testing for lynch syndrome, an inherited cancer of the bowel, endometrium, and ovary," *Reviews in Obstetrics & Gynecology*, vol. 5, no. 1, pp. 42–49, 2012.

[31] C. S. Walsh, A. Blum, A. Walts et al., "Lynch syndrome among gynecologic oncology patients meeting Bethesda guidelines for screening," *Gynecologic Oncology*, vol. 116, no. 3, pp. 516–521, 2010.

[32] C. J. Pouchet, N. Wong, G. Chong et al., "A comparison of models used to predict MLH1, MSH2 and MSH6 mutation carriers," *Annals of Oncology*, vol. 20, no. 4, pp. 681–688, 2009.

[33] R. C. Mercado, H. Hampel, F. Kastrinos et al., "Performance of $PREMM_{1,2,6}$, MMRpredict, and MMRpro in detecting Lynch syndrome among endometrial cancer cases," *Genetics in Medicine*, vol. 14, no. 7, pp. 670–680, 2012.

[34] A. O. Berg, K. Armstrong, J. Botkin et al., "Recommendations from the EGAPP Working Group: genetic testing strategies in newly diagnosed individuals with colorectal cancer aimed at reducing morbidity and mortality from Lynch syndrome in relatives," *Genetics in Medicine*, vol. 11, no. 1, pp. 35–41, 2009.

[35] G. E. Palomaki, M. R. McClain, S. Melillo, H. L. Hampel, and S. N. Thibodeau, "EGAPP supplementary evidence review: DNA testing strategies aimed at reducing morbidity and mortality from Lynch syndrome," *Genetics in Medicine*, vol. 11, no. 1, pp. 42–65, 2009.

[36] J. M. Lancaster, C. Bethan Powell, N. D. Kauff et al., "Society of Gynecologic Oncologists Education Committee statement on risk assessment for inherited gynecologic cancer predispositions," *Gynecologic Oncology*, vol. 107, no. 2, pp. 159–162, 2007.

[37] V. Bonadona, B. Bonaïti, S. Olschwang et al., "Cancer risks associated with germline mutations in *MLH1*, *MSH2*, and *MSH6* genes in lynch syndrome," *The Journal of the American Medical Association*, vol. 305, no. 22, pp. 2304–2310, 2011.

[38] S. N. Westin, R. A. Lacour, D. L. Urbauer et al., "Carcinoma of the lower uterine segment: a newly described association with Lynch syndrome," *Journal of Clinical Oncology*, vol. 26, no. 36, pp. 5965–5971, 2008.

[39] R. R. Broaddus, H. T. Lynch, L.-M. Chen et al., "Pathologic features of endometrial carcinoma associated with HNPCC: a comparison with sporadic endometrial carcinoma," *Cancer*, vol. 106, no. 1, pp. 87–94, 2006.

[40] M. L. Carcangiu, P. Radice, P. Casalini, L. Bertario, M. Merola, and P. Sala, "Lynch syndrome-related endometrial carcinomas show a high frequency of nonendometrioid types and of high FIGO grade endometrioid types," *International Journal of Surgical Pathology*, vol. 18, no. 1, pp. 21–26, 2010.

[41] L. J. Tafe, K. Garg, I. Chew, C. Tornos, and R. A. Soslow, "Endometrial and ovarian carcinomas with undifferentiated components: clinically aggressive and frequently underrecognized neoplasms," *Modern Pathology*, vol. 23, no. 6, pp. 781–789, 2010.

[42] R. A. Soslow, "Endometrial carcinomas with ambiguous features," *Seminars in Diagnostic Pathology*, vol. 27, no. 4, pp. 261–273, 2010.

[43] F. M. Giardiello, J. I. Allen, J. E. Axilbund et al., "Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-society Task Force on colorectal cancer," *The American Journal of Gastroenterology*, vol. 109, no. 8, pp. 1159–1179, 2014.

[44] M. H. Tan, J. L. Mester, J. Ngeow, L. A. Rybicki, M. S. Orloff, and C. Eng, "Lifetime cancer risks in individuals with germline PTEN mutations," *Clinical Cancer Research*, vol. 18, no. 2, pp. 400–407, 2012.

[45] *National Comprehensive Cancer Network (NCCN) Guidelines*, v.1, 2014.

[46] K. Garg, M. M. Leitao Jr., N. D. Kauff et al., "Selection of endometrial carcinomas for DNA mismatch repair protein immunohistochemistry using patient age and tumor morphology enhances detection of mismatch repair abnormalities," *American Journal of Surgical Pathology*, vol. 33, no. 6, pp. 925–933, 2009.

[47] K. Garg, K. Shih, R. Barakat, Q. Zhou, A. Iasonos, and R. A. Soslow, "Endometrial carcinomas in women aged 40 years and younger: tumors associated with loss of DNA mismatch repair proteins comprise a distinct clinicopathologic subset," *The American Journal of Surgical Pathology*, vol. 33, no. 12, pp. 1869–1877, 2009.

[48] R. Zaino, S. G. Carinelli, L. H. Ellenson et al., "Epithelial tumours and precursors," in *WHO Classification of Tumours of the Female Reproductive Organs*, R. J. Kurman, M. L. Carcangiu, C. S. Herrington, and R. H. Young, Eds., pp. 125–135, International Agency for Research on Cancer (IARC), Lyon, France, 2014.

[49] J. Shia, D. Black, A. J. Hummer, J. Boyd, and R. A. Soslow, "Routinely assessed morphological features correlate with microsatellite instability status in endometrial cancer," *Human Pathology*, vol. 39, no. 1, pp. 116–125, 2008.

[50] K. C. Jensen, M. R. Mariappan, G. V. Putcha et al., "Microsatellite instability and mismatch repair protein defects in ovarian epithelial neoplasms in patients 50 years of age and younger," *American Journal of Surgical Pathology*, vol. 32, no. 7, pp. 1029–1037, 2008.

[51] Z. Ketabi, K. Bartuma, I. Bernstein et al., "Ovarian cancer linked to lynch syndrome typically presents as early-onset, non-serous epithelial tumors," *Gynecologic Oncology*, vol. 121, no. 3, pp. 462–465, 2011.

[52] A. S. Bats, H. Roussel, C. Narjoz et al., "Microsatellite instability analysis for the screening of synchronous endometrial and ovarian cancer in lynch syndrome," *Anticancer Research*, vol. 33, no. 9, pp. 3977–3981, 2013.

[53] K. Resnick, J. M. Straughn Jr., F. Backes, H. Hampel, K. S. Matthews, and D. E. Cohn, "Lynch syndrome screening strategies among newly diagnosed endometrial cancer patients," *Obstetrics and Gynecology*, vol. 114, no. 3, pp. 530–536, 2009.

[54] A. N. Bartley, R. Luthra, D. S. Saraiya, D. L. Urbauer, and R. R. Broaddus, "Identification of cancer patients with lynch syndrome: clinically significant discordances and problems in tissue-based mismatch repair testing," *Cancer Prevention Research*, vol. 5, no. 2, pp. 320–327, 2012.

[55] M. Kawaguchi, M. Yanokura, K. Banno et al., "Analysis of a correlation between the BRAF V600E mutation and abnormal DNA mismatch repair in patients with sporadic endometrial cancer," *International Journal of Oncology*, vol. 34, no. 6, pp. 1541–1547, 2009.

[56] I. Modica, R. A. Soslow, D. Black, C. Tornos, N. Kauff, and J. Shia, "Utility of immunohistochemistry in predicting microsatellite instability in endometrial carcinoma," *The American Journal of Surgical Pathology*, vol. 31, no. 5, pp. 744–751, 2007.

[57] M. J. W. Berends, Y. Wu, R. H. Sijmons et al., "Molecular and clinical characteristics of *MSH6* variants: an analysis of 25 index carriers of a germline variant," *The American Journal of Human Genetics*, vol. 70, no. 1, pp. 26–37, 2002.

[58] P. Peltomäki, "Role of DNA mismatch repair defects in the pathogenesis of human cancer," *Journal of Clinical Oncology*, vol. 21, no. 6, pp. 1174–1179, 2003.

[59] A. M. Bellizzi and W. L. Frankel, "Colorectal cancer due to deficiency in DNA mismatch repair function: a review,"

Advances in Anatomic Pathology, vol. 16, no. 6, pp. 405–417, 2009.

[60] S. Gill, N. M. Lindor, L. J. Burgart et al., "Isolated loss of PMS2 expression in colorectal cancers: frequency, patient age, and familial aggregation," *Clinical Cancer Research*, vol. 11, no. 18, pp. 6466–6471, 2005.

[61] J. Moline, H. Mahdi, B. Yang et al., "Implementation of tumor testing for lynch syndrome in endometrial cancers at a large academic medical center," *Gynecologic Oncology*, vol. 130, no. 1, pp. 121–126, 2013.

[62] A. Müller, G. Giuffre, T. B. Edmonston et al., "Challenges and pitfalls in HNPCC screening by microsatellite analysis and immunohistochemistry," *The Journal of Molecular Diagnostics*, vol. 6, no. 4, pp. 308–315, 2004.

[63] A. K. Folkins and T. A. Longacre, "Hereditary gynaecological malignancies: advances in screening and treatment," *Histopathology*, vol. 62, no. 1, pp. 2–30, 2013.

[64] K. Garg and R. A. Soslow, "Endometrial carcinoma in women aged 40 years and younger," *Archives of Pathology & Laboratory Medicine*, vol. 138, no. 3, pp. 335–342, 2014.

[65] C. R. Boland, S. N. Thibodeau, S. R. Hamilton et al., "A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer," *Cancer Research*, vol. 58, no. 22, pp. 5248–5257, 1998.

[66] O. Buhard, N. Suraweera, A. Lectard, A. Duval, and R. Hamelin, "Quasimonomorphic mononucleotide repeats for high-level microsatellite instability analysis," *Disease Markers*, vol. 20, no. 4-5, pp. 251–257, 2004.

[67] M. A. Rodriguez-Bigas, C. R. Boland, S. R. Hamilton et al., "A National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome: meeting highlights and Bethesda guidelines," *Journal of the National Cancer Institute*, vol. 89, no. 23, pp. 1758–1762, 1997.

[68] N. Suraweera, A. Duval, M. Reperant et al., "Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex PCR," *Gastroenterology*, vol. 123, no. 6, pp. 1804–1811, 2002.

[69] J.-F. You, O. Buhard, M. J. L. Ligtenberg et al., "Tumours with loss of MSH6 expression are MSI-H when screened with a pentaplex of five mononucleotide repeats," *British Journal of Cancer*, vol. 103, no. 12, pp. 1840–1845, 2010.

[70] L. Zhang, "Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome: part II. The utility of microsatellite instability testing," *The Journal of Molecular Diagnostics*, vol. 10, no. 4, pp. 301–307, 2008.

[71] F. M. Giardiello, J. I. Allen, J. E. Axilbund et al., "Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US multi-society task force on colorectal cancer," *Gastroenterology*, vol. 147, no. 2, pp. 502–526, 2014.

[72] M. E. Kovacs, J. Papp, Z. Szentirmay, S. Otto, and E. Olah, "Deletions removing the last exon of TACSTD1 constitute a distinct class of mutations predisposing to lynch syndrome," *Human Mutation*, vol. 30, no. 2, pp. 197–203, 2009.

[73] M. S. Daniels, "Genetic testing by cancer site: uterus," *Cancer Journal*, vol. 18, no. 4, pp. 338–342, 2012.

[74] R. H. Sijmons, M. S. Greenblatt, and M. Genuardi, "Gene variants of unknown clinical significance in Lynch syndrome. An introduction for clinicians," *Familial Cancer*, vol. 12, no. 2, pp. 181–187, 2013.

[75] H. Hampel, "NCCN increases the emphasis on genetic/familial high-risk assessment in colorectal cancer," *Journal of the National Comprehensive Cancer Network*, vol. 12, no. 5, supplement, pp. 829–831, 2014.

[76] L. H. Gerritzen, N. Hoogerbrugge, A. L. M. Oei et al., "Improvement of endometrial biopsy over transvaginal ultrasound alone for endometrial surveillance in women with Lynch syndrome," *Familial Cancer*, vol. 8, no. 4, pp. 391–397, 2009.

[77] D. R. Nebgen, K. H. Lu, S. Rimes et al., "Combined colonoscopy and endometrial biopsy cancer screening results in women with Lynch syndrome," vol. 135, no. 1, pp. 85–89, 2014.

[78] H. T. Lynch, P. M. Lynch, J. Pester, and R. M. Fusaro, "The cancer family syndrome. Rare cutaneous phenotypic linkage of Torre's syndrome," *Archives of Internal Medicine*, vol. 141, no. 5, pp. 607–611, 1981.

[79] R. Kruse, A. Rütten, C. Lamberti et al., "Muir-Torre phenotype has a frequency of DNA mismatch-repair-gene mutations similar to that in hereditary nonpolyposis colorectal cancer families defined by the Amsterdam criteria," *The American Journal of Human Genetics*, vol. 63, no. 1, pp. 63–70, 1998.

[80] C. D. South, H. Hampel, I. Comeras, J. A. Westman, W. L. Frankel, and A. de la Chapelle, "The frequency of Muir-Torre syndrome among Lynch syndrome families," *Journal of the National Cancer Institute*, vol. 100, no. 4, pp. 277–281, 2008.

[81] G. Ponti and M. P. de Leon, "Muir-Torre syndrome," *The Lancet Oncology*, vol. 6, no. 12, pp. 980–987, 2005.

[82] S. C. Shalin, S. Lyle, E. Calonje, and A. J. F. Lazar, "Sebaceous neoplasia and the Muir-Torre syndrome: important connections with clinical implications," *Histopathology*, vol. 56, no. 1, pp. 133–147, 2010.

[83] O. Abbas and M. Mahalingam, "Cutaneous sebaceous neoplasms as markers of Muir-Torre syndrome: a diagnostic algorithm," *Journal of Cutaneous Pathology*, vol. 36, no. 6, pp. 613–619, 2009.

[84] H. T. Lynch, W. A. Bardawil, R. E. Harris, P. M. Lynch, H. A. Guirgis, and J. F. Lynch, "Multiple primary cancers and prolonged survival: familial colonic and endometrial cancers," *Diseases of the Colon & Rectum*, vol. 21, no. 3, pp. 165–168, 1978.

[85] T. Tohya, T. Ogura, K. Nishi, H. Nishi, and K. Kuriwaki, "Muir-Torre syndrome associated with endometrial carcinoma," *International Journal of Clinical Oncology*, vol. 13, no. 6, pp. 559–561, 2008.

[86] P. R. Cohen, S. R. Kohn, and R. Kurzrock, "Association of sebaceous gland tumors and internal malignancy: the Muir-Torre syndrome," *The American Journal of Medicine*, vol. 90, no. 5, pp. 606–613, 1991.

[87] B. Jones, C. Oh, E. Mangold, and C. A. Egan, "Muir-Torre syndrome: diagnostic and screening guidelines," *Australasian Journal of Dermatology*, vol. 47, no. 4, pp. 266–269, 2006.

[88] A. Mojtahed, I. Schrijver, J. M. Ford, T. A. Longacre, and R. K. Pai, "A two-antibody mismatch repair protein immunohistochemistry screening approach for colorectal carcinomas, skin sebaceous tumors, and gynecologic tract carcinomas," *Modern Pathology*, vol. 24, no. 7, pp. 1004–1014, 2011.

[89] J. N. Everett, V. M. Raymond, M. Dandapani et al., "Screening for germline mismatch repair mutations following diagnosis of sebaceous neoplasm," *JAMA Dermatology*, 2014.

[90] M. E. Roberts, D. L. Riegert-Johnson, B. C. Thomas et al., "Screening for Muir-Torre syndrome using mismatch repair

protein immunohistochemistry of sebaceous neoplasms," *Journal of Genetic Counseling*, vol. 22, no. 3, pp. 393–405, 2013.

[91] M. A. Scheper, N. G. Nikitakis, E. Sarlani, J. J. Sauk, and T. F. Meiller, "Cowden syndrome: report of a case with immunohistochemical analysis and review of the literature," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*, vol. 101, no. 5, pp. 625–631, 2006.

[92] D.-M. Li and H. Sun, "TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β," *Cancer Research*, vol. 57, no. 11, pp. 2124–2129, 1997.

[93] M. R. Nelen, H. Kremer, I. B. M. Konings et al., "Novel PTEN mutations in patients with Cowden disease: absence of clear genotype-phenotype correlations," *European Journal of Human Genetics*, vol. 7, no. 3, pp. 267–273, 1999.

[94] R. Pilarski and C. Eng, "Will the real Cowden syndrome please stand up (again)? Expanding mutational and clinical spectra of the *PTEN* hamartoma tumour syndrome," *Journal of Medical Genetics*, vol. 41, no. 5, pp. 323–326, 2004.

[95] J. Huang and C. D. Kontos, "PTEN modulates vascular endothelial growth factor-mediated signaling and angiogenic effects," *The Journal of Biological Chemistry*, vol. 277, no. 13, pp. 10760–10766, 2002.

[96] K. A. Waite and C. Eng, "Protean *PTEN*: form and function," *The American Journal of Human Genetics*, vol. 70, no. 4, pp. 829–844, 2002.

[97] J. LoPiccolo, M. S. Ballas, and P. A. Dennis, "PTEN hamartomatous tumor syndromes (PHTS): rare syndromes with great relevance to common cancers and targeted drug development," *Critical Reviews in Oncology/Hematology*, vol. 63, no. 3, pp. 203–214, 2007.

[98] G. L. Mutter, "Histopathology of genetically defined endometrial precancers," *International Journal of Gynecological Pathology*, vol. 19, no. 4, pp. 301–309, 2000.

[99] G. L. Mutter, M.-C. Lin, J. T. Fitzgerald, J. B. Kum, and C. Eng, "Changes in endometrial PTEN expression throughout the human menstrual cycle," *Journal of Clinical Endocrinology and Metabolism*, vol. 85, no. 6, pp. 2334–2338, 2000.

[100] R. Pilarski, J. A. Stephens, R. Noss, J. L. Fisher, and T. W. Prior, "Predicting *PTEN* mutations: an evaluation of cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome clinical features," *Journal of Medical Genetics*, vol. 48, no. 8, pp. 505–512, 2011.

[101] M.-H. Tan, J. Mester, C. Peterson et al., "A clinical scoring system for selection of patients for pten mutation testing is proposed on the basis of a prospective study of 3042 probands," *The American Journal of Human Genetics*, vol. 88, no. 1, pp. 42–56, 2011.

[102] D. L. Riegert-Johnson, F. C. Gleeson, M. Roberts et al., "Cancer and Lhermitte-Duclos disease are common in Cowden syndrome patients," *Hereditary Cancer in Clinical Practice*, vol. 8, no. 1, article 6, 2010.

[103] M.-H. Tan, J. L. Mester, J. Ngeow, L. A. Rybicki, M. S. Orloff, and C. Eng, "Lifetime cancer risks in individuals with germline PTEN mutations," *Clinical Cancer Research*, vol. 18, no. 2, pp. 400–407, 2012.

[104] V. Bubien, F. Bonnet, V. Brouste et al., "High cumulative risks of cancer in patients with *PTEN* hamartoma tumour syndrome," *Journal of Medical Genetics*, vol. 50, no. 4, pp. 255–263, 2013.

[105] M. H. Nieuwenhuis, C. M. Kets, M. Murphy-Ryan et al., "Cancer risk and genotype-phenotype correlations in PTEN hamartoma tumor syndrome," *Familial Cancer*, vol. 13, no. 1, pp. 57–63, 2014.

[106] J. Ngeow, K. Stanuch, J. L. Mester, J. S. Barnholtz-Sloan, and C. Eng, "Second malignant neoplasms in patients with Cowden syndrome with underlying germline PTEN mutations," *Journal of Clinical Oncology*, vol. 32, no. 17, pp. 1818–1824, 2014.

[107] D. Black, F. Bogomolniy, M. E. Robson, K. Offit, R. R. Barakat, and J. Boyd, "Evaluation of germline PTEN mutations in endometrial cancer patients," *Gynecologic Oncology*, vol. 96, no. 1, pp. 21–24, 2005.

[108] H. Mahdi, J. Mester, E. A. Nizialek, J. Ngeow, C. Michener, and C. Eng, "Germline PTEN, SDHB-D, and KLLN alterations in endometrial cancer patients with cowden and cowden-like syndromes: an international, multicenter, prospective study," *Cancer*, 2014.

[109] T. M. Starink, J. P. van der Veen, F. Arwert et al., "The Cowden syndrome: a clinical and genetic study in 21 patients," *Clinical Genetics*, vol. 29, no. 3, pp. 222–233, 1986.

[110] W. D. Baker, A. P. Soisson, and M. K. Dodson, "Endometrial cancer in a 14-year-old girl with Cowden syndrome: a case report," *Journal of Obstetrics and Gynaecology Research*, vol. 39, no. 4, pp. 876–878, 2013.

[111] A. C. ElNaggar, S. L. Spunt, W. Smith, M. Depas, and J. T. Santoso, "Endometrial cancer in a 15-year-old girl: a complication of cowden syndrome," *Gynecologic Oncology Case Reports*, vol. 3, pp. 18–19, 2013.

[112] K. M. Schmeler, M. S. Daniels, A. C. Brandt, and K. H. Lu, "Endometrial cancer in an adolescent: a possible manifestation of Cowden syndrome," *Obstetrics and Gynecology*, vol. 114, no. 2, part 2, pp. 477–479, 2009.

[113] J. M. Edwards, S. Alsop, and S. C. Modesitt, "Coexisting atypical polypoid adenomyoma and endometrioid endometrial carcinoma in a young woman with Cowden Syndrome: case report and implications for screening and prevention," *Gynecologic Oncology Case Reports*, vol. 2, no. 2, pp. 29–31, 2012.

[114] O. S. Salem and W. D. Steck, "Cowden's disease (multiple hamartoma and neoplasia syndrome). A case report and review of the English literature," *Journal of the American Academy of Dermatology*, vol. 8, no. 5, pp. 686–696, 1983.

[115] T. M. Starink and R. Hausman, "The cutaneous pathology of extrafacial lesions in Cowden's disease," *Journal of Cutaneous Pathology*, vol. 11, no. 5, pp. 338–344, 1984.

[116] M. B. Daly, R. Pilarski, J. E. Axilbund et al., "Genetic/familial high-risk assessment: breast and ovarian, version 1.2014," *Journal of the National Comprehensive Cancer Network*, vol. 12, no. 9, pp. 1326–1338, 2014.

[117] R. Nosbaum, K. J. Vogel, and K. Ready, "Susceptibility to breast cancer: hereditary syndromes and low penetrance genes," *Breast Disease*, vol. 27, no. 1, pp. 21–50, 2007.

[118] C. A. Schrager, D. Schneider, A. C. Gruener, H. C. Tsou, and M. Peacocke, "Clinical and pathological features of breast disease in Cowden's syndrome: an underrecognized syndrome with an increased risk of breast cancer," *Human Pathology*, vol. 29, no. 1, pp. 47–53, 1998.

[119] J. Ngeow, J. Mester, L. A. Rybicki, Y. Ni, M. Milas, and C. Eng, "Incidence and clinical characteristics of thyroid cancer in prospective series of individuals with cowden and cowden-like syndrome characterized by germline PTEN, SDH, or KLLN alterations," *Journal of Clinical Endocrinology and Metabolism*, vol. 96, no. 12, pp. E2063–E2071, 2011.

[120] B. Heald, J. Mester, L. Rybicki, M. S. Orloff, C. A. Burke, and C. Eng, "Frequent gastrointestinal polyps and colorectal adenocarcinomas in a prospective series of PTEN mutation carriers," *Gastroenterology*, vol. 139, no. 6, pp. 1927–1933, 2010.

[121] P. P. Stanich, V. L. Owens, S. Sweetser et al., "Colonic polyposis and neoplasia in Cowden syndrome," *Mayo Clinic Proceedings*, vol. 86, no. 6, pp. 489–492, 2011.

[122] T. M. Starink and R. Hausman, "The cutaneous pathology of facial lesions in Cowden's disease," *Journal of Cutaneous Pathology*, vol. 11, no. 5, pp. 331–337, 1984.

[123] S. K. Fistarol, M. D. Anliker, and P. H. Itin, "Cowden disease or multiple hamartoma syndrome—cutaneous clue to internal malignancy," *European Journal of Dermatology*, vol. 12, no. 5, pp. 411–421, 2002.

[124] O. Kovich and D. Cohen, "Cowden's syndrome," *Dermatology Online Journal*, vol. 10, no. 3, p. 3, 2004.

[125] G. J. Carlson, S. Nivatvongs, and D. C. Snover, "Colorectal polyps in Cowden's disease (multiple hamartoma syndrome)," *The American Journal of Surgical Pathology*, vol. 8, no. 10, pp. 763–770, 1984.

[126] A. J. Taylor, W. J. Dodds, and E. T. Stewart, "Alimentary tract lesions in Cowden's disease," *The British Journal of Radiology*, vol. 62, no. 742, pp. 890–892, 1989.

[127] S. Olschwang, O. M. Serova-Sinilnikova, G. M. Lenoir, and G. Thomas, "*PTEN* germ-line mutations in juvenile polyposis coli," *Nature Genetics*, vol. 18, no. 1, pp. 12–14, 1998.

[128] R. Coriat, M. Mozer, E. Caux et al., "Endoscopic findings in Cowden syndrome," *Endoscopy*, vol. 43, no. 8, pp. 723–726, 2011.

[129] P. P. Stanich, R. Pilarski, J. Rock, W. L. Frankel, S. El-Dika, and M. M. Meyer, "Colonic manifestations of PTEN hamartoma tumor syndrome: case series and systematic review," *World Journal of Gastroenterology*, vol. 20, no. 7, pp. 1833–1838, 2014.

[130] J. A. Barletta, A. M. Bellizzi, and J. L. Hornick, "Immunohistochemical staining of thyroidectomy specimens for pten can aid in the identification of patients with cowden syndrome," *The American Journal of Surgical Pathology*, vol. 35, no. 10, pp. 1505–1511, 2011.

[131] J. E. Hall, D. J. Abdollahian, and R. J. Sinard, "Thyroid disease associated with cowden syndrome: a meta-analysis," *Head and Neck*, vol. 35, no. 8, pp. 1189–1194, 2013.

[132] M. H. Brownstein, M. Wolf, and J. B. Bikowski, "Cowden's disease: a cutaneous marker of breast cancer," *Cancer*, vol. 41, no. 6, pp. 2393–2398, 1978.

[133] F. Barone, B. A. Noubari, A. Torrisi, S. Lanzafame, R. Tropea, and P. Mancuso, "Lhermitte Duclos disease and Cowden disease: clinical, pathological and neuroimaging study of a case," *Journal of Neurosurgical Sciences*, vol. 44, no. 4, pp. 234–237, 2000.

[134] D. A. Nowak and H. A. Trost, "Lhermitte-Duclos disease (dysplastic cerebellar gangliocytoma): a malformation, hamartoma or neoplasm?" *Acta Neurologica Scandinavica*, vol. 105, no. 3, pp. 137–145, 2002.

[135] K. Garg, R. R. Broaddus, R. A. Soslow, D. L. Urbauer, D. A. Levine, and B. Djordjevic, "Pathologic scoring of PTEN immunohistochemistry in endometrial carcinoma is highly reproducible," *International Journal of Gynecological Pathology*, vol. 31, no. 1, pp. 48–56, 2012.

[136] O. Maiques, M. Santacana, J. Valls et al., "Optimal protocol for PTEN immunostaining; role of analytical and preanalytical variables in PTEN staining in normal and neoplastic endometrial, breast, and prostatic tissues," *Human Pathology*, vol. 45, no. 3, pp. 522–532, 2014.

[137] B. Djordjevic, B. T. Hennessy, J. Li et al., "Clinical assessment of PTEN loss in endometrial carcinoma: immunohistochemistry outperforms gene sequencing," *Modern Pathology*, vol. 25, no. 5, pp. 699–708, 2012.

[138] B. Djordjevic, B. A. Barkoh, R. Luthra, and R. R. Broaddus, "Relationship between PTEN, DNA mismatch repair, and tumor histotype in endometrial carcinoma: retained positive expression of PTEN preferentially identifies sporadic non-endometrioid carcinomas," *Modern Pathology*, vol. 26, no. 10, pp. 1401–1412, 2013.

[139] D. J. Marsh, J. B. Kum, K. L. Lunetta et al., "PTEN mutation spectrum and genotype-phenotype correlations in Bannayan-Riley-Ruvalcaba syndrome suggest a single entity with Cowden syndrome," *Human Molecular Genetics*, vol. 8, no. 8, pp. 1461–1472, 1999.

[140] I. de Vivo, D. M. Gertig, S. Nagase et al., "Novel germline mutations in the *PTEN* tumour suppressor gene found in women with multiple cancers," *Journal of Medical Genetics*, vol. 37, no. 5, pp. 336–341, 2000.

[141] R. E. Teresi, K. M. Zbuk, M. G. Pezzolesi, K. A. Waite, and C. Eng, "Cowden syndrome-affected patients with PTEN promoter mutations demonstrate abnormal protein translation," *American Journal of Human Genetics*, vol. 81, no. 4, pp. 756–767, 2007.

[142] G. Tate, T. Suzuki, Y. Endo, and T. Mitsuya, "A novel mutation of the PTEN gene in a Japanese patient with Cowden syndrome and bilateral breast cancer," *Cancer Genetics and Cytogenetics*, vol. 184, no. 1, pp. 67–71, 2008.

[143] X.-P. Zhou, D. J. Marsh, C. D. Morrison et al., "Germline inactivation of PTEN and dysregulation of the phosphoinositol-3-kinase/Akt pathway cause human Lhermitte-Duclos disease in adults," *The American Journal of Human Genetics*, vol. 73, no. 5, pp. 1191–1198, 2003.

[144] J. Reifenberger, L. Rauch, M. W. Beckmann, M. Megahed, T. Ruzicka, and G. Reifenberger, "Cowden's disease: clinical and molecular genetic findings in a patient with a novel *PTEN* germline mutation," *British Journal of Dermatology*, vol. 148, no. 5, pp. 1040–1046, 2003.

[145] J. Mester and C. Eng, "Estimate of de novo mutation frequency in probands with PTEN hamartoma tumor syndrome," *Genetics in Medicine*, vol. 14, no. 9, pp. 819–822, 2012.

[146] E. Nizialek, C. Peterson, J. Mester, E. Downes-Kelly, and C. Eng, "Germline and somatic KLLN alterations in breast cancer dysregulate G2 arrest," *Human Molecular Genetics*, vol. 22, no. 12, pp. 2451–2461, 2013.

[147] V. Moyer, "Summaries for patients. Assessing the genetic risk for BRCA-related breast or ovarian cancer in women: recommendations from the U.S. Preventive Services Task Force," *Annals of Internal Medicine*, vol. 160, no. 4, pp. 1–16, 2014.

[148] M. S. Brose, T. R. Rebbeck, K. A. Calzone, J. E. Stopfer, K. L. Nathanson, and B. L. Weber, "Cancer risk estimates for *BCRA1* mutation carriers identified in a risk evaluation program," *Journal of the National Cancer Institute*, vol. 94, no. 18, pp. 1365–1372, 2002.

[149] J. L. Bermejo and K. Hemminki, "Risk of cancer at sites other than the breast in Swedish families eligible for *BRCA1* or *BRCA2* mutation testing," *Annals of Oncology*, vol. 15, no. 12, pp. 1834–1841, 2004.

[150] P. L. Mai, N. Chatterjee, P. Hartge et al., "Potential excess mortality in BRCA1/2 mutation carriers beyond breast, ovarian, prostate, and pancreatic cancers and melanoma," *PLoS ONE*, vol. 4, no. 3, Article ID e4812, 2009.

[151] J. M. Noh, D. H. Choi, H. Baek et al., "Associations between *BRCA* mutations in high-risk breast cancer patients and familial cancers other than breast or ovary," *Journal of Breast Cancer*, vol. 15, no. 3, pp. 283–287, 2012.

[152] K. Yoshida and Y. Miki, "Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage," *Cancer Science*, vol. 95, no. 11, pp. 866–871, 2004.

[153] P. L. Welcsh, K. N. Owens, and M.-C. King, "Insights into the functions of *BRCA1* and *BRCA2*," *Trends in Genetics*, vol. 16, no. 2, pp. 69–74, 2000.

[154] T. Helleday, "The underlying mechanism for the PARP and BRCA synthetic lethality: clearing up the misunderstandings," *Molecular Oncology*, vol. 5, no. 4, pp. 387–393, 2011.

[155] C. C. O'Sullivan, D. H. Moon, E. C. Kohn, and J.-M. Lee, "Beyond breast and ovarian cancers: PARP inhibitors for BRCA mutation-associated and BRCA-like solid tumors," *Frontiers in Oncology*, vol. 4, article 42, 2014.

[156] W. R. Wiedemeyer, J. A. Beach, and B. Y. Karlan, "Reversing platinum resistance in high-grade serous ovarian carcinoma: targeting BRCA and the homologous recombination system," *Frontiers in Oncology*, vol. 4, article 34, 2014.

[157] S. C. Lauchlan, "Tubal (serous) carcinoma of the endometrium," *Archives of Pathology and Laboratory Medicine*, vol. 105, no. 11, pp. 615–618, 1981.

[158] M. Hendrickson, J. Ross, and A. Martinez, "Uterine papillary serous carcinoma: a highly malignant form of endometrial adenocarcinoma," *The American Journal of Surgical Pathology*, vol. 6, no. 2, pp. 93–108, 1982.

[159] S. F. Lax and R. J. Kurman, "A dualistic model for endometrial carcinogenesis based on immunohistochemical and molecular genetic analyses," *Verhandlungen der Deutschen Gesellschaft für Pathologie*, vol. 81, pp. 228–232, 1997.

[160] A. Jhingran, L. M. Ramondetta, D. C. Bodurka et al., "A prospective phase II study of chemoradiation followed by adjuvant chemotherapy for FIGO stage I-IIIA (1988) uterine papillary serous carcinoma of the endometrium," *Gynecologic Oncology*, vol. 129, no. 2, pp. 304–309, 2013.

[161] M. E. Sherman, P. Bitterman, N. B. Rosenshein, G. Delgado, and R. J. Kurman, "Uterine serous carcinoma: a morphologically diverse neoplasm with unifying clinicopathologic features," *The American Journal of Surgical Pathology*, vol. 16, no. 6, pp. 600–610, 1992.

[162] J. P. Geisler, J. I. Sorosky, H.-L. Duong et al., "Papillary serous carcinoma of the uterus: increased risk of subsequent or concurrent development of breast carcinoma," *Gynecologic Oncology*, vol. 83, no. 3, pp. 501–503, 2001.

[163] H. Pere, J. Tapper, M. Seppälä, S. Knuutila, and R. Butzow, "Genomic alterations in fallopian tube carcinoma: comparison to serous uterine and ovarian carcinomas reveals similarity suggesting likeness in molecular pathogenesis," *Cancer Research*, vol. 58, no. 19, pp. 4274–4276, 1998.

[164] C. Kandoth, N. Schultz, A. D. Cherniack et al., "Integrated genomic characterization of endometrial carcinoma," *Nature*, vol. 497, no. 7447, pp. 67–73, 2013.

[165] R. Vang, I. M. Shih, and R. J. Kurman, "Fallopian tube precursors of ovarian low- and high-grade serous neoplasms," *Histopathology*, vol. 62, no. 1, pp. 44–58, 2013.

[166] R. J. Kurman, "Origin and molecular pathogenesis of ovarian high-grade serous carcinoma," *Annals of Oncology*, vol. 24, no. 10, pp. x16–x21, 2013.

[167] G. Hornreich, U. Beller, O. Lavie, P. Renbaum, Y. Cohen, and E. Levy-Lahad, "Is uterine serous papillary carcinoma a BRCA1-related disease? Case report and review of the literature," *Gynecologic Oncology*, vol. 75, no. 2, pp. 300–304, 1999.

[168] R. Goshen, W. Chu, L. Elit et al., "Is uterine papillary serous adenocarcinoma a manifestation of the hereditary breast-ovarian cancer syndrome?" *Gynecologic Oncology*, vol. 79, no. 3, pp. 477–481, 2000.

[169] D. A. Levine, O. Lin, R. R. Barakat et al., "Risk of endometrial carcinoma associated with BRCA mutation," *Gynecologic Oncology*, vol. 80, no. 3, pp. 395–398, 2001.

[170] O. Lavie, G. Hornreich, A. Ben-Arie et al., "*BRCA* germline mutations in Jewish women with uterine serous papillary carcinoma," *Gynecologic Oncology*, vol. 92, no. 2, pp. 521–524, 2004.

[171] T. Biron-Shental, L. Drucker, M. Altaras, J. Bernheim, and A. Fishman, "High incidence of BRCA1-2 germline mutations, previous breast cancer and familial cancer history in Jewish patients with uterine serous papillary carcinoma," *European Journal of Surgical Oncology*, vol. 32, no. 10, pp. 1097–1100, 2006.

[172] O. Lavie, A. Ben-Arie, Y. Segev et al., "BRCA germline mutations in women with uterine serous carcinoma—still a debate," *International Journal of Gynecological Cancer*, vol. 20, no. 9, pp. 1531–1534, 2010.

[173] I. Bruchim, K. Amichay, D. Kidron et al., "BRCA1/2 germline mutations in Jewish patients with uterine serous carcinoma," *International Journal of GynecologicalCancer*, vol. 20, no. 7, pp. 1148–1153, 2010.

[174] K. P. Pennington, T. Walsh, M. Lee et al., "*BRCA1*, *TP53*, and *CHEK2* germline mutations in uterine serous carcinoma," *Cancer*, vol. 119, no. 2, pp. 332–338, 2013.

[175] B. B. Roa, A. A. Boyd, K. Volcik, and C. S. Richards, "Ashkenazi jewish population frequencies for common mutations in BRCA1 and BRCA2," *Nature Genetics*, vol. 14, no. 2, pp. 185–187, 1996.

[176] D. Ford, D. F. Easton, and J. Peto, "Estimates of the gene frequency of BRCA1 and its contribution to breast and ovarian cancer incidence," *The American Journal of Human Genetics*, vol. 57, no. 6, pp. 1457–1462, 1995.

[177] M. E. Sherman, M. Piedmonte, P. L. Mai et al., "Pathologic findings at risk-reducing salpingo-oophorectomy: primary results from gynecologic oncology group trial GOG-0199," *Journal of Clinical Oncology*, 2014.

[178] C. Maheu, A.-D. Bouhnik, C. Nogues et al., "Which factors predict proposal and uptake of psychological counselling after BRCA1/2 test result disclosure?" *Psycho-Oncology*, vol. 23, no. 4, pp. 420–427, 2014.

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Research Article

Clinical Usefulness of Immunohistochemical Staining of $p57^{kip2}$ for the Differential Diagnosis of Complete Mole

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Objective. Can polymer-based immunohistochemical staining of $p57^{kip2}$ replace DNA analysis as an inexpensive means of differentiating complete mole from partial mole or hydropic abortion? *Methods and Materials*. Original paraffin-embedded tissue blocks from 14 equivocal cases were turned over to our laboratory and examined by immunohistochemical staining of $p57^{kip2}$. *Results*. Four of the 14 cases showed clearly negative nuclear staining in cytotrophoblasts and villous stromal cells: these results were fully concordant with the control staining. The remaining 10 cases showed apparently positive staining in cytotrophoblasts and villous stromal cells. Without DNA analysis we are able to clearly differentiate the 4 cases of complete mole among the 14 equivocal cases. During follow-up, secondary low-risk gestational trophoblastic neoplasia (GTN) developed in 1 of the 4 cases of complete mole: the GTN was treated by single-agent chemotherapy. No subsequent changes were observed during follow-up in the other cases. *Conclusion*. Polymer-based immunohistochemical staining of $p57^{kip2}$ (paternally imprinted gene, expressed from maternal allele) is a very effective method that can be used to differentiate androgenetic complete mole from partial mole and hydropic abortion. We might be able to avoid the cost of DNA analysis.

1. Introduction

Today, widespread use of ultrasonography and measurement of serum human chorionic gonadotropin (hCG) can be used to detect blighted ovum in the very early stage of pregnancy. Typical classic hydatidiform mole is now rarely seen. However, we, including pathologists, often face equivocal cases of complete mole versus partial mole that are difficult to diagnose histologically. In such cases, pathologists always notify us that complete mole cannot be ruled out and that strict clinical follow-up should be necessary. Usually, we proceed to DNA polymorphism analysis to obtain an accurate diagnosis in such cases. This requires both the patient's consent and extra expenditures.

We recently read the report that $p57^{kip2}$ gene, which encodes the cyclin-dependent kinase inhibitor (CDKI) $p57^{kip2}$, was located on chromosome 11 p15.5 and that this gene is paternally imprinted but expressed from the maternal allele. In the androgenetic complete mole, this gene is underexpressed or not expressed at all as discussed by Saxena et al. [1].

Several reports [2–7] have been published on the efficacy of immunohistochemical staining of this gene product for differentiation of complete mole, although there have been some exceptions. For many years, we have performed this same examination confirmed by DNA analysis in our laboratory. However, we obtained several false-positive results by immunohistochemical staining of $p57^{kip2}$. We noticed that

the false-positive immunoreaction was induced by endogenous biotin when we applied the standard streptavidin-biotin method that was used in the reported studies.

The polymer-based method is now gaining traction as an improved method in immunohistochemical staining method. With this method, a secondary antibody conjugated with a polymer is used. This polymer method has 10 to 100 times the sensitivity of the standard method, and there is almost no false staining of the target cells. Before starting this study, we used the polymer method in 10 cases each of androgenetic complete mole, partial mole, and biparental spontaneous abortion. These cases have been diagnosed by DNA analysis in our laboratory.

We confirmed completely negative immunohistochemical staining of $p57^{kip2}$ by this polymer method in cytotrophoblasts and villous stromal cells of the complete moles. Further, there was no false negative staining in the 10 cases of partial moles or the 10 cases of abortion, respectively, in this preliminary study.

We report herein the results obtained by polymer-based immunohistochemical staining of $p57^{kip2}$ in 14 equivocal cases.

2. Objective

Can polymer-based immunohistochemical staining of $p57^{kip2}$ replace DNA analysis as an inexpensive means of differentiating complete mole from partial mole or hydropic abortion?

3. Materials and Methods

3.1. Materials. We investigated 14 cases considered equivocal after evacuation by local doctors. All were local cases referred to us in 2012. All cases were initially diagnosed by pathologists working at commercial clinical laboratories. This is because local doctors ask first for pathological examination by commercial clinical laboratories as a matter of routine management. After equivocal results were returned to these doctors, the specimens were sent to us for further evaluation. Original paraffin-embedded tissue blocks from these 14 cases were collected by our laboratory for this project. These sections were made for each case stained with hematoxylin-eosin and then reexamined independently by our three pathologists and reclassified as difficult equivocal cases. Under our pathologists' review, 5 cases were considered as either hydropic abortion or partial mole, and 9 cases were considered partial or complete mole (Table 1). Informed consent was obtained from all patients, and the 14 cases were examined by polymer-based immunohistochemical staining of $p57^{kip2}$.

3.2. Methods. The polymer-based method of immunohistochemical staining is now well known to have 10 to 100 times the sensitivity of the standard streptavidin-biotin method. The immunohistochemical staining was carried out by heat-induced antigen retrieval followed by the polymer method. Duplicated 4 μm thick sections from the formalin-fixed, paraffin-embedded blocks were obtained in each case. Sections were deparaffinized in xylene and alcohol, washed, and rehydrated in distilled water.

After endogenous peroxidase activity was quenched with 3% hydrogen peroxidase solution, antigen retrieval was performed. The sections were immersed in 0.01 M citrate buffer (pH 7.0) with 0.1% Tween-20, kept for 40 minutes at 98°C. The sections were allowed to cool for 20 minutes spontaneously. Next, sections were immersed in 1 mM EDTA (pH 9.0), for 40 minutes at 98°C, and again allowed to cool. Next the sections were immersed again in 1 mg/mL protease XXIV (Sigma-Aldrich. St. Louis, MO, USA) in PBS for 60 minutes at room temperature and then washed in water and PBS. To block nonspecific reactions, these sections were immersed with 5% gout serum for 20 minutes at room temperature. Mouse monoclonal antibody for human $p57^{kip2}$ protein, the primary antibody, was applied to samples for overnight incubation at 4°C (Novocastra Liquid Mouse Monoclonal Antibody for human p57 protein (Product code: NCL-L-p57: Leica Biosystems Newcastle Ltd, Newcastle, UK)). Peroxidase-labeled secondary antibody for anti-mouse immunoglobulin conjugated with amino acid polymer (Nichirei Co, Ltd., Tokyo, Japan) was applied for 60 minutes at room temperature. Sections were then washed three times for 5 minutes each with PBS. The sections were incubated with diaminobenzidine as a chromogen for 10 minutes, then washed in water, and nuclear-counterstained with hematoxylin. Staining patterns on the tissue sections were examined microscopically and compared to those of control sections. The control sections were prepared from the DNA-established androgenetic complete moles, partial moles (triploidy), and abortions of biparental origin and prepared in the same manner as the cases' sections.

Reactivity was judged positive only when distinct nuclear staining of cytotrophoblasts and villous stromal cells was identified. There was no faint nuclear staining observed by polymer-based method through this experiment. Control study showed clearly negative staining of complete moles and positive in partial moles and abortions in cytotrophoblasts and villous stromal cells, respectively. Decidual stromal cells were positive for $p57^{kip2}$ in all cases and provided a reliable internal control (Figure 1). Syncytiotrophoblasts in complete moles, partial moles, and abortions always stained negatively (Figure 1).

4. Results

Duplicate immunohistochemical staining by the polymer method was done for each of the 14 equivocal cases, and the staining patterns were compared with those of the control cases confirmed genetically in our laboratory. Four cases (Cases 2, 5, 6, and 9 indicated by asterisks in Table 1) showed a clearly negative immunoreaction for $p57^{kip2}$. The others stained positively.

Thus, we were able to differentiate these 4 cases as complete moles among the 9 equivocal cases of partial or

Table 1: Fourteen equivocal cases subjected to polymer-based $p57^{kip2}$ immunohistochemistry for differentiation between complete and partial mole or hydropic abortion.

Case/patient	Age (yr)	G-P-A	Clinical Dx*	hCG mIU/mL before evacuation	Histopathologic Dx	$p57^{kip2}$ staining	Final Dx
1	40	1-0-1	7 weeks	6700	Hydropic/partial	+	Hydropic/partial
2**	30	3-3-0	7 weeks	4780	Partial/complete	–	Complete mole
3	40	2-2-0	7 weeks	49100	Partial/complete	+	Partial
4	27	3-2-1	8 weeks	83100	Partial/complete	+	Partial
5**†	48	4-2-2	6 weeks	6590	partial/complete	–	Complete mole
6**	30	3-1-2	7 weeks	28500	Partial/complete	–	Complete mole
7	27	1-1-0	8 weeks	91700	Hydropic/partial	+	Hydropic/partial
8	44	3-1-2	7 weeks	4670	Hydropic/partial	+	Hydropic/partial
9**	27	1-1-0	6 weeks	31200	Partial/complete	–	Complete mole
10	34	2-1-1	7 weeks	7800	Partial/complete	+	Partial
11	34	2-1-1	8 weeks	4400	Partial/complete	+	Partial
12	33	0-0-0	5 weeks	4600	Partial/complete	+	Partial
13	32	1-1-0	6 weeks	6800	Hydropic/partial	+	Hydropic/partial
14	36	3-2-1	6 weeks	5200	Hydropic/partial	+	Hydropic/partial

G-P-A, gravida/para/abortus; hCG, human chorionic gonadotropin; dilation and curettage; Dx, diagnosis.

*All diagnosed clinically as blighted ovum; **clearly differentiated as complete hydatidiform mole by polymer-based immunohistochemistry for $p57^{kip2}$; †hCG elevated to 8740, persistent trophoblastic disease, treated by single-agent chemotherapy.

S. SASAKI 2012.

complete mole. Cases 3, 4, 10, 11, and 12 in Table 1 were considered partial moles.

This staining did not differentiate partial moles from hydropic abortions.

The other cases (Cases 1, 7, 8, 13, and 14 in Table 1) remained equivocal cases of partial mole, or hydropic abortion.

These 4 cases of complete moles as well as the other cases were followed to 24 weeks by weekly serum hCG measurement. Of the 4 cases of complete mole, one (Case 5 in Table 1) developed into a secondary low risk gestational trophoblastic neoplasia (GTN) and was treated with single-agent chemotherapy.

No subsequent changes were observed during follow-up in the other cases.

5. Discussion

As a preliminary study, we performed standard streptavidin-biotin immunohistochemical staining of $p57^{kip2}$ in our DNA-established complete mole and hydropic abortion cases to know how effective the reported method is for differentiation of complete moles from hydropic abortion [5, 6]. In several established moles, however, we observed false positive staining. In reading the previous papers carefully, we learned that the investigators also encountered a small percentage of false positive staining.

With the standard streptavidin-biotin method, endogenous biotin has a positive effect on the staining pattern. So, we then used 3% hydrogen peroxidase solution to quench the endogenous biotin activity. This was 10 times the concentration reported by Jun et al. [6], but we still encountered false positive staining in several established complete moles. Subsequently, we learned that the polymer method of immunohistochemical staining, in which a secondary antibody conjugated with a polymer is used, is much more sensitive (10 to 100 times) than the standard streptavidin-biotin method.

The polymer method is easy and more sensitive, and it is not affected by endogenous biotin.

The secondary antibody conjugated with a polymer can be easily obtained commercially.

The polymer-based method is now described in textbooks as an improved method.

We applied the polymer method to our DNA analysis-established androgenetic complete moles and confirmed that the polymer methods do not produce false-positive or false-negative staining.

We found the method to be a reliable tool that can be used to differentiate complete mole in equivocal cases without the need for DNA analysis of each specimen.

However, there is 1 report of a definitive androgenetic complete mole that stained positively for $p57^{kip2}$ [3].

Of course, we must be vigilant, and we must realize that immunoreaction is not always absolute.

DNA analysis should be done, whenever a case remains questionable. However, there is no doubt that the polymer method is sensitive and effective.

We believe this method to be a very useful tool for differentiation of complete mole, when the results of other tests are equivocal. We would like to recommend that the polymer method of immunohistochemistry be applied first as a routine examination in any equivocal cases, especially

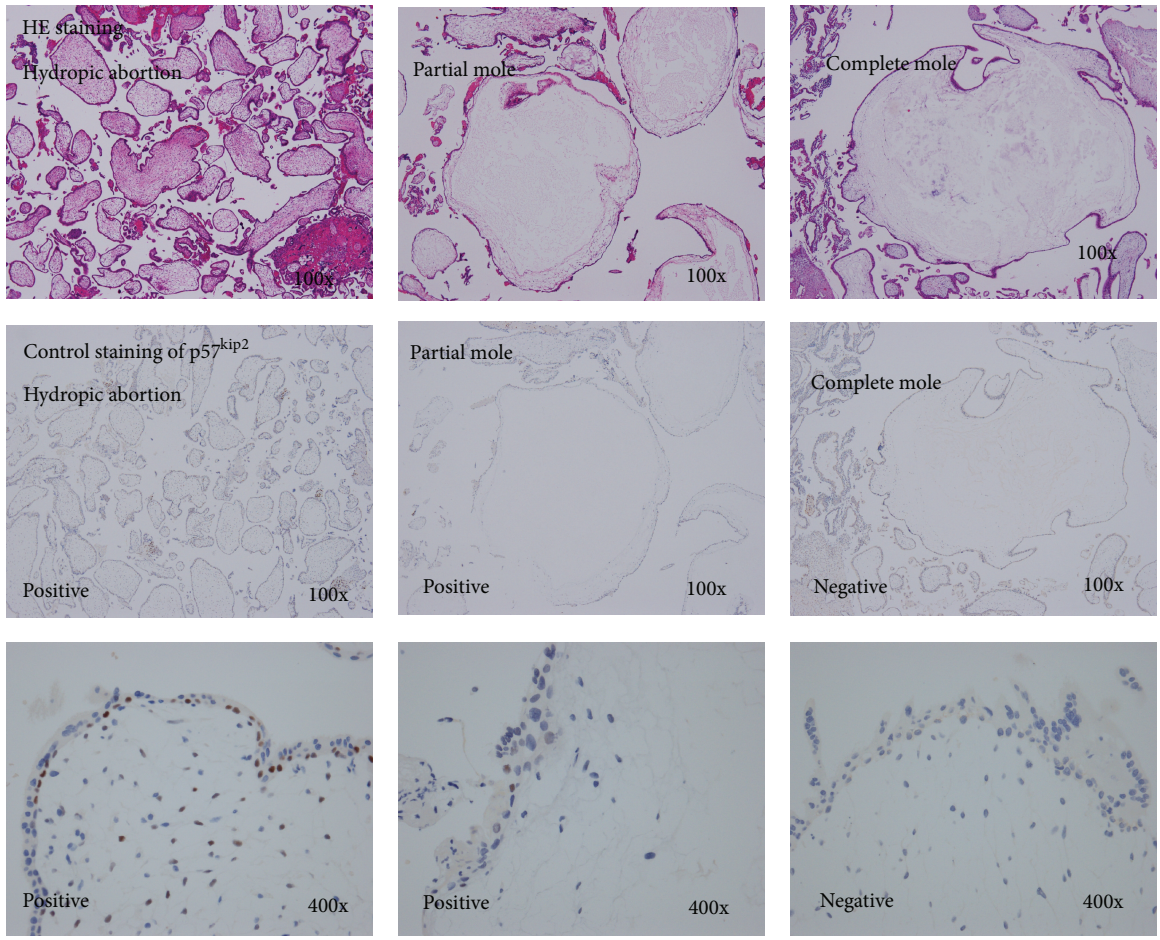

Figure 1: Those figures are the control staining in hydropic abortion (left), partial mole (middle), and complete mole (right). Upper panels show hematoxylin-eosin staining, and middle and lower panels show immunohistochemical staining for $p57^{kip2}$ by the polymer-based method of control hydropic abortion (left), partial mole (middle), and complete mole (right). Magnification are 100x and 400x by microscope, respectively. In cytotrophoblasts and villous stromal cells, strong positive immunoreactive staining for $p57^{kip2}$ is seen in the hydropic abortion (left) and partial mole (middle): staining is absent in the complete mole (right). The genetic origins of the control partial and complete mole were established by DNA polymorphism analysis in our laboratory.

for doctors who work in developing countries, where DNA analysis is far too expensive or even unfeasible. We may be able to avoid the cost of DNA analysis.

Kihara et al. published the first report of perfect concordance between negative $p57^{kip2}$ immunoreactivity and molar tissue of androgenetic origin [8]. They used a polymer system produced by DakoCytomation (Glostrup, Denmark). Our study independently supports their findings.

6. Conclusion

Polymer-based immunohistochemical staining of $p57^{kip2}$ (paternally imprinted gene, expressed from maternal allele) is a very effective method that can be used to differentiate androgenetic complete mole from partial mole and hydropic abortion. We might be able to avoid the cost of DNA analysis.

Ethical Approval

The authors obtained permission to conduct this study from the ethics committee of Iino Hospital, and they obtained informed consent from all 14 study patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] A. Saxena, D. Frank, P. Panichkul, I. B. Van den Veyver, B. Tycko, and H. Thaker, "The product of the imprinted gene IPL marks human villous cytotrophoblast and is lost in complete hydatidiform mole," *Placenta*, vol. 24, no. 8-9, pp. 835–842, 2003.

[2] M. Chilosi, E. Piazzola, M. Lestani et al., "Differential expression of p57(kip2), a maternally imprinted cdk inhibitor, in normal human placenta and gestational trophoblastic disease," *Laboratory Investigation*, vol. 78, no. 3, pp. 269–276, 1998.

[3] D. H. Castrillon, D. Sun, S. Weremowicz, R. A. Fisher, C. P. Crum, and D. R. Genest, "Discrimination of complete hydatidiform mole from its mimics by immunohistochemistry of the paternally imprinted gene product p57KIP2," *American Journal of Surgical Pathology*, vol. 25, no. 10, pp. 1225–1230, 2001.

[4] R. A. Fisher, M. D. Hodges, H. C. Rees et al., "The maternally transcribed gene $p57^{KIP2}$ (CDNK1C) is abnormally expressed in both androgenetic and biparental complete hydatidiform moles," *Human Molecular Genetics*, vol. 11, no. 26, pp. 3267–3272, 2002.

[5] M. Fukunaga, "Immunohistochemical characterization of $p57^{KIP2}$ expression in early hydatidiform moles," *Human Pathology*, vol. 33, no. 12, pp. 1188–1192, 2002.

[6] S.-Y. Jun, J. Y. Ro, and K.-R. Kim, "$p57^{kip2}$ is useful in the classification and differential diagnosis of complete and partial hydatidiform moles," *Histopathology*, vol. 43, no. 1, pp. 17–25, 2003.

[7] H. Crisp, J. L. Burton, R. Stewart, and M. Wells, "Refining the diagnosis of hydatidiform mole: image ploidy analysis and $p57^{KIP2}$ immunohistochemistry," *Histopathology*, vol. 43, no. 4, pp. 363–373, 2003.

[8] M. Kihara, H. Matsui, K. Seki, Y. Nagai, N. Wake, and S. Sekiya, "Genetic origin and imprinting in hydatidiform moles. Comparison between DNA polymorphism analysis and immunoreactivity of $p57^{kip2}$," *The Journal of Reproductive Medicine*, vol. 50, no. 5, pp. 307–312, 2005.

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Research Article

Levonorgestrel Inhibits Human Endometrial Cell Proliferation through the Upregulation of Gap Junctional Intercellular Communication via the Nuclear Translocation of Ser255 Phosphorylated Cx43

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Objects. To assess whether LNG exerts antiproliferation effects on human endometrial cells through changes of GJIC function and the phosphorylated Cx43. *Methods.* Cell proliferation and apoptosis of human endometrial stromal cells (HESCs) and glandular cells (HEGCs) treated with LNG in a dose- and time-dependent manner. GJIC change and further total Cx43 and serine 368 and 255 phosphorylated Cx43 were measured. *Results.* 5×10^{-5} mol/L LNG revealed a time-dependent inhibition of cell proliferation and an increase of apoptosis in both HESCs and HEGCs. Furthermore, these cells demonstrated a significant GJIC enhancement upon treatment with 5×10^{-5} mol/L for 48 hours. The effects of LNG were most noticeable in HESCs rather than in HEGCs. Associated with these changes, LNG induced a relative increase in total Cx43 in a time-dependent manner but not Ser368 phosphorylated Cx43. Moreover, laser scanning confocal microscope confirmed the increased expression of total Cx43 in the cytoplasm and, interestingly, the nuclear translocation of Ser255 phosphorylated Cx43. *Conclusions*. LNG likely inhibits the proliferation and promotes apoptosis in HESCs and HEGCs though an increase in gap junction permeability in vitro, which is achieved through the upregulation of Cx43 expression and the translocation of serine 255 phosphorylated Cx43 from the plasma to the nuclear compartment.

1. Introduction

Endometrial cancer (EC) is the most frequently diagnosed gynecological malignancy, and the incidence is increasing among women with younger and younger age in the last decade, partly owning to the increasing incidence of polycystic ovary syndrome which may cause long-term single estrogen effect in vivo. Endometrial complex atypical hyperplasia (CAH) is a precursor to endometrioid adenocarcinoma, and untreated CAH carries a risk of progression to carcinoma of approximately 25% [1, 2]. Hyperplasic endometrial polyps, in spite of their rare malignant changes, were considered as a risk factor for EC [3, 4]. Recent data from Saccardi et al. showed that there was a strong correlation between previous history of abnormal uterine bleeding (AUB) and hysteroscopic evidence of CAH in patients with a history of breast cancer and subsequent tamoxifen (TAM) therapy [5]. Outpatient hysteroscopy with biopsy has high diagnostic accuracy of EC and is mandatory in all postmenopausal women with AUB [6].

In women who do not have fertility requirements, the therapy choice for CAH is usually hysterectomy, whereas in

younger patients who desire to become pregnant conservative medical or surgical treatments could be offered. Conservative surgical treatment, such as endometrial resection by hysteroscopy, though it is safe and effective, can be suggested to treat focal lesion of CAH [7]. However, in women with diffuse atypical endometrial hyperplasia, high-dose progestin-based medical therapy is an alternate option to regress the lesion. In addition, progestins used are also efficacious in treating early grade endometrial endometrioid adenocarcinoma in women seeking to preserve fertility [1, 2]. Before hormonal therapy, assessment of the extent of malignant lesions, such as depth of myometrial invasion, cervical or parametrial involvement, should be considered. Besides, tumor sizes are easily assessable and useful to estimate the lesion degree and the prognosis of conservative treatment [8]. For advanced EC, progestin therapy results in lower response rates and does not often cure patients.

However, for the high-dose oral progestin treatments, side effects are also accompanied with. A locally high-dose of progestin use that is called levonorgestrel-releasing intrauterine system (LNG-IUS) has been documented for use in the local treatment of endometrial hyperplasia and even CAH or grade-one endometrial cancer, which is considered having treatment effect but not many systemic side effects [2]. In the past few years, we attempted to use LNG-IUS to treat patients with simple and complicated endometrial hyperplasia, and those with CAH and requirement for pregnancy very carefully and with close following-up during clinical practice. The following-up results are promising, and those with CAH treated eventually achieved healthy pregnancy after one or two years of LNG-IUS treatment (unpublished data: the histological regression (glandular atrophy and stroma decidualization) of atypical endometrial hyperplasia using LNG-IUS was observed in the supplemental data in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/758684). A study of 59 patients by Arnes et al. found that LNG-IUS was effective at reducing the occurrence of endometrial polyps [4]. Moreover, a systematic review of literature by Gizzo et al. concluded that LNG-IUS use could decrease the risk of endometrial atypia and polyps in TAM-treated breast cancer survivors [9].

Though it has been reported that progestin could cause atrophy and decidualization of endometrium, the various sources of synthesis progesterone have different biological activities and degrees. Furthermore, the molecular mechanism of progesterone causing these effect remains poorly understood. Therefore, we attempted to study the biological effects of LNG, the main components of LNG-IUS on the primary endometrial cells, through mimicking the local high-dose of LNG on the endometrium, and further the mechanisms that may cause the decidualization of the endometrium.

Connexin 43 (Cx43) is the most widely expressed connexin in the endometrium and is known to be important in a variety of physiological and pathological processes in this tissue [10–13]. Expression of Cx43 has been reported to be regulated by ovarian hormones in the female reproductive tract of rodents. Schlemmer et al. [14] analyzed paraffin-embedded uterine sections from hysterectomy specimens of patients with normal endometrium and from patients diagnosed with grade-one, -two, and -three endometrial cancer and found an inverse correlation between Cx43 expression and tumor grade, which indicated that Cx43 expression may be useful as an adjunctive marker of progression for endometrial carcinoma. In addition, it has been reported that expression of estrogen receptor alpha and progesterone receptors in relation to the expression of Cx26 and Cx43 in endometrial cancer. Disorders of connexin expression and altered distribution pattern occur during endometrial carcinogenesis, and it seems that progesterone receptors could participate in this fact [15]. The expression of Cx43 is positively related to gap junction intercellular communication (GJIC), while the phosphorylation of Cx43 is inversely related to GJIC in endometrial stromal cells. The restoration of GJIC by the induction or transfection of Cx43 was documented to reverse the transformed phenotype of certain cancer types, including those derived from the ovaries, lungs, and cervix [10, 11, 13, 16]. Thus, it appears that the expression and phosphorylation of Cx43 in endometrial cells participate in the maintenance and regulation of endometrial gap junction proteins.

Therefore, we proposed that LNG may suppress the proliferation of endometrial cells via an increase in gap junction permeability in vitro, which is achieved through the upregulation of Cx43 expression and its changed phosphorylated status. In this study, we observed the LNG effects on the cell proliferation and apoptosis of primary human endometrial cells and its role in the changes in the GJIC and Cx43 expression and its phosphorylated status.

2. Materials and Methods

2.1. Cell Cultures. The primary human endometrial stromal cells (HESCs) and glandular cells (HEGCs) were obtained from patients aged 37–45 years. The patients were undergoing uterectomies as a result of myoma of the uterus and went without hormone treatment for the past three months. The separation of HESCs and HEGCs from eutopic endometrial tissue was performed using the procedure developed by modified methods reported by Ryan et al. [17]. Cells were grown and maintained in DMEM/F12 (Gibco) and supplemented with 10% fetal bovine serum, 2 mm of glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, and 1 μg/mL fungizone (complete medium). Cytokeratin and vimentin were detected to determine the successful culture and separation of glandular and stromal cells, respectively, using immunohistochemistry.

2.2. Treatment of Cells. LNG and 17β-estradiol (E_2) were diluted in dimethyl sulfoxide (DMSO) with a stock concentration of 1×10^{-2} mol/L, and 12-O-tetradecanoylphorbol-13-acetate (TPA) was diluted in DMSO with a stock of 1 μg/mL. These hormones were diluted in accordance with the indicated concentration in the standardized amounts for the experiments. The final concentration of DMSO as a solvent was always less than 0.1%. The treatments of the cells with

these hormones were carried out for the time periods and with the concentrations described in our figure legends.

2.3. Cell Proliferation Measured by MTT. HESCs and HEGCs were placed in 200 μl of culture media per well in a 96-well plate with a density of 2×10^4 cells/mL (with 8 wells left empty as blank controls) and incubated overnight to allow the cells to attach to the wells. The cells were then treated with 2 μL of LNG in a concentration course manner (1×10^{-9}, 1×10^{-5}, 2.5×10^{-5}, or 5×10^{-5} mol/L) and in a time course (24 h, 48 h, and 72 h), with E_2 as the opposite control, and DMSO (0.5%) as the negative control. The concentration of LNG displaying an inhibitory effect and that of E_2 exhibiting a stimulatory effect on cell proliferation (both were 5×10^{-5} mol/L) were adopted as the final treating concentrations during the followed experiments determined according to the preliminary experiments. The concentration of 5×10^{-5} mol/L LNG is similar to that in the vivo endometrium in the presence of a mirena with a releasing rate of 20 μg/24 hours of LNG. Afterward, 20 μL of MTT solution was added to each well and incubated (37°C, 5% CO_2) for 2 to 4 hours until a purple precipitate became visible. When the purple precipitate became visible, 200 μL of DMSO was added and left at room temperature in the dark for 2 hours. The cell growth curve was detected in a λ of 597 mm and expressed with an OD value. Finally, the absorbance (optical density) was recorded at 570 nm.

The inhibition rate (IR) was calculated using the following formula:

$$\text{IR} = \frac{\text{Control OD}_{492} - \text{Experiment OD}_{492}}{\text{Control OD}_{492}} \times 100\%. \quad (1)$$

2.4. Apoptosis Measured by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL). HESCs and HEGCs were collected and cultured in six-well plates with glass cover-slips for 48 hours and then treated with 5×10^{-5} mol/L LNG in a time course (24 hs, 48 hs, and 72 hs), with 5×10^{-5} mol/L E_2 as the opposite control, and DMSO (0.5%) as the negative control. The cover-slips for the cells were fixed with 4% paraformaldehyde, sealed with 3% H_2O_2-methanol, and detected using the TUNEL method (cell technology), which was performed in accordance with the recommended protocol. The apoptosis rate (AR) was calculated using the following formula:

$$\text{Apoptosis rate} = \frac{\text{Apoptosis cells}}{\text{Total cells}} \times 100\%. \quad (2)$$

2.5. Analysis of the Cell Cycles Using Propidium Iodide (PI) Staining and Flow Cytometry. HESCs and HEGCs were cultured with a 1×10^5-cell/mL density in 25 mL flasks and then treated with LNG as the experiments above mentioned, with E_2 as the opposite control and DMSO as the negative control. The cells were then digested and suspended in 0.5 mL of PBS, with a density of 1×10^6/mL, combined with 2 mL of 70% cold ethanol and incubated overnight at −20°C. The cells were also centrifuged at 1200 rpm for 5 min to collect the precipitate. For each sample, 50 μL of RNAase and 450 μL of PI solution were added before incubation for 30 min at 4°C. These suspensions were analyzed by passage through a FACSCalibur flow cytometer (Becton Dickinson); 2×10^4 cells were counted. The percentage of S-phase cells and the cell apoptosis index were computed and analyzed.

2.6. Scrape Loading (SL)/Dye Transfer (DT). The levels of GJIC in both the control and treated cultures were determined using the SL/DT technique (22) with a fluorescent dye called Lucifer Yellow (LY) (Molecular Probes, Eugene, OR). The primary endometrial stromal and glandular cells were cultured in 35 mm plates in the manner described above and treated with LNG or E_2 in the manner described in Figure 4 legend, with TPA as the control. Scrape loading was performed with a razor blade by applying a cut on the cell monolayer, and the LY was then added to the cells. Then, an incision across the diameter of the clustered regions was made in the presence of a Lucifer Yellow (LY) CH mixture. The cells were washed thoroughly with PBS. The dye was rinsed away after 5 min. The cells were washed three times with PBS and fixed with 4% paraformaldehyde, and the cells stained with LY were detected by fluorescence emission using a reversed fluorescent microscope equipped with either a camera or a laser scanning confocal microscope (LSCM). Cells that received the LY from the scrape-loaded cells were considered to be communicating. A full-automatic photograph analyzing system (KONTRON IBAS2.5, Germany) was used to analyze a 50×60 mm^2 area of dye transfer on both sides of each cut.

2.7. Total and Phosphorylated Cx43 (pSer368-Cx43) Measured by Western Blot Analysis. The cells were washed twice with PBS and lysed on ice with a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40) containing a complete protease inhibitor cocktail (Roche, Nutley, NJ, USA) and phosphatase inhibitors (1 mM PMSF, 100 mM NaF, and 10 mM Na_3VO_4). Then, the lysis of HESCs was measured by western blot analysis with anti-Cx43 antibodies, the total Cx43 expression, or anti-pS368-Cx43 antibodies for S368 phosphorylation of Cx43. Proteins were resolved on a 4–15% gradient SDS-PAGE and transferred to nitrocellulose membranes (Sigma). Immunoblots were incubated with rabbit polyclonal anti-Cx43 antibodies (Sigma) (1 : 80) for the expression of total Cx43, rabbit polyclonal anti-P-Cx43 (S368) (Cell Signaling Technology) for the phosphorylated level of Cx43, followed by incubation with appropriate secondary IgG antibodies conjugated with HRP (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The immunoreactive bands were visualized by the Enhanced Chemiluminescence System (Cell Signaling Technology). Blots were washed, reprobed with anti-β-actin (Chemicon) antibodies, and developed in an identical manner for assessing β-actin protein levels to ensure even loading.

2.8. Total Cx43 and Phosphorylated Cx43 (pSer255-Cx43) Detected by LSCM. HESCs were cultured in six-well plates with cover-slips. When the cells grew to 50% impletion, they were treated with drugs in the manner described in

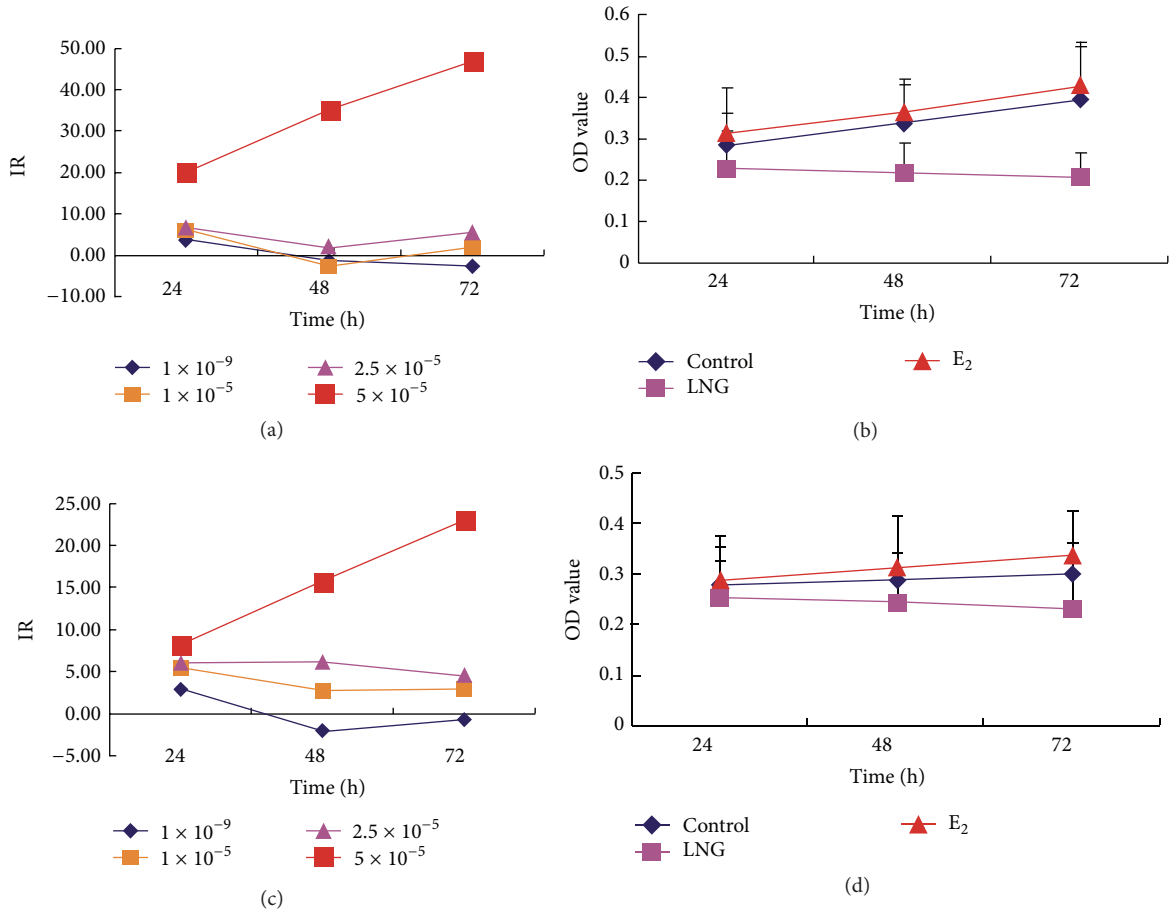

Figure 1: Cell proliferation inhibited by LNG. The MTT cell growth curve was performed to investigate the effects of the treatments on the proliferation of human endometrial stromal cells (HESCs) and glandular cells (HEGCs) treated with LNG, with 5×10^{-5} mol/L E_2 as the opposite control and DMSO (0.5%) as the blank control. (a) The inhibition rate (IR) of the HESCs treated with LNG in various concentrations in a time course manner (24 hs, 48 hs, and 72 hs); (b) MTT cell growth curve of HESCs treated with 5×10^{-5} mol/L LNG in a time course manner. (c) IR of the HEGCs treated with LNG in various concentrations in a time course manner; (d) MTT cell growth curve of HEGCs treated with 5×10^{-5} mol/L LNG in a time course manner; $^{*}P < 0.05$; there was a significant difference. Each experiment was repeated for 20 patients.

the figure legends. The glass cover-slips containing cells were fixed with 4% paraformaldehyde, sealed with goat serum at room temperature for 30 min, and incubated with anti-mouse Cx43 antibodies (1 : 80) overnight at 4°C, with PBS as the negative control and an unrelated antibody as the control for antibody specificity. After being thoroughly washed with PBS, a FITC-conjugated secondary antibody (1 : 200) was added before incubating the cells for 1 h at 37°C in order to detect total Cx43. For phosphorylatedCx43 (Ser255) detection, the first antibody used was pS255-Cx43 (Santa Gruz) (1 : 80), and the secondary antibody used was Cy3-conjugated IgG (Sigma) (1 : 50). A laser scanning confocal microscope (Zeiss LSCM510 META, Germany) was used to localize different fluorescent-labeled antibodies bound to HESCs. Immunostained cells were scanned with a laser at 488 nm for a green fluorescent of total Cx43 detection or 569–574 nm for a red fluorescent of pS255-Cx43, and optical sections were made either at 0.2 mm or at 1 to 2 mm increments from the top of the cell to the point of contact with the culture plate by means of a stepper motor. Images were obtained from 5–7 scans using Kalman averaging to increase the signal-to-noise ratio; they were viewed on screen and printed via a Sony videoprinter. Fluorescence within a defined region was analyzed by a point counting routine of pixel intensity. Specific counts were added from all sections and then divided by the total pixel areas of the cells to quantify the specific staining of antigens.

2.9. Ethics Statement. The institutional review board of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University specifically approved this study. And the written informed consents from the patients on the endometrium collection have been obtained.

2.10. Statistical Analysis. All of the experiments have a minimum of three determinants. The data were expressed as mean ± SD. In some figures, the data are from a representative experiment that was qualitatively similar to the replicate experiments. Statistical significance ($P < 0.05$) was determined with Student's t-test (two-tailed) between an

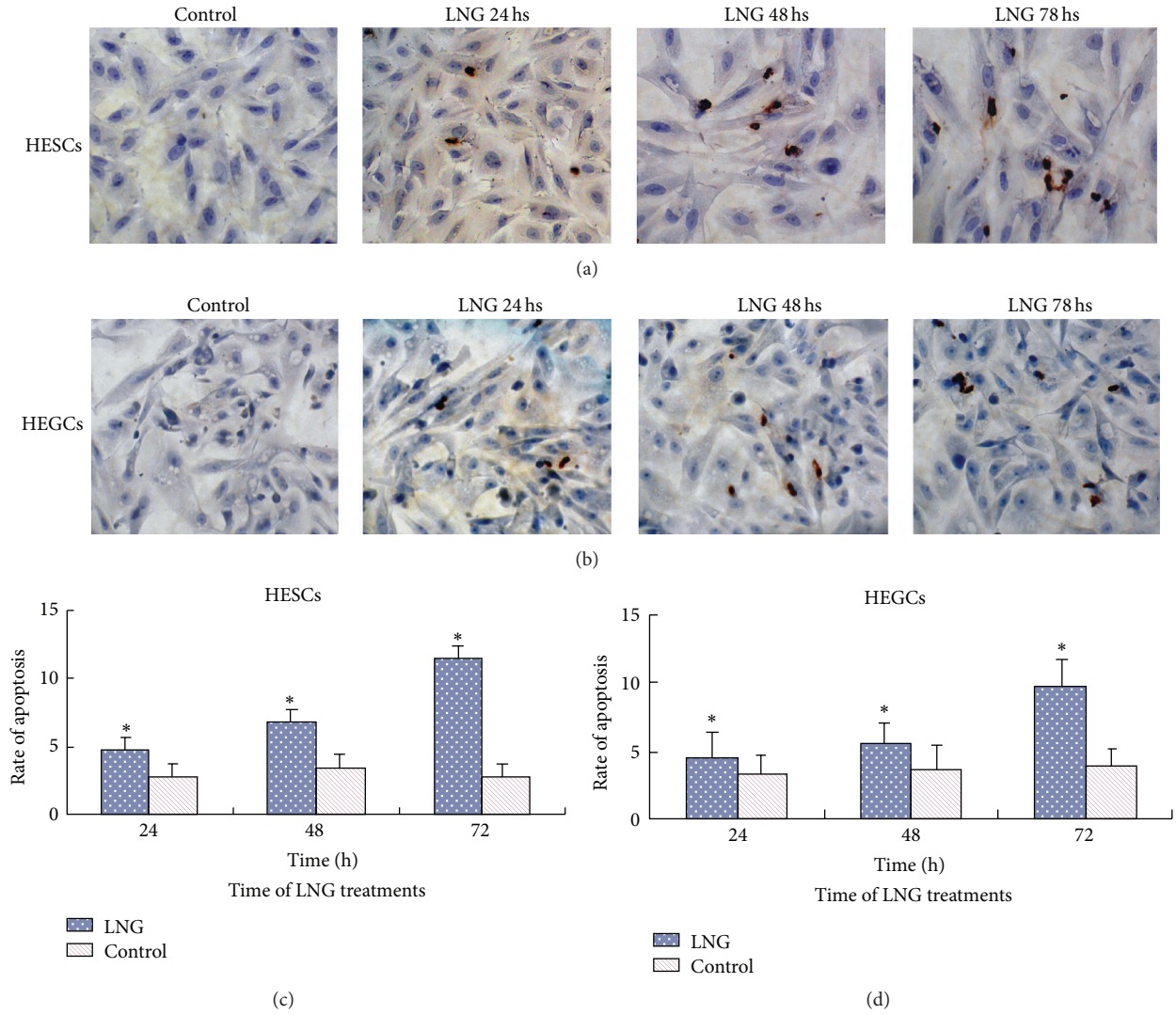

FIGURE 2: Effects of LNG on endometrial cell apoptosis determined by TUNEL. HESCs (a) or HEGCs (b) were treated in the absence (control) or presence of 5×10^{-5} mol/L LNG for 24 hs, 48 hs, and 72 hs, measured using TUNEL techniques. The brown staining showed the apoptotic body. The histogram represents the mean ± S.D. of the apoptosis rate calculated for HESCs (c) and HEGCs (d) in 6 patients. $^{*}P < 0.05$; there was a significant difference compared to the control.

individual experimental group and the corresponding control condition set as 100% (one-sample t-test).

3. Results

3.1. LNG Inhibits the Proliferation of Human Endometrial Cells. The cell growth curve study by MTT demonstrated that 5×10^{-5} mol/L LNG inhibited the cell proliferation of primary HESCs (Figures 1(a) and 1(b)) significantly over time (24 hs, 48 hs, and 72 hs). In a concentration of 5×10^{-5} mol/L, the inhibitory effect on primary HEGCs (Figures 1(c) and 1(d)) was significant but slightly weaker than that found in HESCs. For the opposite control of E_2, at the concentration of 2.5×10^{-5} mol/L, E_2 significantly stimulated the proliferation of both HESCs and HEGCs, and this stimulation was stronger for the concentration of 5×10^{-5} mol/L (Figures 1(a) and 1(c)).

3.2. LNG Promotes the Apoptosis of Human Endometrial Cells. TUNEL showed that the apoptosis rate increased after the treatment with 5×10^{-5} mol/L LNG for 24 hs, with a greater increase after 72 hs ($P < 0.01$) in both HESCs (Figures 2(a) and 2(c)) and HEGCs (Figures 2(b) and 2(d)). The apoptosis rate was more significant in HESCs than HEGCs. There was no significant increase in the apoptosis rate after the treatment with E_2. The propidium iodide (PI) staining and flow cytometry study demonstrated that 5×10^{-5} mol/L LNG significantly increased the apoptosis rates of both HESCs (Figures 3(a) and 3(c)) and HEGCs (Figures 3(b) and 3(d)), and the rates increased over time. Moreover, 5×10^{-5} mol/L E_2 had no significant effect on the apoptosis rates in both types of cells.

3.3. LNG Enhances GJIC. To determine the mechanisms responsible for the inhibitory and stimulatory effects on

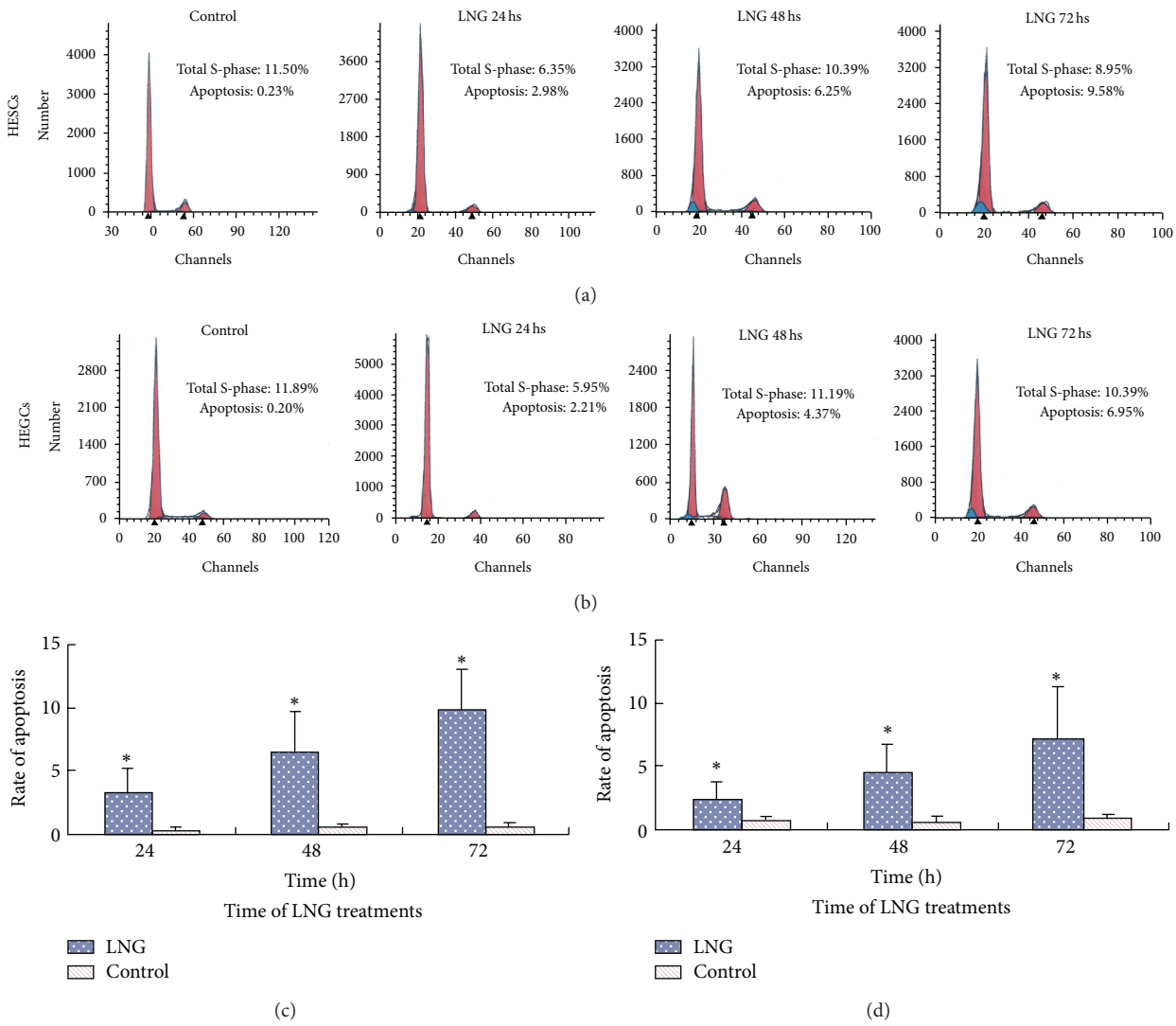

Figure 3: Effects of LNG on the apoptosis of endometrial cells analyzed by propidium iodide (PI) staining and flow cytometry. HESCs (a) and HEGCs (b) were treated in the absence (control) or presence of 5×10^{-5} mol/L LNG for 24 hs, 48 hs, and 72 hs. The cell cycle was analyzed by PI staining and flow cytometry, with 2×10^4 cells counted. The histogram represents the mean ± S.D. of the apoptosis rate for HESCs (c) and HEGCs (d) in 6 patients. $^*P < 0.05$; there was a significant difference, compared to the control.

the proliferation and apoptosis of LNG, respectively, and determine which mechanism was related to GJIC changes, we performed SL/DT assays using the gap junction permeable fluorescent dye LY. We found that 5×10^{-5} mol/L LNG significantly enhanced the GJIC in the HESCs (Figure 4(a)) compared to the control. The opposite control of the TPA treatment demonstrated that TPA could significantly inhibit GJIC in the HESCs (Figure 4(a)). There was no significant change in the GJIC in the HESCs after 5×10^{-5} mol/L E_2 treatments (Figures 4(a) and 4(c)). The response of GJIC to these drug treatments was similar in the HEGCs (Figures 4(b) and 4(d)).

3.4. LNG Enhances the Total Expression of Cx43 but Not the Level of p-S368 Cx43. Using western blotting, we investigated the effects of LNG on the protein expression and phosphorylation status of Cx43. Total Cx43 (Figure 5(a)), including nonphosphorylated Cx43 (P0) and phosphorylated Cx43 (P1 and P2), was more strongly expressed after treatment with 5×10^{-5} mol/L LNG for 24, 48, and 72 hours in HESCs (Figure 5(c)), with an increased expression over time, while after the LNG treatment for 96 hours, these increased expressions subsided. Furthermore, to test which phosphorylation site was associated with increased levels of P1 and P2, we measured the expression of S368 phosphorylated Cx43 after the treatment with LNG. However, we found that no significant expressive change of p-S368 Cx43 was present (Figures 5(b) and 5(d)).

3.5. LNG Promotes the Plasma Expression of Total Cx43 and the Nuclear Translocation of p-S255 Cx43. We also

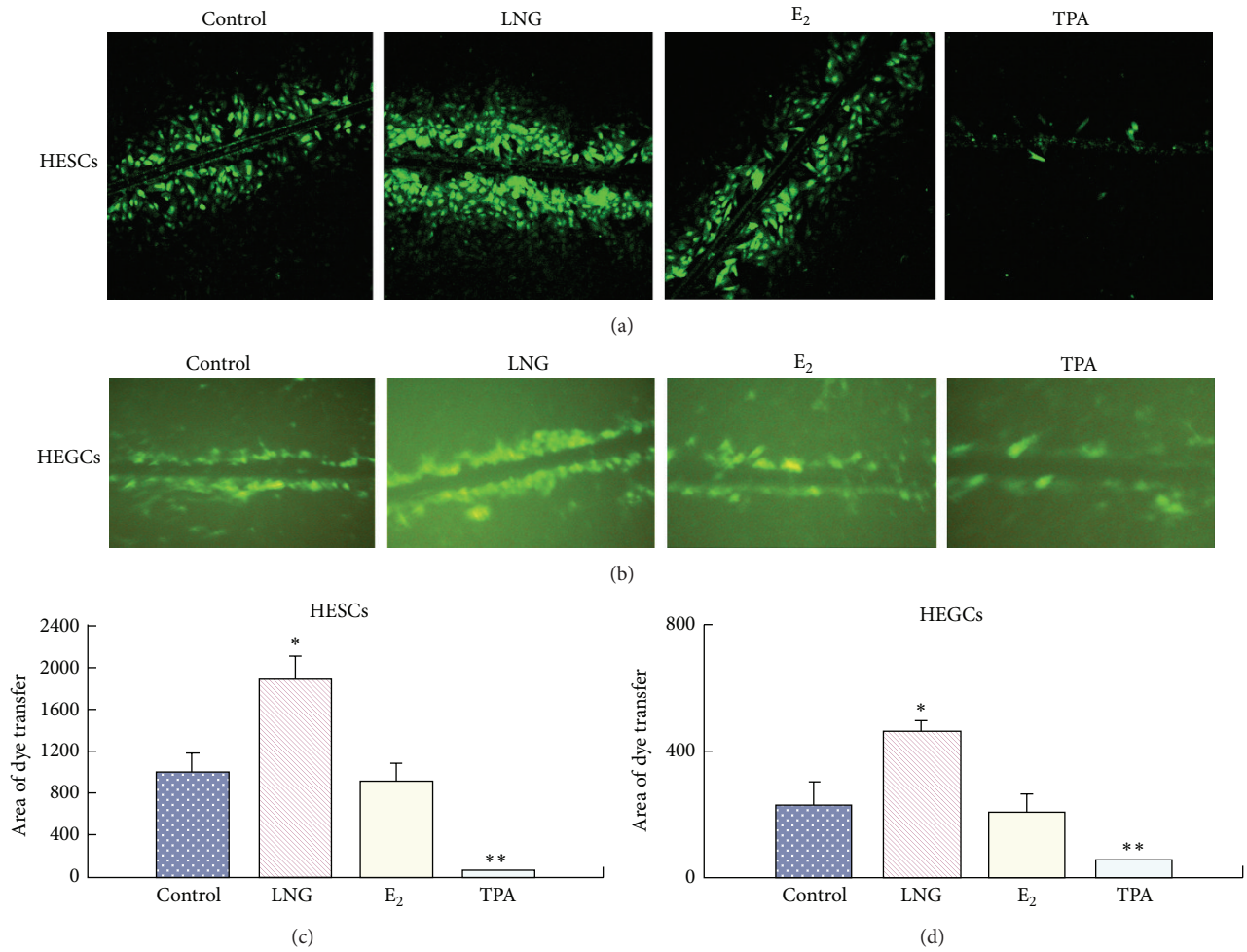

FIGURE 4: GJIC changes in endometrial cells, measured by scrape loading (SL)/dye transfer (DT). HESCs and HEGCs cells were treated in the absence (control) or presence of 5×10^{-5} mol/L LNG or 5×10^{-5} mol/L E_2 for 48 hs or in 10 ng/mL TPA for 2 hs. (a) Lucifer Yellow CH-recipient HESCs, observed by LSCM; (b) Lucifer Yellow CH-recipient HEGCs, observed by a reversed fluorescent microscope; ((c), (d)) The histogram representing the mean ± S.D. of the area of dye transfer for HESCs (c) or HEGCs (d) of 6 patients; $^{*}P < 0.05$, $^{**}P < 0.01$; there was a significant difference.

detected the expression and location of total Cx43 and S255 phosphorylated Cx43 in HESCs using LSCM. Total Cx43 was expressed in both the nuclear compartment and cytoplasm (Figure 6(a)). Moreover, the LSCM confirmed the stronger expression of total Cx43 in the cytoplasm after treatment with 5×10^{-5} mol/L LNG for 48 hours (Figure 6(b)). Interestingly, before the LNG treatment, S255 phosphorylated Cx43 was present in the cytoplasm and in some parts of the nuclear compartment, but for the LNG treatment, the p-S255 Cx43 protein was found to be translocated to the nucleus (Figures 6(c) and 6(d)).

4. Discussion

In this study, we found that LNG inhibits the cell proliferation and promotes apoptosis in normal human endometrial stromal and glandular cells through the enhancement of GJIC's function in these cells, which may be mediated through the overexpression of total Cx43 and the nuclear translocation of S255 phosphorylated Cx43. This study is the first report on the effect of LNG on the apoptosis of normal human endometrial cells in vitro and on the function and mechanisms of GJIC, of which the abstract was published in a conference proceeding [18].

The inhibition of cell growth and the promotion of cell apoptosis in response to 5×10^{-5} mol/L LNG treatment in a time-dependent manner (24, 48, and 72 hours) are consistent with our previous study and other studies suggesting that LNG could lead to the atrophy of the human glandular endometrium in vivo and the histological regression of endometrial hyperplasia and early stage endometrial carcinoma during or at the end of LNG-IUS treatment. Carcinogenesis is a multistage process, and different pathways can lead to cancer. The decreased expression of connexins and alterations in GJIC correlate with tumorigenesis [19]. Connexins in the intracellular (cytoplasmic or nuclear) compartment may control tumor progression, modulating the

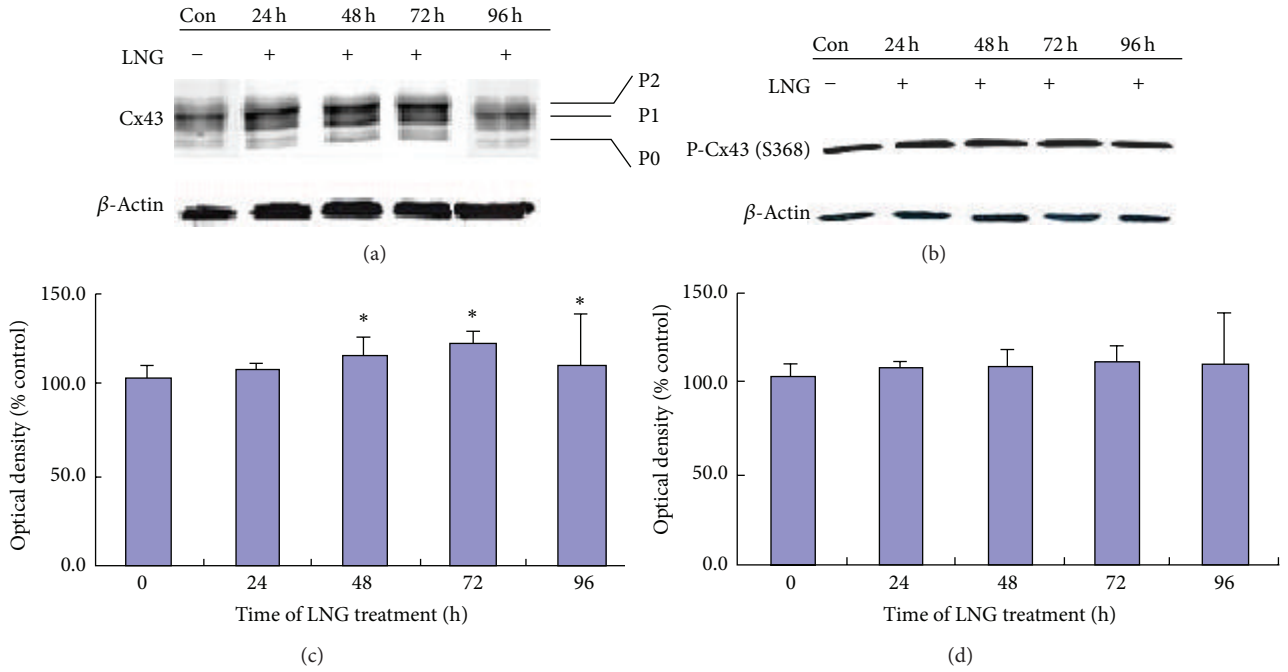

Figure 5: LNG increases connexin 43 protein levels and does not affect the level of p-connexin 43 at the S368 site, as measured by western blot analysis. HESCs were treated with either 5×10^{-5} mol/L LNG or the negative control (0.5% DMSO) for 24 hs, 48 hs, 72 hs, and 96 hs; then the protein lysis was measured by western blot ($n = 11$). ((a), (b)) Expression of total connexin 43 (Cx43) (a) and p-S368 Cx43 (b) treated with 5×10^{-5} mol/L LNG in a time-dependent manner; ((c), (d)) Comparison of the expression of total Cx43 (c) and p-S368 Cx43 (d) treated with 5×10^{-5} mol/L LNG; $^{*}P < 0.05$; there was a significant difference.

expression of the genes responsible for cell growth regulation, differentiation and apoptosis, and other functions of cancerous cells. Several studies have suggested that gap junction proteins in endometrial stromal cells play a regulatory role in maintaining normal levels of GJIC in glandular cells [20]. Our study also indicated that LNG could enhance the GJIC function, which is more pronounced in HESCs than HEGCs, suggesting that in addition to connecting the stromal cells, Cx43 is also involved in the inhibition of glandular cells' growth and differentiation. In addition, it has been reported that Cx43 expression is associated with an overexpressed connective tissue growth factor/nephroblastoma (CYR61/CTFG/NOV) family of growth regulators (CNN) [21], such as cyr61, which is an immediate, early gene that encodes a cysteine-rich, heparin-binding protein and a proangiogenic factor that mediates diverse roles in development, cell proliferation, and tumorigenesis. Moreover, Cx43 is originally found synthesized in its nonphosphorylated form and then inserted into the plasma membrane, where it is converted to its phosphorylated forms [22]. A lack of Cx43 expression and the aberrant localization of Cx43 have been associated with a lack of GJIC between tumor cells [23]. Our study found that LNG enhanced the GJIC function via the elevated expression of nonphosphorylated Cx43 (P0). Further investigations of LSCM demonstrated that the expression of Cx43 located in both cytoplasmic and nuclear compartments of the human endometrial stromal cells occurred in a punctuated pattern, which corresponds with the study conducted by McCulloch et al. [24]. After LNG treatment, the level of Cx43 inserted into the plasma membrane increased.

It has been reported that Cx43 can be phosphorylated on at least 14 of the 21 serines and two of the tyrosines in the cytoplasmic tail region (amino acids 245–382). Ser368 is one of the major phosphorylation sites in the carboxyl-terminus of Cx43 by protein kinase C (PKC) [25]. Sáez et al. [26] found that the protein kinase inhibitor, staurosporine, had an inhibitory effect on cell coupling and Cx43 phosphorylation, which is reversed by acute treatment with TPA. Following the study of Richards et al. [27], which showed that the increased amount of Cx43 in S368 phosphorylation reached maximum levels in the skin 24 hours after the wounding and returned to the baseline level by 72 hours, we further measured the effect of LNG on pSer368-Cx43 in a time-dependent manner. In contrast to the effect of TPA, in our study, the expression of pSer368-Cx43 did not change significantly upon LNG treatment, even over a prolonged period of time. On the other hand, Cx43 is reported to be phosphorylated in granulosa cells through a MAPK-dependent mechanism on serines 255, 262, and 279/282 [28] in response to the luteinizing hormone (LH), causing a decrease in the gap junction permeability between the granulosa cells and contributing to the resumption of meiosis in the oocyte [29]. Phosphorylation at these sites is transient and is no longer phosphorylated by 5 hours after the LH treatment [28]. On the basis of these

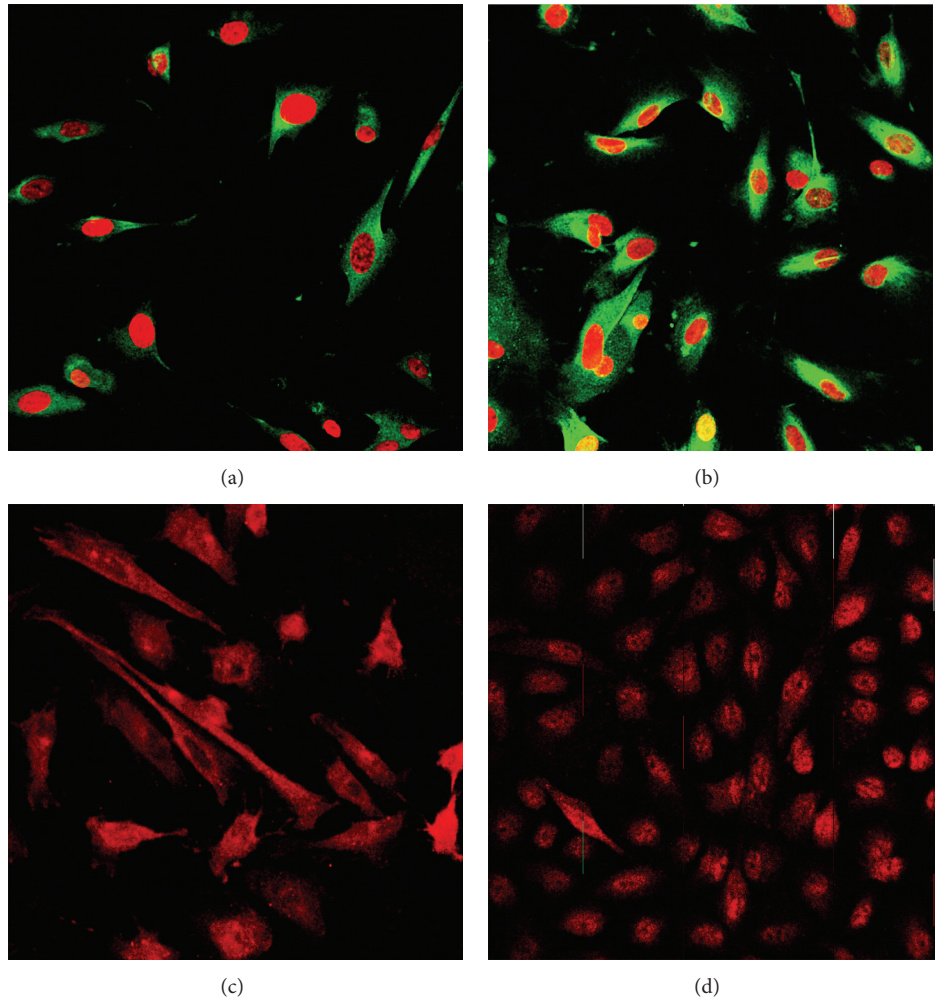

Figure 6: LNG promotes the increased level of total Cx43 in the plasma and the nuclear translocation of p-Cx43 at the S255 site, as measured by LSCM. HESCs were treated with 5×10^{-5} mol/L LNG or with the negative control (0.5% DMSO) for 48 hs to detect total Cx43 protein levels or for 5 hs to detect the expression and localization of the p255 Cx43 protein by using LSCM ($n = 5$). ((a), (b)) Expression and localization of total Cx43, treated with 5×10^{-5} mol/L LNG or DMSO (control) for 48 hs with PI staining. ((c), (d)) Expression and localization of pS255 Cx43, treated with 5×10^{-5} mol/L LNG or DMSO (control) for 5 hs.

characteristics of pS255 Cx43 over time, we detected the serines 255 phosphorylation of Cx43 upon LNG treatment using LCSM. In our study, more cells expressed the Ser255 phosphorylated Cx43 and the translocation of pS255 Cx43 from the plasma to the nuclear compartment in human endometrial stromal cells. The increased phosphorylation of Cx43 mediated by two serine/threonine protein kinase families, protein kinase C (PKC) [25] and MAPK [28], was reported to be causally linked with the disruption of GJIC. Although raloxifene could also work on cellular enhanced attachment and migration, it plays a role by interfering the recruitment of the Ga13/RhoA/ROCK/moesin cascade [30] and as a selective estrogen receptor modulator. Therefore, LNG may have a completely different molecular mechanism from raloxifene, working as a potent progesterone on the progesterone receptor. Based on this theory, our observations led to the demonstration that LNG increases the gap junction permeability in endometrial stromal cells via the translocation of Ser255 phosphorylated Cx43 from the plasma to nuclear compartment, consistent with the increased, nonphosphorylated Cx43 levels in the plasma.

5. Conclusions

In conclusion, LNG could inhibit the cell proliferation of and promotes apoptosis in human endometrial stromal and glandular cells though increasing the gap junction permeability in vitro study. The complete connection among the stromal cells is important in the normal growth of endometrial cells. The stronger GJIC function may be achieved by the upregulation of Cx43 expression and the translocation of serine 255 phosphorylated Cx43 from the plasma to the nuclear compartment. The data suggest a novel mechanism by which LNG can influence endometrial cell biology, which provides a theory basis for the use of LNG-IUS in

the conservative treatment of endometrial cancer of early stage as an optional fertility sparing approach [30].

List of Abbreviations

GJIC: Gap junction intercellular communication
Cx: Connexin
LNG-IUS: Levonorgestrel-releasing intrauterine system
HESCs: Human endometrial stromal cells
HEGCs: Human endometrial glandular cells
EC: Endometrial cancer
PCOS: Polycystic ovary syndrome
CAH: Endometrial complex atypical hyperplasia
ER alpha: Estrogen receptor alpha
PRs: Progesterone receptors
AR: Apoptosis rate
LSCM: Laser scanning confocal microscope.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xiaomiao Zhao and Xuelian Tang contributed as co-first authors.

Acknowledgments

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References

[1] L. Minig, D. Franchi, S. Boveri, C. Casadio, L. Bocciolone, and M. Sideri, "Progestin intrauterine device and GnRH analogue for uterus-sparing treatment of endometrial precancers and well-differentiated early endometrial carcinoma in young women," *Annals of Oncology*, vol. 22, no. 3, pp. 643–649, 2011.

[2] S. Haimovich, M. A. Checa, G. Mancebo, P. Fusté, and R. Carreras, "Treatment of endometrial hyperplasia without atypia in peri- and postmenopausal women with a levonorgestrel intrauterine device," *Menopause*, vol. 15, no. 5, pp. 1002–1004, 2008.

[3] P. Gambadauro, M. A. Martinez-Maestre, J. Schneider, and R. Torrejon, "Malignant and premalignant changes in the endometrium of women with an ultrasound diagnosis of endometrial polyp," *Journal of Obstetrics and Gynaecology*, vol. 34, no. 7, pp. 611–615, 2014.

[4] M. Arnes, B. Hvingel, and A. Orbo, "Levonorgestrel-impregnated Intrauterine device reduces occurrence of hyperplastic polyps: a population-based follow-up cohort study," *Anticancer Research*, vol. 34, no. 5, pp. 2319–2324, 2014.

[5] C. Saccardi, S. Gizzo, T. S. Patrelli et al., "Endometrial surveillance in tamoxifen users: role, timing and accuracy of hysteroscopic investigation: observational longitudinal cohort study," *Endocrine-Related Cancer*, vol. 20, no. 4, pp. 455–462, 2013.

[6] P. Litta, F. Merlin, C. Saccardi et al., "Role of hysteroscopy with endometrial biopsy to rule out endometrial cancer in postmenopausal women with abnormal uterine bleeding," *Maturitas*, vol. 50, no. 2, pp. 117–123, 2005.

[7] P. Litta, C. Bartolucci, C. Saccardi et al., "Atypical endometrial lesions: hysteroscopic resection as an alternative to hysterectomy," *European Journal of Gynaecological Oncology*, vol. 34, no. 1, pp. 51–53, 2013.

[8] R. Berretta, T. S. Patrelli, C. Migliavacca et al., "Assessment of tumor size as a useful marker for the surgical staging of endometrial cancer," *Oncology Reports*, vol. 31, no. 5, pp. 2407–2412, 2014.

[9] S. Gizzo, S. di Gangi, A. Bertocco et al., "Levonorgestrel intrauterine system in adjuvant tamoxifen treatment: balance of breast risks and endometrial benefits—systematic review of literature," *Reproductive Sciences*, vol. 21, no. 4, pp. 423–431, 2014.

[10] K. Cesen-Cummings, M. J. Fernstrom, A. M. Malkinson, and R. J. Ruch, "Frequent reduction of gap junctional intercellular communication and connexin43 expression in human and mouse lung carcinoma cells," *Carcinogenesis*, vol. 19, no. 1, pp. 61–67, 1998.

[11] T. J. King, L. H. Fukushima, A. D. Hieber, K. A. Shimabukuro, W. A. Sakr, and J. S. Bertram, "Reduced levels of connexin43 in cervical dysplasia: Inducible expression in a cervical carcinoma cell line decreases neoplastic potential with implications for tumor progression," *Carcinogenesis*, vol. 21, no. 6, pp. 1097–1109, 2000.

[12] B.-Y. Zhang, X.-W. Dai, Q.-Y. Chen et al., "Expression of epithelial-cadherin, CD44v6 and connexin43 in hepatocellular carcinoma," *Chinese Journal of Pathology*, vol. 35, no. 10, pp. 616–619, 2006.

[13] Z. Q. Zhang, W. Zhang, N. Q. Wang, M. Bani-Yaghoub, Z. X. Lin, and C. C. Naus, "Suppression of tumorigenicity of human lung carcinoma cells after transfection with connexin43," *Carcinogenesis*, vol. 19, no. 11, pp. 1889–1894, 1998.

[14] S. R. Schlemmer, D. B. Novotny, and D. G. Kaufman, "Changes in connexin 43 protein expression in human endometrial carcinoma," *Experimental and Molecular Pathology*, vol. 67, no. 3, pp. 150–163, 1999.

[15] H. Yamasaki, V. Krutovskikh, M. Mesnil, T. Tanaka, M. L. Zaidan-Dagli, and Y. Omori, "Role of connexin (gap junction) genes in cell growth control and carcinogenesis," *Comptes Rendus de l'Academie des Sciences*, vol. 322, no. 2-3, pp. 151–159, 1999.

[16] M. J. Fernstrom, L. D. Koffler, G. Abou-Rjaily, P. D. Boucher, D. S. Shewach, and R. J. Ruch, "Neoplastic reversal of human ovarian carcinoma cells transfected with Connexin43," *Experimental and Molecular Pathology*, vol. 73, no. 1, pp. 54–60, 2002.

[17] I. P. Ryan, E. D. Schriock, and R. N. Taylor, "Isolation, characterization, and comparison of human endometrial and endometriosis cells in vitro," *The Journal of Clinical Endocrinology & Metabolism*, vol. 78, no. 3, pp. 642–649, 1994.

[18] X. Zhao, X. Tang, and M. Xie, "Levonorgestrel inhibits human endometrial cell proliferation through the up-regulation of gap junctional intercellular communication via the increased expression of Connexin43 and the nuclear translocation of its Ser255 phosphorylation," *Endocrine Abstracts*, vol. 32, article P591, 2013.

[19] J. Czyz, "The stage-specific function of gap junctions during tumourigenesis," *Cellular & Molecular Biology Letters*, vol. 13, no. 1, pp. 92–102, 2008.

[20] S. R. Schlemmer and D. G. Kaufman, "Endometrial stromal cells regulate gap-junction function in normal human endometrial epithelial cells but not in endometrial carcinoma cells," *Molecular Carcinogenesis*, vol. 28, no. 2, pp. 70–75, 2000.

[21] C. S. Wun, J. F. Bechberger, W. J. Rushlow, and C. C. Naus, "Dose-dependent differential upregulation of CCN1/Cyr61 and CCN3/NOV by the gap junction protein connexin43 in glioma cells," *Journal of Cellular Biochemistry*, vol. 103, no. 6, pp. 1772–1782, 2008.

[22] L. S. Musil and D. A. Goodenough, "Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques," *Journal of Cell Biology*, vol. 115, no. 5, pp. 1357–1374, 1991.

[23] M. Mesnil, S. Crespin, J.-L. Avanzo, and M.-L. Zaidan-Dagli, "Defective gap junctional intercellular communication in the carcinogenic process," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1719, no. 1-2, pp. 125–145, 2005.

[24] F. McCulloch, R. Chambrey, D. Eladari, and J. Peti-Peterdi, "Localization of connexin 30 in the luminal membrane of cells in the distal nephron," *American Journal of Physiology: Renal Physiology*, vol. 289, no. 6, pp. F1304–F1312, 2005.

[25] P. D. Lampe, E. M. TenBroek, J. M. Burt, W. E. Kurata, R. G. Johnson, and A. F. Lau, "Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication," *Journal of Cell Biology*, vol. 149, no. 7, pp. 1503–1512, 2000.

[26] J. C. Sáez, A. C. Nairn, A. J. Czernik, G. I. Fishman, D. C. Spray, and E. L. Hertzberg, "Phosphorylation of connexin43 and the regulation of neonatal rat cardiac myocyte gap junctions," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 8, pp. 2131–2145, 1997.

[27] T. S. Richards, C. A. Dunn, W. G. Carter, M. L. Usui, J. E. Olerud, and P. D. Lampe, "Protein kinase C spatially and temporally regulates gap junctional communication during human wound repair via phosphorylation of connexin43 on serine368," *The Journal of Cell Biology*, vol. 167, no. 3, pp. 555–562, 2004.

[28] R. P. Norris, M. Freudzon, L. M. Mehlmann et al., "Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption," *Development*, vol. 135, no. 19, pp. 3229–3238, 2008.

[29] Y. Kalma, I. Granot, D. Galiani, A. Barash, and N. Dekel, "Luteinizing hormone-induced connexin 43 down-regulation: inhibition of translation," *Endocrinology*, vol. 145, no. 4, pp. 1617–1624, 2004.

[30] S. Gizzo, C. Saccardi, T. S. Patrelli et al., "Update on raloxifene: mechanism of action, clinical efficacy, adverse effects, and contraindications," *Obstetrical and Gynecological Survey*, vol. 68, no. 6, pp. 467–481, 2013.

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Research Article

Interleukin 16- (IL-16-) Targeted Ultrasound Imaging Agent Improves Detection of Ovarian Tumors in Laying Hens, a Preclinical Model of Spontaneous Ovarian Cancer

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Limited resolution of transvaginal ultrasound (TVUS) scanning is a significant barrier to early detection of ovarian cancer (OVCA). Contrast agents have been suggested to improve the resolution of TVUS scanning. Emerging evidence suggests that expression of interleukin 16 (IL-16) by the tumor epithelium and microvessels increases in association with OVCA development and offers a potential target for early OVCA detection. The goal of this study was to examine the feasibility of IL-16-targeted contrast agents in enhancing the intensity of ultrasound imaging from ovarian tumors in hens, a model of spontaneous OVCA. Contrast agents were developed by conjugating biotinylated anti-IL-16 antibodies with streptavidin coated microbubbles. Enhancement of ultrasound signal intensity was determined before and after injection of contrast agents. Following scanning, ovarian tissues were processed for the detection of IL-16 expressing cells and microvessels. Compared with precontrast, contrast imaging enhanced ultrasound signal intensity significantly in OVCA hens at early ($P < 0.05$) and late stages ($P < 0.001$). Higher intensities of ultrasound signals in OVCA hens were associated with increased frequencies of IL-16 expressing cells and microvessels. These results suggest that IL-16-targeted contrast agents improve the visualization of ovarian tumors. The laying hen may be a suitable model to test new imaging agents and develop targeted anti-OVCA therapeutics.

1. Introduction

The global yearly rate of death of women due to ovarian cancer (OVCA) is approximately 140,200 women and that of the USA is approximately 15,000 [1, 2] making OVCA one of the lethal gynecological malignancies. Because of the lack of an effective early detection test, OVCA in most cases is detected at late stages. Development of resistance to currently available chemotherapeutics and frequent recurrences when detected at late stages decrease 5-year survival rate of OVCA patients to <20%. In contrast, OVCA can be cured in >90% cases when it is detected at early stage. Therefore, early detection of OVCA is crucial and an effective early detection test is urgently needed. Serum levels of CA-125 alone or in combination with traditional transvaginal ultrasound (TVUS) imaging are the currently available test for the detection of OVCA [3]. However, neither the CA-125 nor the TVUS can detect OVCA at early stage specifically as serum CA-125 level is

elevated in patients with several benign gynecological as well as nongynecological abnormalities. On the other hand, although TVUS is the currently available preferred method for noninvasive imaging of ovarian abnormalities, unfortunately, with its limited resolution, traditional TVUS cannot detect OVCA at early stage [4]. In addition, a combination of serum CA-125 levels together with traditional TVUS imaging also failed to detect early OVCA as no imaging target in the ovary corresponding to the elevated serum CA-125 levels is established [4]. Thus a fresh approach is needed.

Extensive studies have been performed on the establishment of serum biomarkers for the detection of OVCA at early stage and a plethora of serum based marker(s) have been suggested. However, due to their lack of specificity and sensitivity, none of these markers was successful in detecting OVCA at early stage indicating that serum marker(s) alone may not be able to detect OVCA at early stage. Thus, an imaging target related to the malignant transformation of the ovary needs to be established and the current detection limit of traditional TVUS needs to be enhanced to detect early OVCA-related changes in the ovary. Moreover, to facilitate early detection of OVCA specifically, this imaging target(s) should also be associated with a surrogate maker(s) to be detectable in the serum. Contrast agents have been developed to enhance the visualization of tumors by several imaging modalities including TVUS scanning [5–9]. Imaging agents targeting $\alpha v\beta 3$-integrins and vascular endothelial growth factor receptor 2 (VEGFR-2) have been developed for contrast enhanced ultrasound imaging [10, 11]. However, very few reports are available on the ability of these targeted contrast agents in detecting OVCA at early stage. Moreover, absence of a corresponding serum surrogate marker reduces the specificity and sensitivity of these imaging agents. Thus additional imaging target(s) associated with malignant transformation needs to be established and imaging agents need to be developed to detect these new imaging targets for early detection of OVCA with high specificity.

Inflammation has been suggested as a risk factor for malignant transformation [12]. Unresolved inflammation leads to hypoxic conditions accompanied by changes in inflammatory cytokines including interleukin 16 (IL-16) [12, 13]. Ovulation is an inflammatory process which exposes ovarian surface (at the site of ovulatory rupture) and fimbrial epithelium (the site of reception of the ovulated ovum) to inflammatory factors including IL-16 secreted by immune cells. Exposure of the ovary and tubal epithelium to inflammatory agents due to frequent ovulation leads to the development of oxidative stress and longstanding unresolved oxidative stress has been suggested to cause malignant transformation. On the other hand, expression of IL-16 by the tumor epithelium and its serum levels has been reported to increase in association with ovarian tumor development [14, 15]. Moreover, IL-16 has also been reported as a proangiogenic factor [16] and may also be expressed by the endothelium of tumor-associated microvessels. Thus IL-16 represents a potential marker of early OVCA and IL-16 expressing tissues in the ovary can be detected by ultrasound imaging provided an IL-16-targeted ultrasound imaging agent can be developed.

Identification and access to patients with early stage OVCA are the significant barriers to develop and test the efficacy of contrast enhancing imaging agents in detecting spontaneous OVCA at early stage. Most of the available contrast agents were developed using rodents and thus are difficult to translate in human OVCA [11, 17–19], because rodents do not develop OVCA spontaneously and induced ovarian carcinomas in rodents are histopathologically not similar to those of spontaneous OVCA in humans [20]. Recently, laying hens have been shown to develop OVCA spontaneously with high incidence rates. Spontaneous OVCA in hens are remarkably similar to human OVCA with regard to tumor histopathology and expression of several molecular markers [14, 20–26]. Furthermore, methods for the imaging of hen ovaries and ovarian tumors by TVUS scanning have been adapted [27–29]. Moreover, similar to humans, expression of IL-16 by ovarian tumors has been reported to be increased in association with tumor development and progression in hens [14, 15]. Thus the laying hen represents a highly innovative model to test the feasibility of IL-16-targeted imaging agents for the detection of spontaneous OVCA at an early stage by noninvasive TVUS imaging. Therefore, the goal of this study was to examine whether IL-16-targeted contrast agents enhance the intensity of traditional TVUS imaging and improve the early detection of spontaneous ovarian tumors in laying hens, a preclinical model of OVCA.

2. Materials and Methods

2.1. Animals. A flock of 3-4-year-old commercial strains of White Leghorn laying hens (*Gallus domesticus*) were maintained under standard poultry care and management and provided with feed and water *ad libitum*. Egg laying rates of the hens were recorded on a daily basis. Egg laying rates in a hen are used as a relative indicator of ovulation rates in hens. The normal rate of egg laying by a commercial laying hen is more than 250 eggs per year and less than 50% of the normal laying rate is considered a low egg laying rate [27]. 150 hens with normal, low, or irregular egg laying rates and those that stopped laying with no large preovulatory follicle, with or without solid mass in the ovary and abdominal distention (a sign of possible ovarian tumor-associated ascites), were selected for IL-16-targeted contrast enhanced imaging agents. The incidence of ovarian cancer in laying hens of this age group was reported to be approximately 10% to 20% and is associated with low laying rates or complete cessation of egg laying [20, 21, 27]. All procedures were performed according to the Institutional Animal Care and Use Committee approved protocol.

2.2. IL-16-Targeted Contrast Enhanced Ultrasound Imaging Agents. IL-16-targeted imaging agents were prepared by conjugating anti-chicken IL-16 antibodies with Targestar containing microbubbles (Targeson, Inc., San Diego, CA). Targestar SA is a targetable ultrasound contrast agent coated with streptavidin. Biotinylated antibodies can be easily conjugated to the microsphere surface, enabling target-specific

retention for molecular imaging. The agent remains acoustically active up to 15 minutes. Agents are administered as an intravenous bolus injection. Microbubbles preparation, ligand conjugation, characterization of labeled microbubbles, and their binding specificity of tumor tissues were similar to those reported earlier [10].

2.3. Ultrasound Imaging

2.3.1. Precontrast Traditional Transvaginal Ultrasound (TVUS) Imaging. All hens were scanned using an instrument equipped with a 1 to 7.5 MHz transvaginal transducer (MicroMaxx, SonoSite, Inc., Bothell, WA) as reported previously with little modification [27, 29]. Hens were immobilized and gently restrained by an assistant. Transmission gel was applied to the surface of the transducer; the transducer was covered by a cover and to ensure uninterrupted conductance of the sound waves, gel was reapplied to the covered probe. The transducer was inserted approximately at a 30° angle to the body, 3 to 5 cm into the vagina, and 2-dimensional (2D) gray scale and pulsed Doppler sonography were performed. Young egg laying hens (as the ovaries of these hens contain more developing follicles compared to old hens) were used as standard controls for mechanical adjustment to reveal and characterize the fully functional normal ovaries of hens. The area of a tumor to be imaged was determined according to 3 conditions as reported previously [27, 29]: (a) the whole tumor, if possible, should be seen on the image; (b) the sectional plane should contain the solid part (wall, septa, and papillae) of the tumor; and (c) the most vascularized area was selected. For normal ovaries, ovaries without any detectable tumor, and atrophic ovaries, the region surrounding the ovary was scanned and the transducer was swept through the entire area for complete scanning of the ovary. Gray scale morphologic evaluation of the ovarian mass was performed with attention to the number of preovulatory follicles, the presence of abnormal-looking follicles, septations, papillary projections or solid areas, and echogenicity. After morphologic evaluation, color Doppler mode was activated for identification of vascular color signals. Once a vessel was identified on color Doppler imaging, pulsed Doppler was activated to obtain a flow velocity waveform.

2.3.2. Injection of IL-16-Targeted Contrast Agents and Contrast Enhanced Ultrasound Imaging. Contrast imaging was performed following precontrast scanning. A preliminary experiment was conducted with IL-16-targeted or isotype control microbubbles using 10 animals containing fully functional ovaries to adjust the mechanical setup and determine the optimum dosage of microbubbles. The dose of 10 μL/kg body weight was found optimal for better resolution in the preliminary experiment. Microbubbles containing contrast agents were prepared before injection. Briefly, the vial containing the microbubble suspension was inverted and gently rotated to resuspend the microspheres completely. The suspension was transferred from the vial by an injection syringe with a 19-gauge needle to a angiocatheter (small-vein infusion set, female luer, 12-in. tubing, 25-gauge needle; Kawasumi Laboratories, Tampa, FL) containing 100 μL of 0.9% sodium chloride previously inserted into the left wing vein (brachial vein) of the hen and followed by the reloading of 100 μL of a 0.9% sodium chloride solution. The loading of the sodium chloride solution before and after injection of microbubbles helped maintain the vascular patency and airtight condition, in addition to flushing the bubbles from the hen's circulation.

The area imaged during precontrast scanning was imaged again after contrast microbubble injection. Following injection of contrast agents and before postcontrast imaging, time was allowed for microbubbles to bind with their targets and retention of bounded microbubbles in the tumor as well as wash-out of unbound microbubbles. The timing of contrast imaging was determined through an initial experiment using different time points including 2, 5, 7, and 10 min. Imaging after 7 min of contrast agent injection was found optimum with minimum background signals. Then a destructive pulse was delivered and images were taken again. The difference in the intensity of ultrasound imaging between the images at 7 min after injection and images after the delivery of destructive pulse confirms that the signal acquired after contrast agent injection was from microbubbles-bounded target tissue. For an individual hen, the same imaging plane and same size of ROI were used for measuring the precontrast and postcontrast intensity of ultrasound imaging. All images (screenshots) were archived digitally in a still format as well as real-time clips on single-sided recordable digital video disks (DVD+R format; Maxell Corporation of America, Fair Lawn, NJ) readable on a personal computer.

2.3.3. Evaluation of the Effects of IL-16-Targeted Contrast Agents. The effect of contrast agents was evaluated visually during the examination and the enhancement of tumor detection by contrast imaging was assessed afterward from reviewing the archived video clips. After reviewing the complete clip the image containing the stroma of normal hens or containing the tumor was selected and used as region of interest (ROI) for measuring the precontrast and postcontrast intensity of ultrasound imaging. In normal hens, areas containing large developing follicles were avoided during the selection of images containing the ROIs. The intensity of the pixels in the selected area was measured using a computer-assisted software program (MicroSuite version 5, Olympus Corporation, Tokyo, Japan) and expressed as arbitrary values. The intensity of the ROI (sum of the arbitrary values from the pixels within the region of interest) was measured from the precontrast and contrast image and expressed as the mean ± SD in 40,000-pixel area. The net contrast enhancement (CE = Ct − Cpt) was determined and the CE ratio (CER) was calculated using the following equation: $\mathrm{CER} = [(\mathrm{Ct} - \mathrm{Cpt})/\mathrm{Cpt}] \times 100\%$, where Cpt = values from ROI of precontrast image and Ct = values from ROI of contrast image. As mentioned above, Ct is the difference between the intensity of ultrasound imaging from images taken at 7 min after injection of contrast agents and after the delivery of a destructive pulse.

2.4. Ovarian Gross Morphologic Evaluation. All hens were euthanized after contrast imaging and examined for the

presence of a solid mass in the ovary as well as in any other organs, ascitic fluid, preovulatory follicles, and atrophy of the ovary, as reported previously [21]. Gross observation was compared with the sonographic evaluations and photographed. A normally functional ovary had viable preovulatory follicles (more detailed information on hen ovarian physiology has been published elsewhere [21, 27]), whereas no large follicles or visible lesions were found in normal hens that stopped egg laying. Tumor staging was performed according to the gross metastatic status as reported previously [21]. Briefly, early OVCA was characterized by detectable formation of solid tumor limited to the ovary. Late stages of OVCA were characterized by tumor metastasis to distant organs with moderate to extensive ascites.

2.5. Histologic Evaluation and Immunohistochemical Detection of Ovarian IL-16 Expressing Cells and Microvessels. Representative portions of a solid ovarian mass or the whole ovary (in cases of atrophic or grossly normal-appearing ovaries) were divided into several blocks, processed for paraffin or frozen sections, and stained with hematoxylin-eosin. Microscopic tumor (if present) in any part of the ovary was detected by routine histologic examination with hematoxylin-eosin staining, and tumor types were determined by light microscopy, as reported previously [21].

After histopathologic examination, paraffin sections (5 μm thick) of normal and malignant ovaries of all stages and types were processed for routine immunohistochemistry to assess the frequency of IL-16 expressing cells and microvessels using rabbit anti-chicken IL-16 polyclonal antibodies as reported earlier [14, 15]. The frequencies of IL-16 expressing cells and microvessels were determined from the stroma of the ovarian tumors or ovarian stroma of normal hens (excluding the follicular areas), as reported earlier [28, 30] using a light microscope attached to digital imaging stereological software (MicroSuite version 5; Olympus Corporation) with little modification. Briefly, immunostained slides were examined at low-power magnification (×10 objective and ×10 ocular) to identify the areas with maximum IL-16 expressing cells or microvessels. Vessels with thick, regular, and complete muscular walls as well as vessels with large lumina were excluded from the count, as reported previously [28]. In each section, the 5 highly immunostained areas for IL-16 expressing cells or microvessels were chosen and immunopositive cells or microvessels (with leaky, incomplete, and thin vessel wall) were counted. The number of immunopositive cells or microvessels in a 20,000 μm^2 area was counted at ×40 objective and ×10 ocular magnification. The averages of these sections were expressed as the number of immunopositive cells or microvessels in a 20,000 μm^2 area of a normal ovary or ovary with tumor. Tumor histology and immunohistochemical observations were compared to the sonographic predictions.

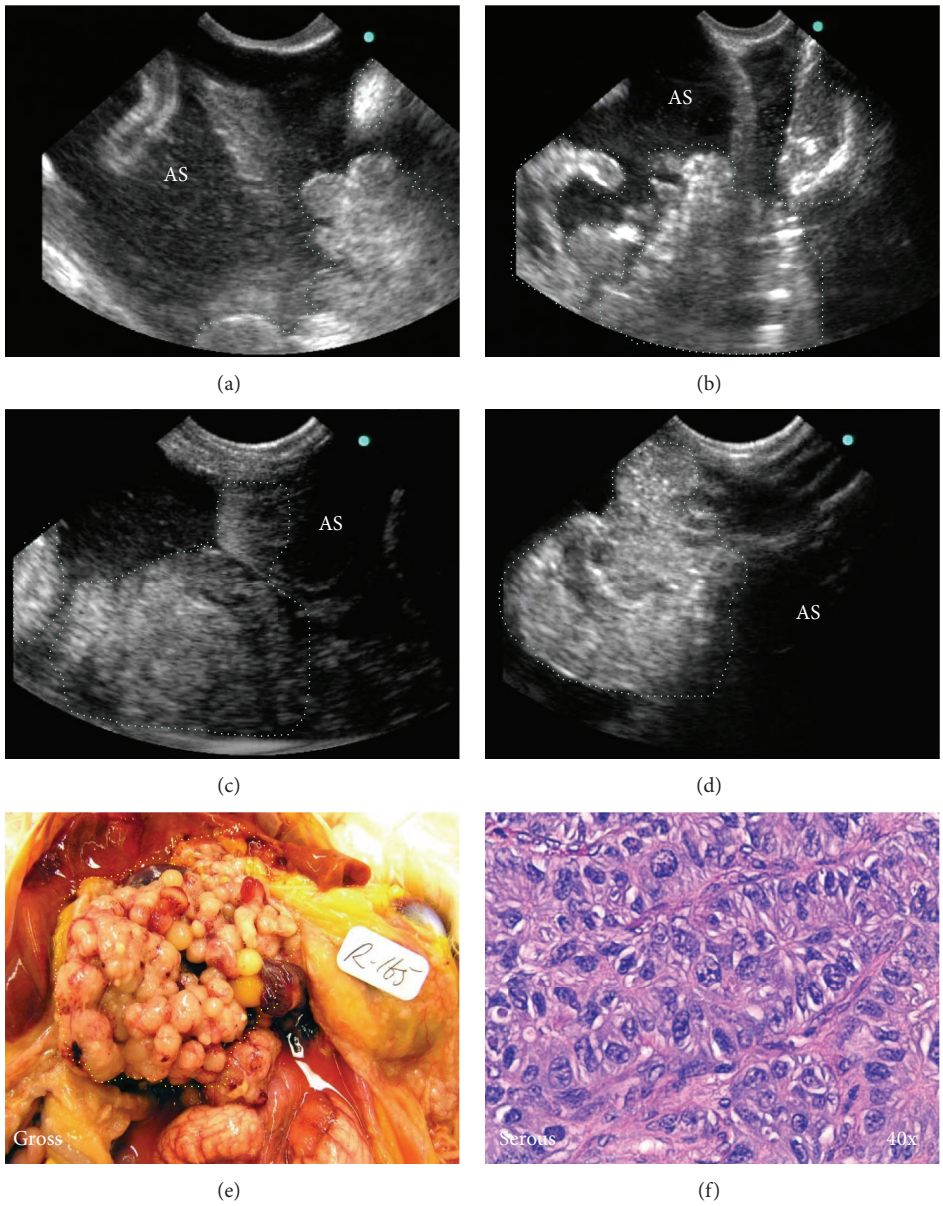
2.6. Statistical Analysis. Descriptive statistics for imaging parameters were determined, and statistical analysis was performed in SPSS version 15 (SPSS Inc., Chicago, IL). The differences in the net intensities of ultrasound imaging and the frequencies of IL-16 expressing cells and microvessels among normal hens or hens with early and late stage OVCA were analyzed by the two-sample t test. The association between the intensity of ultrasound imaging and the frequency of IL-16 expressing cells or microvessels was examined by Pearson coefficient of correlations. $P < 0.05$ was considered significant. All reported P values are 2 sided.

3. Results

3.1. Evaluation of Noninvasive Contrast Enhanced Ultrasound Imaging. In normal hens with functional ovaries, multiple preovulatory follicles and small growing stromal follicles were observed on precontrast and contrast imaging. Compared to precontrast ovaries, visualization of solid ovarian masses with or without projected septa and papillary structures or both were enhanced remarkably in the ovaries of 23 hens. Of these 23 hens, 16 had solid masses in the ovary together with profuse ascites and were predicted to have late stage OVCA (Figures 1(a)–1(d)). In the remaining 7 hens, solid masses were limited to a part of the ovary with no or little ascites, and they were provisionally categorized as early stage OVCA (Figures 2(a) and 2(b)). Compared with precontrast scanning, IL-16-targeted contrast enhanced imaging improved the visualization of ovarian tumor masses in these 23 hens on gray scale (Figures 1 and 2). All of these hens were categorized as "hens with suspected ovarian cancer."

All hens were euthanized following IL-16-targeted contrast imaging and sonographic predictions and stages of the tumor were confirmed by gross examination of hens at necropsy (Figures 1(e) and 2(d)). Ovarian morphology including ovarian follicles and their sizes, oviducts, presence of solid mass in the ovary, levels of tumor metastasis, OVCA stages, and accompanying ascites were recorded and tissues were processed as mentioned above. Tumor types were determined by routine hematoxylin and eosin staining (H&E) of paraffin sections (Figure 1(f)). Staging of ovarian tumors was performed as reported previously [21]. As observed during targeted imaging, late stage OVCA ($n = 16$ hens including 7 serous, 6 endometrioid, and 3 mucinous) was associated with moderate to profuse ascites and metastasized to peritoneal and abdominal organs. Tumors in early stage OVCA ($n = 7$ including 4 serous, 2 endometrioid, and 1 mucinous) were limited to the ovary with no or little ascites.

Overall, mean signal intensity (mean ± SD) of IL-16-targeted imaging in normal healthy hens with low egg laying rates was $27.7 \times 10^5 \pm 3.3 \times 10^5$ which was 1.06-fold higher than the precontrast signal intensities (Figure 3). However the difference was not statistically significant. On the other hand, compared with precontrast ($39.9 \times 10^5 \pm 10.8 \times 10^5$) imaging, the mean signal intensity increased significantly ($P < 0.05$) to $61.9 \times 10^5 \pm 21.2 \times 10^5$ in postcontrast imaging in hens with tumor masses limited to the ovary (early stage). Thus, IL-16-targeted contrast enhanced imaging increased ultrasound signal intensity to 1.55-fold in hens with early stage OVCA (Figure 3). Similarly, in hens with late stage OVCA, the mean signal intensity (mean ± SD) increased



(a) (b) (c) (d) (e) (f)

Figure 1: Enhancement of signal intensity of ultrasound imaging of hen ovarian tumors by IL-16-targeted contrast agents. (a) Case 1: precontrast gray scale ultrasonogram of a hen ovary showing solid mass (dotted lines) with septa and accompanied ascites (AS). (b) Postcontrast gray scale ovarian sonogram of the same hen showing enhanced visualization of the solid tumor mass. (c) Case 2: precontrast gray scale sonogram depicting a suspected ovarian mass (dotted lines) in another hen. (d) Gray scale sonogram of the same ovary (shown in (c)), depicting solid tumor mass with enhanced signal intensity after the injection of targeted imaging agents. (e) Gross presentation confirmed the imaging prediction of an ovarian tumor (shown in (c)-(d), appeared as cauliflower-shaped, yellow circled). (f) Histological examination showed a serous malignant tumor with cells containing large pleomorphic nuclei surrounded by a sheath of fibromuscular tissues. H&E staining.

significantly ($P < 0.001$) from $50.88 \times 10^5 \pm 10.37 \times 10^5$ in precontrast imaging to $67.89 \times 10^5 \pm 10.86 \times 10^5$ in postcontrast imaging (Figure 3). Pre- as well as postcontrast ultrasound signal intensities did not differ significantly among different histological subtypes of ovarian tumors.

3.2. Immunohistochemical Detection of IL-16 Expressing Cells and Microvessels. IL-16 expressing cells were detected in the stroma of normal or tumor-bearing ovaries and in the tumor vicinity including spaces between tumor glands (Figure 4, top panel). A number of epithelial cells (not all) in normal or tumor glands were also positive for IL-16 (Figure 4, top panel (B) and (C)). Very few IL-16 expressing cells were seen in the ovarian stroma and the follicular theca layer of normal healthy hens with low egg laying rates (Figure 4, top panel (A)). Compared with normal hens many IL-16 expressing cells were localized in hens with OVCA (Figure 4, top panel (B)-(C)). The frequency of stromal IL-16 expressing cells was

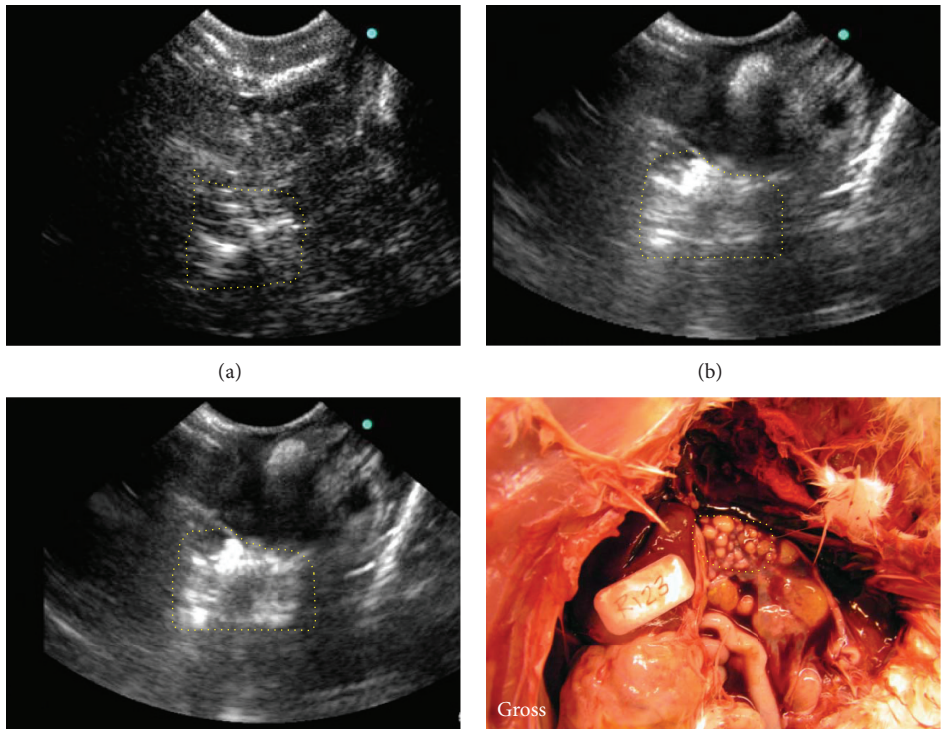


Figure 2: Detection of spontaneous ovarian tumors at early stage in hens by IL-16-targeted contrast enhanced ultrasound imaging. (a) Precontrast ovarian sonogram showing low intensity of ultrasound imaging. Presence of tumor-related solid mass in the ovary is inconclusive. (b)-(c) Corresponding contrast enhanced sonogram with enhanced visualization of ultrasound imaging at 5 min and 7 min after the injection of contrast agents, respectively, suggesting the presence of a small solid mass (yellow dotted lines) in the ovary. (d) Gross morphology shows the presence of a tissue mass (yellow dotted line) limited to a part of the ovary accompanied with a little ascites.

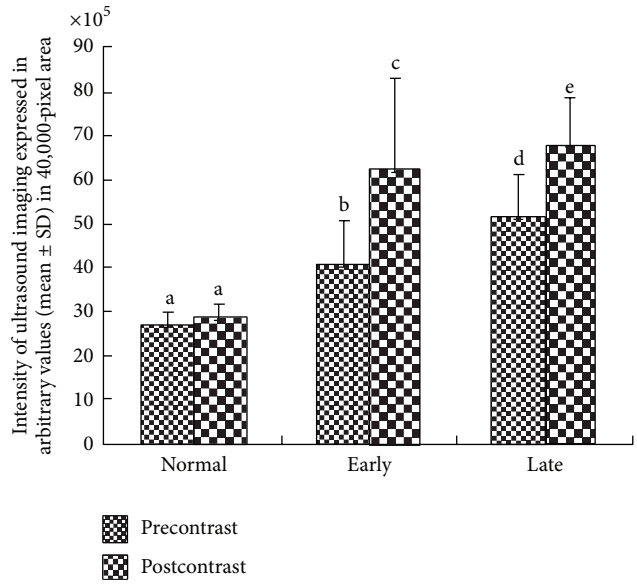


Figure 3: Changes in the signal intensity of ultrasound imaging by IL-16-targeted contrast agent in the ovary of laying hens with or without ovarian cancer (OVCA). Compared with precontrast imaging, IL-16-targeted contrast agents enhanced the intensities of ultrasound imaging significantly in hens with early stage OVCA as well as in late stage OVCA. However, significant differences were not observed between the pre- and postcontrast imaging in healthy hens. Different letters denote significant differences in the intensities of ultrasound imaging between the pre- and postcontrast imaging within the same group including hens with normal ovaries and with early and late stages of OVCA.

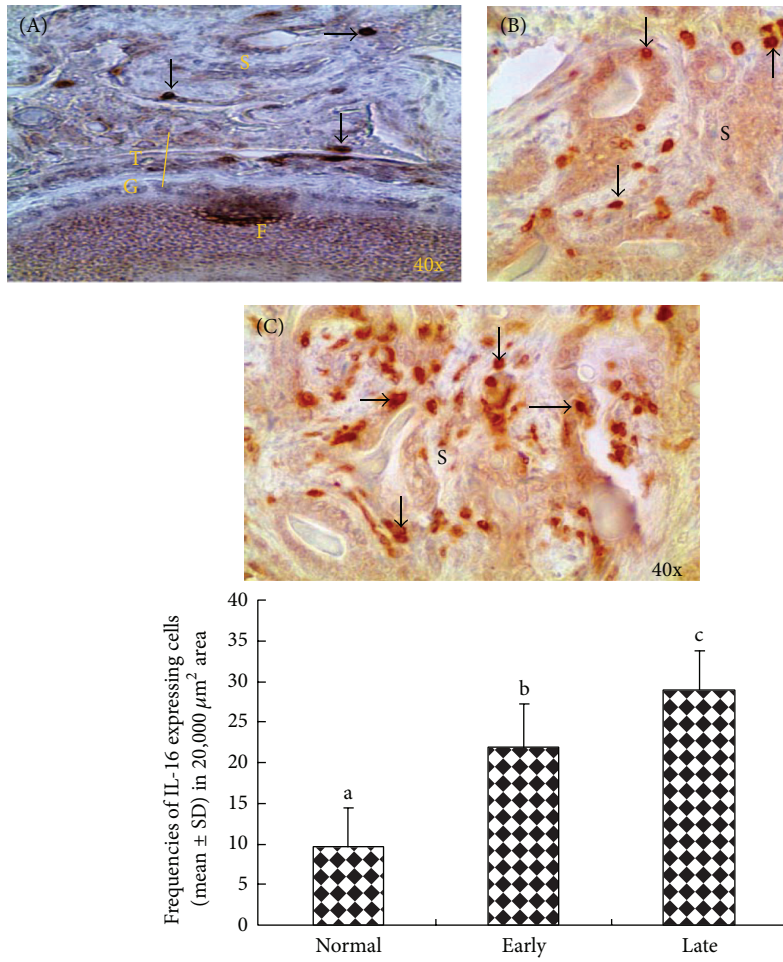


Figure 4: Immunohistochemical localization of IL-16 expressing cells in the ovaries of hens predicted to be normal or cancerous by IL-16-targeted contrast enhanced ultrasound imaging. *Top Panel*. (A) Section of a normal hen ovary showing few IL-16 expressing cells in the ovarian stroma (S) and the follicular (F) theca (T). (B)-(C) Sections of tumor ovaries at early (B) and late (C) stages of OVCA. Compared with normal ovary, many IL-16 expressing cells are seen in OVCA hens. S = stroma; arrows indicate the examples of IL-16 expressing cells. *Bottom Panel*. Compared with normal hens, the frequency of IL-16 expressing cells increased significantly ($P < 0.001$) with tumor development and progression to late stages. Bars with different letters indicate significant differences in the frequencies of IL-16 expressing cells among hens with normal, early stage, and late stage OVCA.

significantly ($P < 0.0001$) higher in hens with early stage OVCA (mean ± SD = 21.85 ± 5.42 in 20,000 μm^2 of tumor tissue) than in normal hens (9.56 ± 4.87 in 20,000 μm^2 of ovarian stromal tissue) and increased further in hens with late stage of OVCA (28.56 ± 5.08 in 20,000 μm^2 of tumor tissue) (Figure 4, bottom panel).

IL-16 expressing microvessels were detected in both normal ovaries and ovaries with tumor (Figure 5, top panel (A)–(C)). In normal ovaries, very few IL-16 expressing microvessels were seen in ovarian stroma (Figure 5, top panel (A)). Compared with normal ovary, many IL-16 expressing microvessels were localized in the stroma of ovaries with tumor (Figure 5, top panel (B)-(C)). The frequencies of IL-16 expressing microvessels were significantly ($P < 0.0001$) greater in hens with early stage OVCA (mean ± SD = 7.0 ± 1.29 in 20,000 μm^2 of tumor tissue) than in normal hens (1.71 ± 0.49 in 20,000 μm^2 of ovarian stromal tissue) and increased further ($P < 0.0001$) in hens with late stage of OVCA (10.33 ± 2.38 in 20,000 μm^2 of tumor tissue) (Figure 5, bottom panel). Differences in the frequencies of IL-6 expressing microvessels were not observed among different histological subtypes of malignant ovarian tumors in hens.

Increases in signal intensities due to IL-16-targeted contrast imaging were positively correlated with the frequencies of IL-16 expressing microvessels in ovarian tumors at early stage ($r = 0.46$) and late stage ($r = 0.70$). These results support the predictions of IL-16-targeted contrast imaging that enhanced signal intensity due to the contrast imaging in hens with tumors was due to the increased IL-16 expressing cells and microvessels in the ovaries with tumors.

4. Discussion

This study examined, for the first time, suitability of IL-16-targeted contrast agent, a newly developed ultrasound imaging agent, in improving the *in vivo* visualization of ovarian

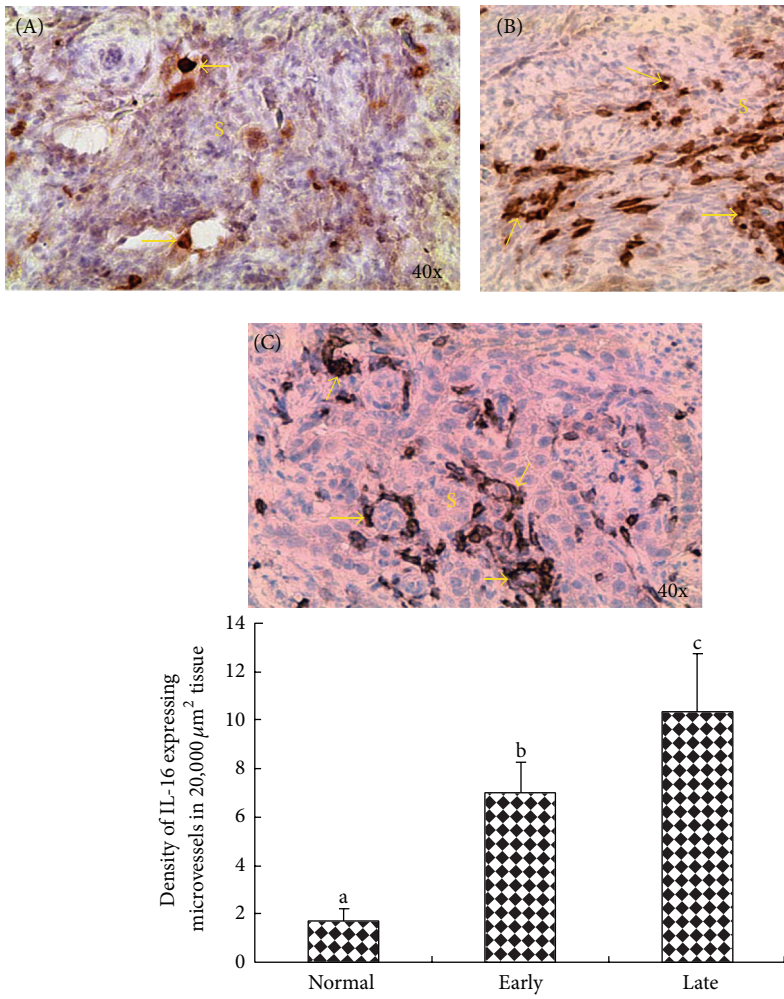


Figure 5: Expression of IL-16 by microvessels in the ovaries of hens with or without ovarian tumors scanned by targeted ultrasound imaging. *Top Panel*. (A) Section of a normal ovary showing few IL-16 expressing microvessels in the stroma (S). (B) and (C) Sections of malignant ovaries at early (B) and late (C) stages of OVCA. Compared with normal hens (A), more IL-16 expressing microvessels are seen in OVCA hens. S = stroma; arrows indicate examples of IL-16 expressing microvessels. *Bottom Panel*. Compared with normal hens, the frequency of IL-16 expressing microvessels was significantly ($P < 0.001$) high in OVCA hens at early and late stages. Bars with different letters indicate significant differences in the frequencies of IL-16 expressing microvessels among hens with normal, early stage, and late stage OVCA.

tumors in laying hens, a preclinical model of spontaneous OVCA. The results of this study demonstrated that IL-16-targeted contrast imaging agents bound with their targets expressed by ovarian tumors at early and late stages in hens and enhanced the intensities of ultrasound imaging signals from these tumors.

Increased expression of IL-16, a proinflammatory cytokine, has been reported to be associated with the development and progression of several malignancies including OVCA [14, 15, 31]. In addition to stromal cells of the tumor, tumor epithelium has also been reported to express IL-16 [14, 15]. Thus IL-16 expressing cells in ovarian tumors represent a potential target for ultrasound imaging for noninvasive detection of OVCA at early stage provided a targeted imaging agent is developed. In this study, compared with precontrast, IL-16-targeted contrast enhanced imaging increased the ultrasound signal intensity remarkably from hens with ovarian tumors at both early and late stages. These results suggest that IL-16-targeted contrast agents bound with their targets in the tumor tissues. In addition, as reported earlier for humans and hens [14, 15], this study also showed significant increase in the frequency of IL-16 expressing cells in hens with early and late OVCA compared to normal hens. Thus, higher signal intensities in hens with early and late stage OVCA than in normal hens may be, in part, due to the increased frequency of targets (IL-16 expressing cells) in OVCA hens which bound with their ligands (IL-16-targeted imaging agents).

IL-16 is a proangiogenic factor suggested to stimulate tumor-associated angiogenesis [16]. Furthermore, the frequency of IL-16 expressing cells was reported to be positively correlated with the frequencies of smooth muscle actin (SMA) expressing microvessels [14] during OVCA development and progression in hens. In this study, endothelial

cells of microvessels expressed IL-16. Furthermore, this study also showed that the density of IL-16 expressing microvessels increased significantly with the development of OVCA and increased further as the tumor progressed to late stages. The frequencies of tumor-associated microvessels expressing $\alpha_v\beta_3$-integrins and VEGFR-2 have also been suggested to increase contrast enhanced ultrasound signal intensities [32, 33]. Thus, in addition to malignant cells, increase in the frequency of IL-16 expressing microvessel might also be a reason for the increased signal intensities during contrast enhanced imaging in hens with OVCA.

The results observed in the current study have, from translational point of view, some exceptional aspects. First, most of the contrast agents so far developed including two most extensively studied agents $\alpha_v\beta_3$-integrins and VEGFR-2 have limited success as expression of these targets was mainly limited to the blood vessels. Moreover, no corresponding serum markers of these imaging targets specific to OVCA have been established making their application difficult for early detection of OVCA. In contrast, in addition to the expression of IL-16 by the tumor epithelium and the microvessels, IL-16 is also secreted into the circulation. Serum levels of IL-16 have been reported to be increased significantly in association with OVCA development and progression [14, 15]. Thus, serum IL-16 levels offer a potential marker to be used in conjunction with IL-16-targeted contrast enhanced ultrasound imaging for the detection of OVCA at early stage. Second, most of the previous studies used rodent models with induced tumors. On the other hand, this study used laying hens, the only widely available and easily accessible spontaneous model of OVCA. Rodents do not develop OVCA spontaneously and the histopathology of induced OVCA is not similar to those of spontaneous OVCA. Moreover, anatomical differences in the location of induced rodent models (subcutaneous tumor) compared with deeper tissue like the ovary may also affect the transduction of ultrasound signals as well as the behavior of contrast agents. Thus information on the binding ability and detection of spontaneous OVCA by contrast agents (as seen for IL-16) is essential. Third, chickens are easy to access to test and develop targeted imaging agents as well as anti-OVCA drugs for the detection and treatment of spontaneous OVCA. Moreover, because of the lower cost of hens, this model is also suitable for toxicological studies of newly developed imaging agent or therapeutic in a cost-effective way. Presently, studies with hens are ongoing in which animals are being monitored prospectively with IL-16-targeted contrast agents together with serum IL-16 levels to detect spontaneous ovarian tumor development at relatively earlier stages. This study has also some limitations. We did not use animals with benign ovarian tumors. Small sample size specially the number of hens with ovarian tumors may also be a limitation of this study.

5. Conclusion

Overall, the results of the present study suggest that the IL-16-targeted contrast agents bind with their targets expressed by the spontaneous ovarian tumors in hens and enhance the visualization of tumors at early and late stages. This study also suggests that laying hens offer a new avenue for testing and development of new contrast agents and targeted antiangiogenic therapeutics.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] American Cancer Society, "Cancer Facts & Figures 2013," 2013, http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2013/index.

[2] R. Siegel, J. Ma, Z. Zou, and A. Jemal, "Cancer statistics, 2014," *CA: A Cancer Journal for Clinicians*, vol. 64, no. 1, pp. 9–29, 2014.

[3] U. Menon, M. Griffin, and A. Gentry-Maharaj, "Ovarian cancer screening—current status, future directions," *Gynecologic Oncology*, vol. 132, no. 2, pp. 490–495, 2014.

[4] S. S. Buys, E. Partridge, A. Black et al., "Effect of screening on ovarian cancer mortality: the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Randomized Controlled Trial," *The Journal of the American Medical Association*, vol. 305, no. 22, pp. 2295–2302, 2011.

[5] J. S. Abramowicz, "Ultrasonographic contrast media: has the time come in obstetrics and gynecology?" *Journal of Ultrasound in Medicine*, vol. 24, no. 4, pp. 517–531, 2005.

[6] S. Dutta, F.-Q. Wang, A. C. Fleischer, and D. A. Fishman, "New frontiers for ovarian cancer risk evaluation: proteomics and contrast-enhanced ultrasound," *American Journal of Roentgenology*, vol. 194, no. 2, pp. 349–354, 2010.

[7] A. C. Fleischer, A. Lyshchik, R. F. Andreotti, M. Hwang, H. W. Jones III, and D. A. Fishman, "Advances in sonographic detection of ovarian cancer: depiction of tumor neovascularity with microbubbles," *The American Journal of Roentgenology*, vol. 194, no. 2, pp. 343–348, 2010.

[8] A. C. Fleischer, A. Lyshchik, H. W. Jones III et al., "Diagnostic parameters to differentiate benign from malignant ovarian masses with contrast-enhanced transvaginal sonography," *Journal of Ultrasound in Medicine*, vol. 28, no. 10, pp. 1273–1280, 2009.

[9] A. C. Fleischer, A. Lyshchik, H. W. Jones Jr. et al., "Contrast-enhanced transvaginal sonography of benign versus malignant ovarian masses: preliminary findings," *Journal of Ultrasound in Medicine*, vol. 27, no. 7, pp. 1011–1018, 2008.

[10] C. R. Anderson, J. J. Rychak, M. Backer, J. Backer, K. Ley, and A. L. Klibanov, "ScVEGF microbubble ultrasound contrast agents: a novel probe for ultrasound molecular imaging of tumor angiogenesis," *Investigative Radiology*, vol. 45, no. 10, pp. 579–585, 2010.

[11] J. K. Willmann, R. Paulmurugan, K. Chen et al., "US imaging of tumor angiogenesis with microbubbles targeted to vascular

endothelial growth factor receptor type 2 in mice," *Radiology*, vol. 246, no. 2, pp. 508–518, 2008.

[12] A. Macciò and C. Madeddu, "Inflammation and ovarian cancer," *Cytokine*, vol. 58, no. 2, pp. 133–147, 2012.

[13] W. W. Cruikshank, H. Kornfeld, and D. M. Center, "Interleukin-16," *Journal of Leukocyte Biology*, vol. 67, no. 6, pp. 757–766, 2000.

[14] A. Yellapa, J. M. Bahr, P. Bitterman et al., "Association of interleukin 16 with the development of ovarian tumor and tumor-associated neoangiogenesis in laying hen model of spontaneous ovarian cancer," *International Journal of Gynecological Cancer*, vol. 22, no. 2, pp. 199–207, 2012.

[15] A. Yellapa, P. Bitterman, S. Sharma et al., "Interleukin 16 expression changes in association with ovarian malignant transformation," *American Journal of Obstetrics & Gynecology*, vol. 210, no. 3, pp. 272.e1–272.e10, 2014.

[16] N. L. Mathy, W. Scheuer, M. Lanzendörfer et al., "Interleukin-16 stimulates the expression and production of pro-inflammatory cytokines by human monocytes," *Immunology*, vol. 100, no. 1, pp. 63–69, 2000.

[17] D. J. Lee, A. Lyshchik, J. Huamani, D. E. Hallahan, and A. C. Fleischer, "Relationship between retention of a vascular endothelial growth factor receptor 2 (VEGFR2)-targeted ultrasonographic contrast agent and the level of VEGFR2 expression in an in vivo breast cancer model," *Journal of Ultrasound in Medicine*, vol. 27, no. 6, pp. 855–866, 2008.

[18] A. Lyshchik, A. C. Fleischer, J. Huamani, D. E. Hallahan, M. Brissova, and J. C. Gore, "Molecular imaging of vascular endothelial growth factor receptor 2 expression using targeted contrast-enhanced high-frequency ultrasonography," *Journal of Ultrasound in Medicine*, vol. 26, no. 11, pp. 1575–1586, 2007.

[19] J. K. Willmann, A. M. Lutz, R. Paulmurugan et al., "Dual-targeted contrast agent for US assessment of tumor angiogenesis in vivo," *Radiology*, vol. 248, no. 3, pp. 936–944, 2008.

[20] C. Rodríguez-Burford, M. N. Barnes, W. Berry, E. E. Partridge, and W. E. Grizzle, "Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention," *Gynecologic Oncology*, vol. 81, no. 3, pp. 373–379, 2001.

[21] A. Barua, P. Bitterman, J. S. Abramowicz et al., "Histopathology of ovarian tumors in laying hens: a preclinical model of human ovarian cancer," *International Journal of Gynecological Cancer*, vol. 19, no. 4, pp. 531–539, 2009.

[22] T. N. Fredrickson, "Ovarian tumors of the hen," *Environmental Health Perspectives*, vol. 73, no. 1, pp. 35–51, 1987.

[23] E. Jackson, K. Anderson, C. Ashwell, J. Petitte, and P. E. Mozdziak, "CA125 expression in spontaneous ovarian adenocarcinomas from laying hens," *Gynecologic Oncology*, vol. 104, no. 1, pp. 192–198, 2007.

[24] K. Stammer, S. L. Edassery, A. Barua et al., "Selenium-Binding Protein 1 expression in ovaries and ovarian tumors in the laying hen, a spontaneous model of human ovarian cancer," *Gynecologic Oncology*, vol. 109, no. 1, pp. 115–121, 2008.

[25] J. R. Giles, H. L. Shivaprasad, and P. A. Johnson, "Ovarian tumor expression of an oviductal protein in the hen: a model for human serous ovarian adenocarcinoma," *Gynecologic Oncology*, vol. 95, no. 3, pp. 530–533, 2004.

[26] M. F. Khan, J. M. Bahr, A. Yellapa et al., "Expression of leukocyte inhibitory immunoglobulin-like transcript 3 receptors by ovarian tumors in laying hen model of spontaneous ovarian cancer," *Translational Oncology*, vol. 5, no. 2, pp. 85–91, 2012.

[27] A. Barua, J. S. Abramowicz, J. M. Bahr et al., "Detection of ovarian tumors in chicken by sonography: a step toward early diagnosis in humans?" *Journal of Ultrasound in Medicine*, vol. 26, no. 7, pp. 909–919, 2007.

[28] A. Barua, P. Bitterman, J. M. Bahr et al., "Detection of tumor-associated neoangiogenesis by Doppler ultrasonography during early-stage ovarian cancer in laying hens: a preclinical model of human spontaneous ovarian cancer," *Journal of Ultrasound in Medicine*, vol. 29, no. 2, pp. 173–182, 2010.

[29] A. Barua, P. Bitterman, J. M. Bahr et al., "Contrast-enhanced sonography depicts spontaneous ovarian cancer at early stages in a preclinical animal model," *Journal of Ultrasound in Medicine*, vol. 30, no. 3, pp. 333–345, 2011.

[30] A. Barua, A. Yellapa, J. M. Bahr et al., "Expression of death receptor 6 by ovarian tumors in laying hens, a preclinical model of spontaneous ovarian cancer," *Translational Oncology*, vol. 5, no. 4, pp. 260–268, 2012.

[31] J. Richmond, M. Tuzova, W. Cruikshank, and D. Center, "Regulation of cellular processes by interleukin-16 in homeostasis and cancer," *Journal of Cellular Physiology*, vol. 229, no. 2, pp. 139–147, 2014.

[32] A. Barua, A. Yellapa, J. M. Bahr et al., "Enhancement of ovarian tumor detection with $\alpha v\beta 3$ integrin-targeted ultrasound molecular imaging agent in laying hens: a preclinical model of spontaneous ovarian cancer," *International Journal of Gynecological Cancer*, vol. 24, no. 1, pp. 19–28, 2014.

[33] A. Barua, T. Qureshi, P. Bitterman et al., "Abstract 2455: molecular targeted imaging of vascular endothelial growth factor receptor (VEGFR)-2 and anti-NMP autoantibodies detect ovarian tumor at early stage," *Cancer Research*, vol. 72, no. 8, supplement 1, p. 2455, 2012.