Gynecologic Oncology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Assessment of DNA Ploidy in the ProMisE molecular subgroups of endometrial cancer

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HIGHLIGHTS

- Abnormal DNA ploidy was significantly higher in the p53 abn molecular group of EC, compared to the other molecular groups.
- Abnormal DNA ploidy correlated with worse PFS, lower BMI, higher grade and non-endometrioid histotypes.
- In the MMR-D group, DNA ploidy provided additional prognostic value, which merits further study in a larger series of EC.

ARTICLE INFO

Article history:
Received 24 May 2017
Received in revised form 15 June 2017
Accepted 15 June 2017
Available online xxxx

Keywords:
Endometrial carcinoma
DNA ploidy
Aneuploidy
Image cytometry
Molecular
The Cancer Genome Atlas
TCGA

ABSTRACT

Objective. We sought to determine whether DNA ploidy correlates with the four molecular subgroups of endometrial carcinoma (EC) as determined using ProMisE (Proactive Molecular Risk Classifier for Endometrial Cancer).

Methods. 90 cases of EC previously characterized by clinicopathological parameters, outcomes, and ProMisE molecular subgroup (*POLE* EDM, MMR-D, p53 wt or p53 abn) were assessed for DNA ploidy using image cytometry. Associations of ploidy with traditional clinicopathological parameters were also tested.

Results. Abnormal DNA ploidy status differed amongst the ProMisE groups ($p < 0.001$) and was found in 80.9% (17/21) of p53 abn, 37.0% (10/27) of p53 wt, 28.6% (4/14) of *POLE* EDM and 14.3% (4/28) of MMR-D. Abnormal DNA content was significantly associated with lower BMI ($p = 0.034$) and grade 3 tumors ($p = 0.001$). In the entire cohort, abnormal DNA content was significantly associated with worse progression free survival ($p = 0.0094$) but not disease specific survival ($p = 0.249$) or overall survival ($p = 0.187$). When examining ploidy within each of the ProMisE groups, abnormal DNA content correlated with worse overall survival ($p = 0.041$) and progression free survival ($p = 0.011$) in the MMR-D group. No statistically significant relationship was seen in the remaining 3 groups.

Conclusion. Abnormal DNA ploidy status did correlate with the molecular subgroups of EC; abnormal DNA content was seen in the large majority of p53 abn cases. Abnormal ploidy however was also seen in smaller numbers in the p53 wt, *POLE* EDM and MMR-D groups; therefore abnormal DNA content was not a specific marker for any one molecular group. The addition of ploidy to the ProMisE molecular categories conferred additional prognostic value within the MMR-D group, which merits further study.

1. Introduction

Endometrial cancer (EC) is the fourth most common cause of cancer in women, and the most prevalent gynaecologic malignancy in the developed world [1]. Paralleling a global trend, in the last decade the

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http://dx.doi.org/10.1016/j.ygyno.2017.06.020
0090-8258/

incidence rate of EC in Canada has increased by 2.5% a year [2], likely attributable to an aging population and the obesity epidemic [3]. Although the majority of women are diagnosed at an early stage and have good outcomes, even within clinical stage 1 disease, the 5-year survival ranges from 42% to 90% [4]. This highlights the heterogeneity of the disease and the need to accurately predict high-risk cases.

Currently, pathological factors used to guide the need for adjuvant treatment include surgical FIGO stage (International Federation of Gynaecology and Obstetrics), histotype, tumor grade and the presence or absence of lymphovascular invasion [3,4]. Using these clinical parameters, multiple systems of risk prediction have been developed with the intention of guiding appropriate surgical and adjuvant treatment [5–9]. It is becoming increasingly evident however, that these pathological markers are not always reproducible and can be subject to marked interobserver variability [10–13]. This knowledge has served as a catalyst for the identification of objective, molecular-based prognostic determinants.

Using integrated genomic, transcriptomic and proteomic data, the Cancer Genome Atlas (TCGA) Research Network classified endometrial cancers into four prognostic molecular categories [14]. Since then two groups, including our own, have developed more pragmatic and cost-effective methods to replicate these groups [15,16]. ProMisE (Proactive Molecular Risk Classifier for Endometrial Cancer) uses an iterative combination of protein expression (immunohistochemistry) and focused sequencing to assign EC patients to one of four risk groups: MMR-D (Mismatch Repair Deficient), *POLE* EDM (Polymerase Epsilon Exonuclease Domain Mutated), p53 abn (p53 abnormal) and p53 wt (p53 wild-type), which are analogous albeit not identical to the TCGA MSI (microsatellite instability) hypermutated, *POLE* ultramutated, copy-number high and copy-number low categories.

Copy number alterations in tumor cells can be due to aneuploidies (whole or segmental) or smaller intragenic copy number variations. Large-scale copy number alterations are most often due to aneuploidy, which is also the most common genetic abnormality seen in malignant cells [17]. The prognostic value of DNA ploidy in endometrial cancer was first published by Atkin in 1959 [18]. Since then, multiple studies have evaluated the significance of aneuploidy or non-diploid DNA content in endometrial cancer [19]. DNA ploidy has been correlated with lymphovascular invasion [20] as well as lymph node involvement [21]. Ploidy has been shown to be an adverse prognosticator in microsatellite stable tumors [22] and is an independent prognostic marker in both early stage endometrioid and serous EC's [23,24]. Moreover, the methods used to test tumor ploidy have evolved from using traditionally flow cytometry (requiring the suspension of individual cells and thus making the use of archival formalin-fixed paraffin embedded tissues challenging) to automated image-based cytometry. As a result, ploidy has gained popularity over the last decade, and has been shown to be a valuable prognosticator in a wide spectrum of tumor types [25].

The primary goal of our study was to determine if DNA ploidy could be used as a surrogate marker for any of the ProMisE molecular subgroups, particularly if DNA ploidy could be used to distinguish between the p53 abn and p53 wt groups. Secondary goals of our study were to determine if abnormal DNA content (aneuploidy or tetraploidy) correlated with any traditional clinicopathologic variables and if ploidy could add additional prognostic information to any one of the four ProMisE molecular groups of endometrial cancer.

2. Methods

2.1. Selection and classification of EC cases

From a prior series of over 400 endometrial carcinomas from the Vancouver General Hospital OvCaRe Tissue Bank Repository, sequencing for the exonuclease domain (EDM) of polymerase epsilon (*POLE*) and immunohistochemistry (IHC) for DNA mismatch repair (MMR) proteins and p53 were performed as previously described [16]. Using ProMisE, cases were assigned to one of four molecular groups designated as: 1. MMR-D, 2. *POLE* EDM, 3. p53 abn or 4. p53 wt, as previously described by Talhouk et al. [16]. From this cohort, 90 of the most recent cases of EC were analyzed for DNA ploidy: 14 *POLE* EDM, 28 MMR-D, 27 p53 wt, and 21 p53 abn, with an attempt to acquire fair representation from all 4 groups. IHC for estrogen receptor (ER) and progesterone receptor (PR) were also performed on tissue microarrays. Any intensity of staining in >1% of tumor cells was considered positive.

Clinical and pathological parameters collected included age, body mass index (kg/m^2), stage (updated according to FIGO 2009 classification), grade, histological subtype, lymphovascular space invasion, nodal status, and adjuvant therapy. Clinical risk groups were assigned according to the European Society of Medical Oncologists (ESMO) criteria [26] by two clinicians. Discordant results were discussed and a consensus was reached.

Research ethics approval for the Tissue/Biospecimen Bank and this project was granted from the University of British Columbia Institutional Review Board and all patients underwent informed written consent for the use of their biospecimens for research purposes.

2.2. Image cytometric DNA ploidy analysis

DNA ploidy analyses were performed at the Institute for Cancer Genetics and Informatics, Radiumhospitalet, Oslo University Hospital. For DNA ploidy analyses, monolayers were prepared from 50 μm thick sections of tumor tissue obtained from paraffin-embedded tissue blocks as detailed [27]. Briefly, the sections were deparaffinized, rehydrated, treated with protease (Sigma P5380) and stirred with magnetic stirring bars to disaggregate the cells. After filtering and centrifuging the cell suspensions, monolayers were made on poly L-lysin coated slides using the pellets. Subsequently, the monolayers were air-dried, fixed in 4% formaldehyde, hydrolysed in 5 M HCl and stained with Schiff's solution.

Using the Ploidy Work Station (PWS) Grabber (Room4 Ltd., Crowborough, East Sussex, UK) and a Zeiss Axioplan microscope equipped with a 546-nm green filter and a monochrome high-resolution digital camera (Axiocam MRM, Zeiss, Jena, Germany), images of minimum 1500 Feulgen stained nuclei were captured automatically. The images were automatically sorted into galleries: nuclei-of-interest for measurement, lymphocytes, plasma cells and fibroblast as reference cells. The automatically sorted nuclei were manually verified and edited to discard cut, overlapped and pyknotic nuclei using PWS Classifier (Room4 Ltd., Crowborough, East Sussex, UK). Integrated optical density of each nucleus was calculated. Histograms, generated using integrated optical density, were classified as diploid, tetraploid or aneuploid. A histogram was classified as diploid if only one peak with DNA index between 0.95 and 1.05 was present, the number of nuclei with DNA index between 1.9 and 2.1 did not exceed 10% of the total number of nuclei and the number of nuclei with a DNA content more than 5c did not exceed 1%. A histogram was classified as tetraploid if a peak with DNA index between 1.9 and 2.1 contained >10% of the nuclei-of-interest. A histogram was classified as aneuploid when one or more non-euploid peaks were present (DNA index <0.95, 1.05–1.89 or >2.1) or the number of nuclei not representing euploid populations with a DNA content more than 5c exceeded 1%.

2.3. Statistical analysis

We analyzed the univariable association between DNA ploidy and each clinicopathological feature using a Chi-squared test for binary and categorical variables and a one-way analysis of variance (using Welch's *t*-test) for continuous variables. Statistical significance was set at = 0.05. Univariable survival analyses (overall survival [OS], disease-specific survival [DSS], and progression-free survival [PFS]) for DNA ploidy, ProMisE and other clinicopathologic features of interest were

Please cite this article as: L. Proctor, et al., Assessment of DNA Ploidy in the ProMisE molecular subgroups of endometrial cancer, Gynecol Oncol (2017), http://dx.doi.org/10.1016/j.ygyno.2017.06.020