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JNCI J Natl Cancer Inst (2018) 110(12): djy063

doi: 10.1093/jnci/djy063
First published online April 18, 2018
Article

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Association Between Proportion of Nuclei With High Chromatin Entropy and Prognosis in Gynecological Cancers

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Abstract

Background: Nuclear texture analysis measuring differences in chromatin structure has provided prognostic biomarkers in several cancers. There is a need for improved cell-by-cell chromatin analysis to detect nuclei with highly disorganized chromatin. The purpose of this study was to develop a method for detecting nuclei with high chromatin entropy and to evaluate the association between the presence of such deviating nuclei and prognosis.
Methods: A new texture-based biomarker that characterizes each cancer based on the proportion of high–chromatin entropy nuclei (<25% vs ≥25%) was developed on a discovery set of 175 uterine sarcomas. The prognostic impact of this biomarker was evaluated on a validation set of 179 uterine sarcomas, as well as on independent validation sets of 246 early-stage ovarian carcinomas and 791 endometrial carcinomas. More than 1 million images of nuclei stained for DNA were included in the study. All statistical tests were two-sided.
Results: An increased proportion of high–chromatin entropy nuclei was associated with poor clinical outcome. The biomarker predicted five-year overall survival for uterine sarcoma patients with a hazard ratio (HR) of 2.02 (95% confidence interval [CI] = 1.43 to 2.84), time to recurrence for ovarian cancer patients (HR = 2.91, 95% CI = 1.74 to 4.88), and cancer-specific survival for endometrial cancer patients (HR = 3.74, 95% CI = 2.24 to 6.24). Chromatin entropy was an independent prognostic marker in multivariable analyses with clinicopathological parameters (HR = 1.81, 95% CI = 1.21 to 2.70, for sarcoma; HR = 1.71, 95% CI = 1.01 to 2.90, for ovarian cancer; and HR = 2.03, 95% CI = 1.19 to 3.45, for endometrial cancer).
Conclusions: A novel method detected high–chromatin entropy nuclei, and an increased proportion of such nuclei was associated with poor prognosis. Chromatin entropy supplemented existing prognostic markers in multivariable analyses of three gynecological cancer cohorts.

Genomic instability is central in the multistep development of cancer (1,2), and the assessment of large-scale genomic alterations in cancer cell nuclei is useful for predicting clinical outcomes in cancer patients (3). There is a complex relation between genomic alterations and large-scale rearrangement of interphase nuclear chromatin. Chromatin structure is central in both transcriptional regulation and maintenance of genomic stability (4). Chromatin is continually remodeled, and targeted chromatin remodeling determines transcriptional control (5). Modification of chromatin structure also has a regulatory function in DNA repair, replication, and chromosome segregation (5).

Nuclear texture analysis (Nucleotyping) refers to the characterization of chromatin structure based on digital microscope images of cell nuclei (6). Prior to imaging, the nuclei are stained with a DNA-specific stain, and the gray levels in the images thus correspond to DNA content. Nucleotyping describes the changes in chromatin structure in cancer nuclei by measuring

Received: June 23, 2017; **Revised:** December 6, 2017; **Accepted:** March 13, 2018

the spatial arrangement of the pixel gray levels in small subregions of the nuclear images. Entropy is very useful for such quantification of local heterogeneity in the chromatin structure (6–8) and is found to be a prognostic marker in several gynecological cancers (9–11). The Nucleotyping biomarker used in these previous studies was based on average values computed from all measured nuclei from a patient, and was therefore influenced by the majority of the nuclei. It is generally accepted that subpopulations of cells constitute the driving force and have the ability to metastasize during carcinogenesis, and therefore there is a need for improved cell-by-cell chromatin analysis (1,3,9).

The aim of the present study was to test the hypotheses that there exists a subpopulation of cells with high chromatin entropy and that there is an association between the presence of such cell nuclei and clinical outcome.

Methods

Patient Cohorts

Uterine Sarcoma

A total of 587 uterine sarcomas were registered from 1970 to 2000 at the Norwegian Cancer Registry, which gathers information on all cancer events in Norway (12). Survival dates were provided by the Cancer Registry on October 31, 2007, for all patients. The tumors were reclassified by an experienced gynecological pathologist according to World Health Organization (WHO) recommendations (13), and the diagnosis of uterine sarcoma was confirmed in 419 patients (12). Tissue samples from 354 patients were available for analysis (Supplementary Figure 1A, available online) (10,14). This study was approved by the Regional Ethics Committee (REK, No. S-04298), which also approved the decision to not obtain written informed consent for deceased patients.

Early-Stage Ovarian Cancer

Another study included tissue samples from 246 patients treated during 1982–1989 for ovarian cancer classified as International Federation of Gynecology and Obstetrics (FIGO) stage I (Supplementary Figure 1B, available online) (11). Generally, surgery was performed at county hospitals, and the patients were admitted to The Norwegian Radium Hospital for evaluation and further treatment. All patients were followed up until death or December 31, 1998. Follow-up information was also achieved from the National Statistical Bureau, which keeps records of all inhabitants in Norway (15). All histological sections were reviewed by a single pathologist, and the histological classification was performed using WHO criteria (15). The study was in accordance with Norwegian law, which at the time did not require written informed consent to analyze this type of anonymized data.

Endometrial Cancer

A total of 1046 endometrial cancer patients from the international multicenter trial Molecular Markers in Treatment of Endometrial Cancer (MoMaTEC1, Clinical Trial identifier NCT00598845) were treated in the period from 2001 to 2012. Of these, 402 patients were treated at the Haukeland University Hospital, Norway, and 644 patients were included from nine other hospitals. Information about cancer-specific survival was collected from patient records and correspondence with physicians responsible for outpatient follow-up (16). As described earlier, 791 patients were available for analysis (Supplementary Figure 1C, available online) (9). The last follow-up date for these patients was September 12, 2013. Written informed consent was obtained from all patients. The study was approved by REK (REKIII No. 052.01).

Discovery and Validation Cohorts

Uterine sarcomas were divided into discovery (175 patients) and validation (179 patients) data sets (Supplementary Materials, available online) (10). In order to design a classifier for prognostication of cancer patients, the discovery set was grouped into two prognostic classes. The patients who survived for at least five years were defined as good prognosis, whereas the patients who died within five years were defined as poor prognosis (10).

The methodology was developed on the discovery set and then the trained classifier was evaluated on the corresponding validation set, and further evaluated on the independent validation sets of ovarian and endometrial cancer. As we have previously shown that nuclear texture varies with nuclear area and that prognostication could be enhanced by grouping the nuclear images by their areas (17), the biomarker was computed using only nuclei within a certain size range (nuclear area between 54 μm^2 and 134 μm^2), involving 158 868 and 844 003 nuclei in the discovery and validation sets, respectively (Supplementary Materials, available online). Sample preparation and imaging are described in the Supplementary Materials (available online).

Nuclear Texture Analysis

Each pixel in a nuclear image was characterized by the gray level of the pixel and the entropy computed from the gray levels in a small neighborhood around the pixel (Figure 1, A–C). The occurrence of pairs of gray level and entropy values in the nucleus was counted and accumulated in a gray level entropy matrix and then normalized by dividing each element by the total number of nuclear pixels (Figure 1D; Supplementary Materials, available online).

We propose to characterize a given gray level entropy matrix by the center of mass of the distribution (center of mass in gray level and entropy) together with the concentration measure relative matrix area, containing the peak(s) of the distribution (Figure 1, D–F; Supplementary Materials, available online). These three features were computed for all nuclei representing a patient, resulting in a 3D feature plot for each patient (Figure 1, G and H).

In the design phase of the novel biomarker, center of mass in gray level and entropy and relative matrix area were computed from all nuclei in the discovery set, and the resulting 3D feature space was clustered into five subgroups by k-means clustering. Each cluster corresponds to points with similar feature values (ie, similar chromatin texture), and the clusters were sorted according to increasing mean relative matrix area (ie, clusters 1–5 represented nuclei with increasing chromatin entropy) (Supplementary Figure 2, available online). Cluster 5, which contained 10.8% of the nuclei, was defined to represent the high–chromatin entropy nuclei.

The biomarker classifies each patient as low or high chromatin entropy based on the proportion of high–chromatin entropy nuclei (Figure 1H). The threshold of 25% was selected based on training in the discovery set (Supplementary Materials and Supplementary Figures 3–5, available online).

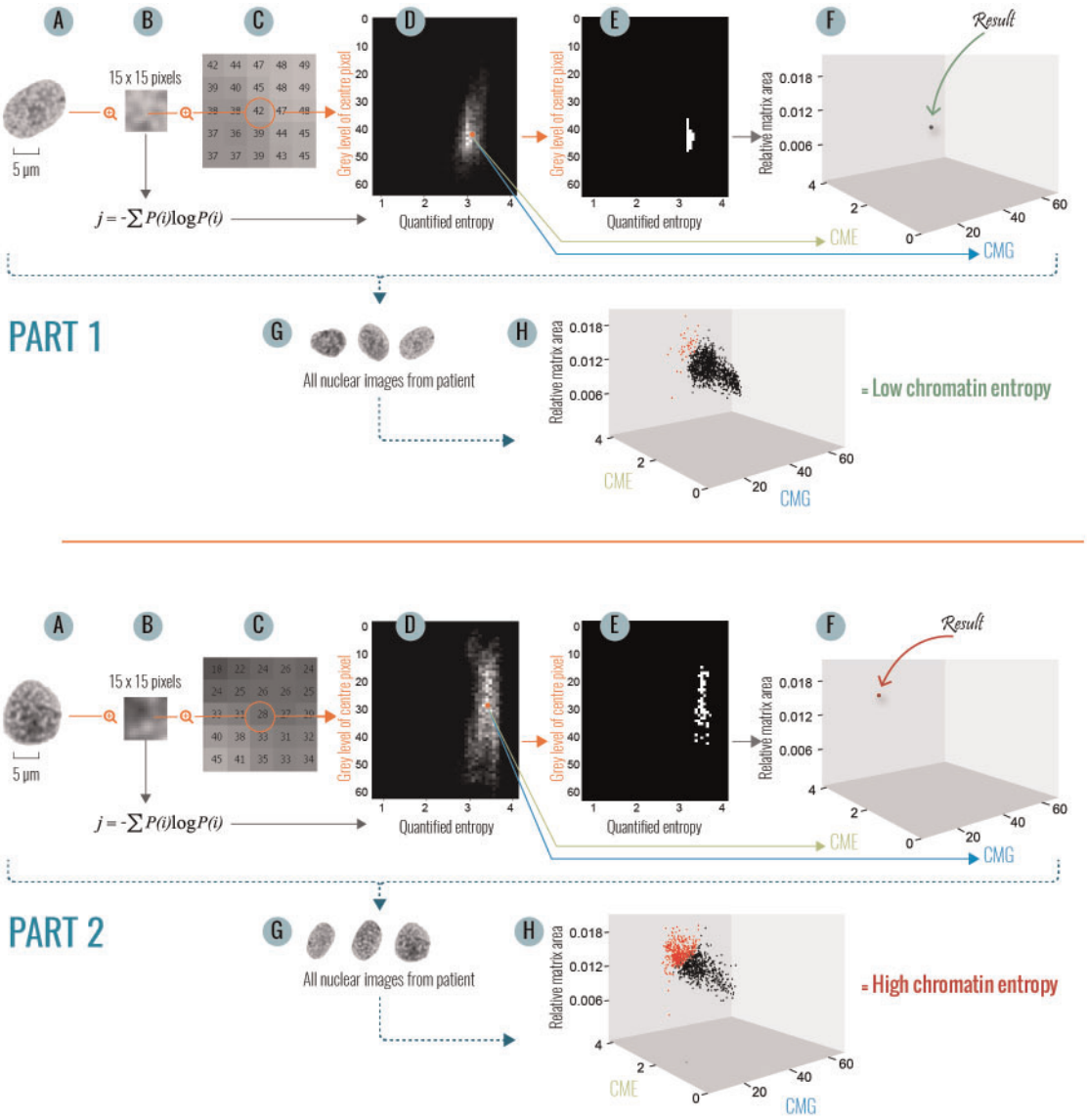

Figure 1. Quantification of chromatin entropy. **A)** A digital image of a cell nucleus. **B)** A quadratic window (15 × 15 pixels) is centered on each pixel in the nucleus, and then the pixel is characterized by the gray level *i* (eg, i = 42) and the entropy *j* computed from the gray levels included in the window (eg, *j* = 3.1), where *P*(*i*) is the normalized frequency of gray level *i* within the window. **C)** Gray levels in a subregion of the window. **D)** The *i* and (quantified) *j* values are used as indices in a two-way table, named a gray level entropy matrix (GLEM), and the frequencies of different (*i*,*j*) combinations are accumulated. The final GLEM is normalized by dividing each element in the table by the total number of nuclear pixels, providing a bivariate probability mass function. **E)** The probability values in the GLEM were sorted in descending order and then summed (starting with the highest value) until the total sum was greater than or equal to 0.25. The concentration of the (*i*,*j*) values in the GLEM was measured as the number of matrix elements included in the summation (shown as **white pixels**) divided by the total number of matrix elements. A smaller number of white matrix elements corresponds to a condensed GLEM, while a larger number of white matrix elements corresponds to a more scattered GLEM. **F)** A three-dimensional feature plot. The coordinates of the **point** are the center of mass in gray levels and in entropy values computed from the GLEM in **(D)** and the relative matrix area from **(E)**. **G)** The steps shown in **(A–F)** were performed for all the cell nuclei representing a given patient. **H)** The data points in the 3D feature space correspond to all measured nuclei from the given patient. **Red points** correspond to nuclei with high chromatin entropy. Based on the proportion of such nuclei (<25% vs ≥25%), patients were classified as low chromatin entropy (eg, the patient in Part 1 who had 4.0% nuclei with high chromatin entropy) or high chromatin entropy (eg, the patient in Part 2 who had a very high proportion [37.7%] of such nuclei). CME = center of the mass in gray levels; CMG = center of the mass in entropy values.

Statistical Analysis

The clinical end points were the same as used in previous studies on the same materials and different for the three materials. In the uterine sarcoma material, overall survival (OS) was calculated from date of diagnosis to death or end of (five-year) follow-up (10). In the ovarian cancer material, time to recurrence (TTR) was calculated from start of treatment to relapse or end of the study period (11,15). The cancer-specific survival (CSS) in the endometrial cancer material was calculated from primary treatment to last follow-up or death (9,16). The end points were defined as proposed by Punt et al. (18). A subsequent analysis of overall survival of uterine sarcomas without truncating at five years was also performed. As we consider cancer-specific survival to be the clinically most relevant end point common to all three cohorts, we also performed subsequent analyses using CSS as the end point for the uterine sarcoma and ovarian cancer cohorts. The Mantel-Cox log-rank test

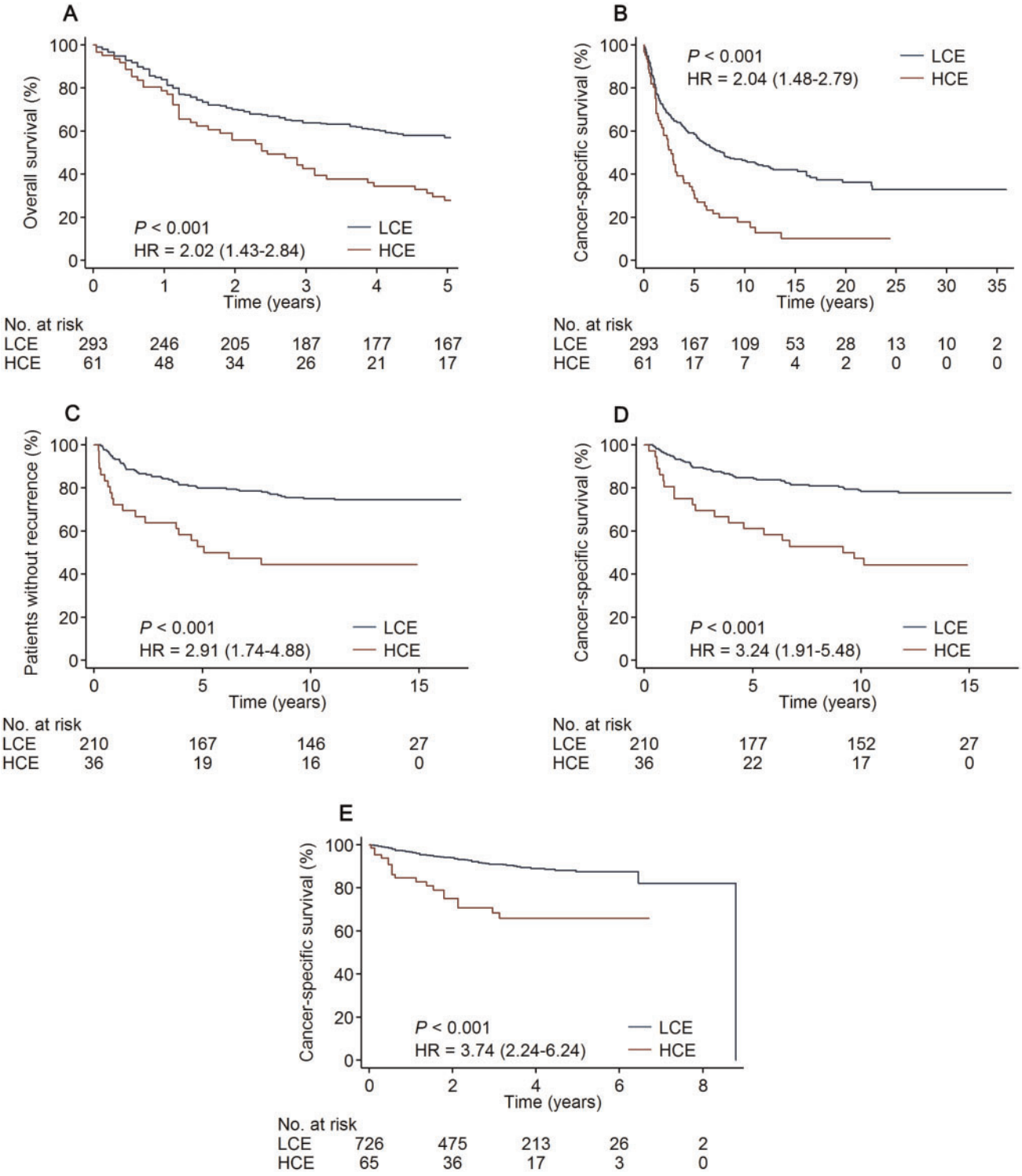

Figure 2. Kaplan-Meier survival curves according to the chromatin entropy marker. The **curves** are based on the complete data sets of **(A and B)** uterine sarcoma (354 patients), **(C and D)** ovarian cancer (246 patients), and **(E)** endometrial cancer (791 patients). The *P* values were calculated using the two-sided Mantel-Cox log-rank test. HCE = high chromatin entropy; HR = hazard ratio; LCE = low chromatin entropy.

was used to test equality of survival distributions. The Wald χ^2 test was used in a Cox proportional hazards model in multivariable analysis. The assumption of proportionality was verified using the Schoenfeld residual plot and test, and no major model violation was observed. The clinical and pathological variables included in multivariable analysis on each cohort were the same as used in previous studies (9–12,14–16). Backward selection was used in stepwise Cox regression analysis of the uterine sarcoma cohort (exclusion criterion $P > .05$), and all excluded variables were eventually tested for model inclusion. The Mann-Whitney *U* test (in MATLAB R2015a) was used to estimate if there was a statistically significant

difference in the proportion of high–chromatin entropy nuclei between the prognostic groups of each of the three materials. Associations between variables were evaluated by Spearman rank correlation. A two-sided P value of less than .05 was considered statistically significant. Texture analysis was performed in MATLAB R2015a (The MathWorks, Inc., Natick, Massachusetts, USA), and survival analysis was performed using the SPSS statistical package (IBM SPSS Statistics version 23, IBM Corp., Armonk, NY, USA).

Results

Patient characteristics are given in Supplementary Tables 1–3 (available online). Classification results of the chromatin entropy marker are given in Supplementary Table 4 (available online). Statistics on the proportion of high–chromatin entropy nuclei for patients in the different prognostic groups are given in Supplementary Table 5 (available online).

Discovery Cohort

The five-year overall survival of sarcoma patients in the discovery set was statistically significantly lower for high–chromatin entropy patients compared with low–chromatin entropy patients (27.6%, 95% confidence interval [CI] = 13.1 to 44.3, vs 57.5%, 95% CI = 49.1 to 65.1, of the patients survived for at least five years; hazard ratio [HR] = 2.13, 95% CI = 1.30 to 3.49).

Validation Cohorts

Uterine Sarcoma

When evaluated in the sarcoma validation set, there was also a statistically significant difference in survival between these two patient groups (28.1%, 95% CI = 14.0 to 44.1, vs 56.5%, 95% CI = 48.1 to 64.0, survived for at least five years; HR = 1.91, 95% CI = 1.18 to 3.08).

The five-year overall survival of all uterine sarcoma patients was 57.0% (95% CI = 51.1% to 62.4%) for low–chromatin entropy patients and 27.9% (95% CI = 17.3% to 39.4%) for high–chromatin entropy patients (HR = 2.02, 95% CI = 1.43 to 2.84) (Fig. 2A). Chromatin entropy was statistically significant in multivariable analysis with established clinicopathological variables (HR = 1.81, 95% CI = 1.21 to 2.70) (Table 1; Supplementary Table 6, available online). Supplementary Figure 6 (available online) shows the prognostic impact of the marker in subgroups of these variables.

Chromatin entropy was also a statistically significant prognostic marker in overall survival (Supplementary Figure 7 and Supplementary Table 7, available online) and in cancer-specific survival analyses (Figure 2B; Supplementary Table 8, available online). There was a positive correlation between proportion of high–chromatin entropy nuclei and cellular atypia (ρ = .53, 95% CI = 0.45 to 0.60, P < .001).

Univariate analyses were performed separately among stage I leiomyosarcomas. Chromatin entropy could be combined with mitoses and tumor size to obtain an improved risk classification with five-year overall survival of 81.6% (95% CI = 67.7% to 90.0%), 47.4% (95% CI = 37.1% to 57.0%), and 9.5% (95% CI = 1.6% to 26.1%), for the low-, medium-, and high-risk groups, respectively, compared with 75.0% (95% CI = 62.5% to 83.9%), 45.1% (95% CI = 34.2% to 55.5%), and 10.5% (95% CI = 1.8% to 28.4%), for an earlier proposed risk stratification defined by mitoses and tumor size (Figure 3) (12).

Table 1. Multivariable five-year overall survival analysis of the chromatin entropy marker and established clinicopathological variables among 354 uterine sarcomas

Feature	HR (95% CI)	P*
Chromatin entropy		.004
Low chromatin entropy	Ref	
High chromatin entropy	1.81 (1.21 to 2.70)	
Histological subtype		.01
Leiomyosarcoma	Ref	
Endometrial stromal sarcoma	0.59 (0.28 to 1.23)	
Adenosarcoma	1.38 (0.52 to 3.70)	
Undifferentiated uterine sarcoma	0.62 (0.26 to 1.47)	
Other sarcomas	2.68 (1.39 to 5.15)	
Mitotic index, high-power field		<.001
≤10	Ref	
>10	2.49 (1.70 to 3.63)	
Tumor extent		<.001
Confined to the uterus	Ref	
Spread outside the uterus	2.57 (1.74 to 3.80)	
Tumor size, cm		.001
≤10	Ref	
>10	1.88 (1.28 to 2.77)	
Tumor margins		.24
Pushing	Ref	
Infiltrating	1.30 (0.84 to 2.02)	
Cellular atypia		.29
Mild	Ref	
Moderate	1.55 (0.83 to 2.90)	
Severe	1.27 (0.66 to 2.46)	
Tumor necrosis		.17
Absent	Ref	
Present	1.44 (0.85 to 2.44)	
Hyaline necrosis		.52
Absent	Ref	
Present	1.13 (0.78 to 1.62)	
Vascular invasion		.08
Absent	Ref	
Present	1.40 (0.97 to 2.01)	

*The P values were calculated using the two-sided Wald χ^2 test. CI = confidence interval; HR = hazard ratio.

Early-Stage Ovarian Cancer

The recurrence rate of ovarian cancer was statistically significantly higher for high–chromatin entropy patients compared with low–chromatin entropy patients (HR = 2.91, 95% CI = 1.74 to 4.88; 55.6%, 95% CI = 40.4% to 72.0%, vs 24.9%, 95% CI = 19.6% to 31.4%, of the patients relapsed within ten years) (Figure 2C).

Chromatin entropy was an independent prognostic marker in multivariable analysis with histological grade and FIGO stage (HR = 1.71, 95% CI = 1.01 to 2.90) (Table 2). Supplementary Figure 8 (available online) shows the prognostic impact of the marker in subgroups of FIGO stage and histological grade, in addition to subgroups of a clinically relevant risk stratification defined by stage and grade (19,20). Among patients classified as high risk by stage and grade, the time to recurrence was statistically significantly shorter for high–chromatin entropy patients compared with low–chromatin entropy patients (ten-year time to recurrence was 36.7%, 95% CI = 20.1% to 53.4%, vs 57.2%, 95% CI = 47.0% to 66.2%) (Figure 4B). In the combined low/medium-risk group, there was no statistically significant difference in recurrence between low– and high–chromatin entropy patients (Figure 4A).

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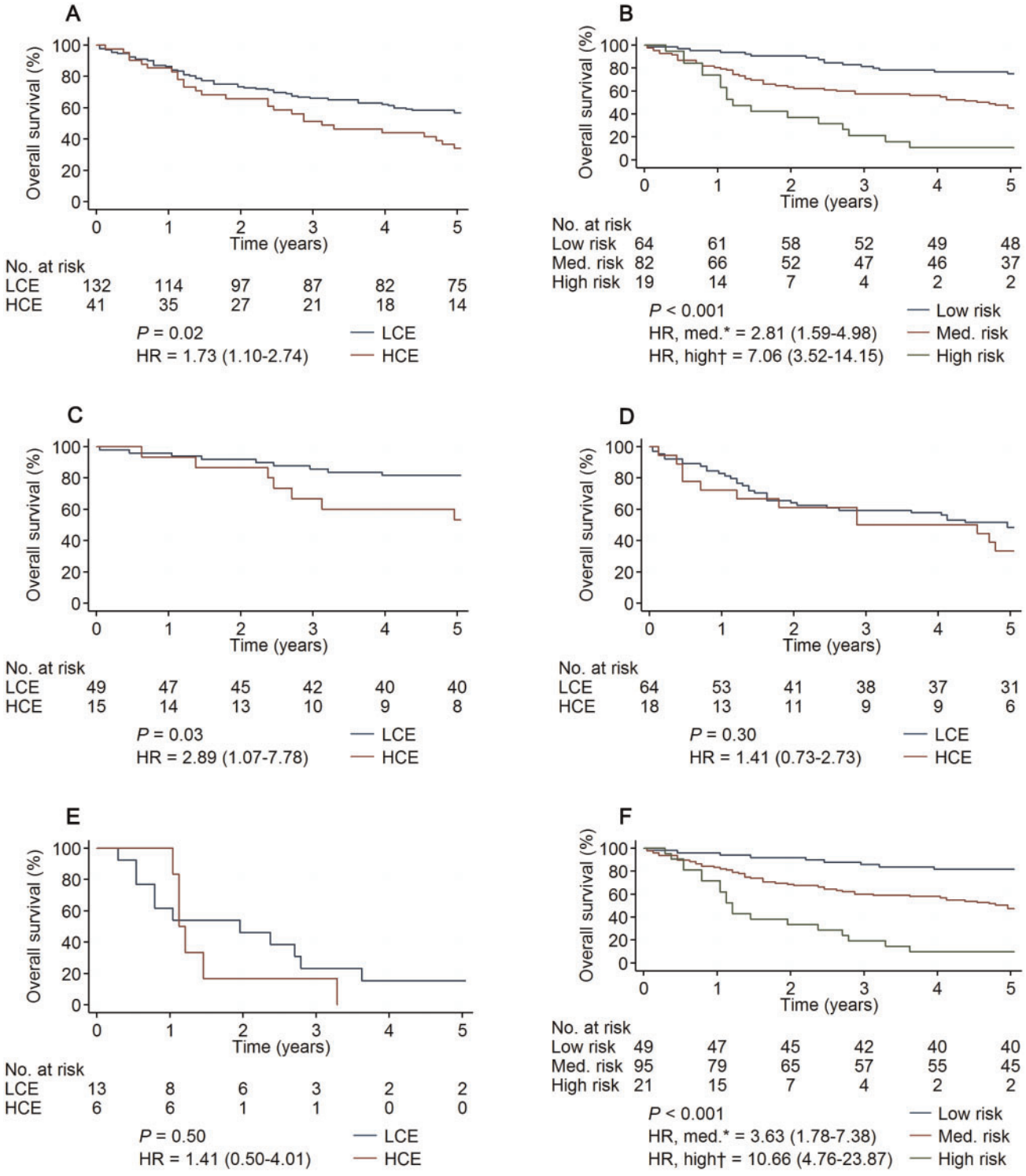

Figure 3. Kaplan-Meier five-year overall survival curves among leiomyosarcoma stage I patients. The survival curves are based on **(A)** chromatin entropy, **(B)** a risk classification defined by tumor size and mitoses (12), chromatin entropy within the **(C)** low-risk, **(D)** medium-risk, and **(E)** high-risk groups defined by tumor size and mitoses, and **(F)** a novel risk classification defined by tumor size, mitoses, and chromatin entropy. Risk classification defined by tumor size and mitoses: low risk: tumor size $\leq$ 10 cm and MI $\leq$ 10 per high-power field (HPF); medium risk: tumor size $\leq$ 10 cm and MI $>$ 10 per HPF or tumor size $>$ 10 cm and MI $\leq$ 10 per HPF; high risk: tumor size $>$ 10 cm and MI $>$ 10 per HPF. Proposed risk classification defined by tumor size, mitoses, and chromatin entropy: low risk: tumor size $\leq$ 10 cm, MI $\leq$ 10 per HPF, and low chromatin entropy; medium risk: tumor size $\leq$ 10 cm, MI $\leq$ 10 per HPF, and high chromatin entropy or tumor size $\leq$ 10 cm and MI $>$ 10 per HPF or tumor size $>$ 10 cm, MI $\leq$ 10 per HPF, and low chromatin entropy; high risk: tumor size $>$ 10 cm, MI $\leq$ 10 per HPF, and high chromatin entropy or tumor size $>$ 10 cm and MI $>$ 10 per HPF. The *P* values were calculated using the two-sided Mantel-Cox log-rank test. *HR of medium risk vs low risk in survival analysis of the three risk groups. †HR of high risk vs low risk in survival analysis of the three risk groups. HCE = high chromatin entropy; HR = hazard ratio; LCE = low chromatin entropy.

Chromatin entropy was also a statistically significant prognostic marker in analysis of cancer-specific survival (Figure 2D; Supplementary Table 9, available online). There was a positive correlation between proportion of high–chromatin entropy nuclei and histological grade (ρ = .37, 95% CI = 0.24 to 0.49, P < .001, clear cell excluded).

Endometrial Cancer

The cancer-specific survival of endometrial cancer patients was statistically significantly shorter for high–chromatin entropy tumors compared with low–chromatin entropy tumors (HR = 3.74, 95% CI = 2.24 to 6.24); five-year cancer-specific survival rates were 65.8% (95% CI = 51.3% to 77.0%) and 87.5% (95% CI = 83.8% to 90.4%), respectively (Figure 2E).

Chromatin entropy was an independent prognostic marker in multivariable analysis with the other preoperative available variables (HR = 2.03, 95% CI = 1.19 to 3.45) (Table 3). Supplementary Figure 9 (available online) shows the prognostic impact of the novel marker in subgroups of both preoperative and postoperative variables. There was a positive correlation between the proportion of high–chromatin entropy nuclei and histological grade (ρ = .42, 95% CI = 0.36 to 0.48, P < .001). Table 4 shows the prognostic value of the chromatin entropy marker within subgroups of primary and adjuvant treatment.

Table 2. Multivariable time to recurrence analysis of the chromatin entropy marker and established variables among 246 stage I ovarian cancer patients*

Feature	HR (95% CI)	P†
Chromatin entropy		.05
Low chromatin entropy	Ref	
High chromatin entropy	1.71 (1.01 to 2.90)	
FIGO stage		.008
Ia	Ref	
Ib–c	2.20 (1.23 to 3.96)	
Histological grade		<.001
1–2	Ref	
3 or not graded (clear cell)	4.82 (2.79 to 8.34)	

*Clear cell tumors are not graded at our institution, and because there was little difference in time to recurrence between patients with clear cell and poorly differentiated tumors, these patients were categorized together in previous analyses (15). For the same reason, patients with FIGO stage Ib and Ic were categorized together (15). CI = confidence interval; HR = hazard ratio.
†The P values were calculated using the two-sided Wald χ^2 test.

Discussion

There is currently no consensus on optimal treatment of uterine sarcoma patients (21,22). Leiomyosarcomas, which is the most common subtype, are very aggressive tumors and the influence of adjuvant therapy on survival is uncertain (22). Radiotherapy may be useful in controlling local recurrences, chemotherapy with doxorubicin or docetaxel/gemcitabine is currently used for advanced or recurrent disease, and some patients may respond to hormonal treatment (22). The proposed new risk stratification identified stage I leiomyosarcoma patients (medium and high risk) who may be candidates for adjuvant chemotherapy. Randomized clinical trials that evaluate the benefit of different treatment strategies in these three risk groups are warranted.

The aim of surgery for early-stage ovarian cancer is to resect the tumor and to undertake adequate staging (23). Relevant postoperative treatment strategies for these patients are observation, single-agent carboplatin, and combination chemotherapy, but there is no clear consensus regarding systemic treatment (19). The European Society for Medical Oncology (ESMO) recommends adjuvant chemotherapy to be offered to suboptimally staged patients and also to optimally staged patients at higher risk of recurrence (23), although also for patients at low or medium risk, a small benefit of chemotherapy cannot be excluded (19). Chromatin entropy offered prognostic information in patients defined as high risk by stage and grade (19,20), and could thus possibly aid in selecting patients who could be treated with single-agent carboplatin and patients who could be candidates for more aggressive combination chemotherapy. Chromatin entropy could also possibly be used to select low-risk patients who should be considered for adjuvant chemotherapy, but this remains uncertain because our data did

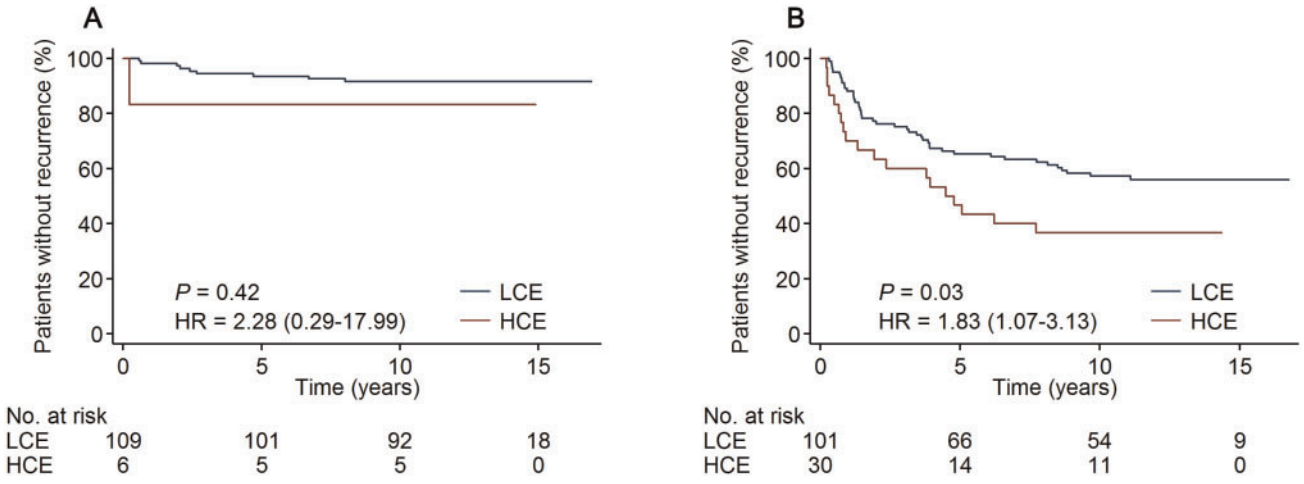

Figure 4. Kaplan-Meier recurrence-free survival curves among 246 early-stage ovarian cancer patients. Chromatin entropy is computed within the **(A)** combined low/medium- and **(B)** high-risk groups, defined by stage and grade (19,20). High-risk ovarian carcinoma was defined as either clear cell histology, poorly differentiated tumor, or the combination of moderately differentiated tumor and International Federation of Gynaecology and Obstetrics (FIGO) stage Ib or Ic; otherwise, the risk was assessed as low (well-differentiated tumor and FIGO stage Ia) or medium (well-differentiated tumor and FIGO stage Ib or Ic, or moderately differentiated tumor and FIGO stage Ia). The P values were calculated using the two-sided Mantel-Cox log-rank test. HCE = high chromatin entropy; HR = hazard ratio; LCE = low chromatin entropy.

not allow reliable evaluation in this subgroup. Randomized clinical trials are needed to assess the benefit of different chemotherapeutic regimes within groups defined by stage, grade, and chromatin entropy.

One of the key challenges in clinical care of endometrial cancer patients is to correctly identify high-risk patients before primary surgery to more precisely tailor the surgical treatment and thus avoid unnecessary invasive surgery of low-risk patients (24). The established preoperative risk assessment based on histological type and grade and diagnostic imaging (ultrasound, computed tomography [CT], magnetic resonance imaging, positron emission tomography–CT) needs to be improved by including new preoperative biomarkers (24). Within the group of patients defined as low risk by curettage histology, high–chromatin entropy patients had a statistically significantly higher risk for cancer-specific death compared with low–chromatin entropy patients. The hazard ratio increased within the group of low-risk patients treated by hysterectomy without receiving adjuvant treatment, also when stratifying on whether lymphadenectomy was performed. The established treatment for presumed low-risk patients is total hysterectomy with bilateral salpingo-oophorectomy (24). Pelvic or para-aortic lymph node metastasis occurs in 11% of presumed stage I endometrial carcinoma, and therefore several centers have proposed extensive lymph node sampling in all patients (25). However, the procedure for lymphadenectomy is not standardized and is associated with increased morbidity, and the benefits from this procedure are uncertain (25). We suggest that chromatin entropy could be combined with the preoperative curettage histology classification, such that patients with low-risk curettage histology and low chromatin entropy could be treated with total hysterectomy and bilateral salpingo-oophorectomy alone, whereas patients with high-risk curettage histology and/or high chromatin entropy could be referred to highly specialized units for more extensive surgery and possibly adjuvant therapy, and warrant evaluation of such treatment recommendations with respect to survival and cost-benefit in randomized clinical trials.

As a part of the study design, the Nucleotyping biomarker was developed on the uterine sarcoma discovery set, which is the most different type of the three materials, and then evaluated on two epithelial cancer validation sets, in addition to the sarcoma validation set. Although all three materials are gynecological cancers located in the same area, they are histologically very different and behave biologically very differently. However, we have previously observed that the average difference in chromatin structure between good and poor patients in each cohort was similar across the three cohorts (9–11). Based on these observations, we wanted to evaluate the novel biomarker in all three gynecological cohorts. The biomarker was not only a statistically significant prognostic marker in the sarcoma validation set, but also in the ovarian and endometrial cancer validation sets. The ability to independently predict multiple end points further demonstrates the robustness of the method. This altogether indicates that the marker could easily and reliably be measured in other laboratories and in clinical practice.

Table 3. Multivariable cancer-specific survival analysis of the chromatin entropy marker and other preoperatively available variables among 791 endometrial cancer patients

Feature	HR (95% CI)	P*
Chromatin entropy		.009
Low chromatin entropy	Ref	
High chromatin entropy	2.03 (1.19 to 3.45)	
Curettage histology†		<.001
Low risk	Ref	
High risk	4.59 (2.92 to 7.23)	
Age, y		<.001
<66	Ref	
≥66	2.75 (1.67 to 4.54)	

*The *P* values were calculated using the two-sided Wald χ^2 test. CI = confidence interval; HR = hazard ratio.
†Curettage histology classified as low risk if benign, hyperplasia or endometrioid grade 1–2, and high risk if nonendometrioid or endometrioid grade 3.

Table 4. Univariate cancer-specific survival analyses of the chromatin entropy marker among endometrial cancer patients within subgroups of primary and adjuvant treatment*

Patient subgroups	No.	HR (95% CI)	P†	5-y CSS (95% CI), %	
				LCE	HCE
All patients	791	3.74 (2.24 to 6.24)	<.001	87.5 (83.8 to 90.4)	65.8 (51.3 to 77.0)
Low-risk curettage histology	610	3.66 (1.52 to 8.80)	.002	92.2 (88.5 to 94.7)	75.7 (53.3 to 88.4)
High-risk curettage histology	175	1.76 (0.93 to 3.35)	.08	64.0 (51.0 to 74.4)	58.2 (37.7 to 74.0)
All patients treated with H	767	3.42 (1.94 to 6.05)	<.001	88.5 (84.8 to 91.3)	70.3 (55.2 to 81.2)
Low-risk curettage histology	602	3.75 (1.56 to 9.04)	.002	92.3 (88.6 to 94.8)	75.7 (53.3 to 88.4)
High-risk curettage histology	161	1.62 (0.76 to 3.45)	.21	67.9 (54.2 to 78.3)	66.2 (43.7 to 81.5)
H + no AT	526	12.70 (5.18 to 31.13)	<.001	96.5 (93.5 to 98.1)	67.7 (45.1 to 82.6)
Low-risk curettage histology	464	18.38 (4.93 to 68.51)	<.001	97.9 (94.8 to 99.2)	74.9 (45.6 to 89.9)
High-risk curettage histology	58	4.81 (1.35 to 17.23)	.007	82.4 (62.7 to 92.2)	53.3 (17.7 to 79.6)
H + no AT + L	406	24.65 (7.13 to 85.26)	<.001	97.6 (94.1 to 99.1)	70.4 (42.6 to 86.6)
Low-risk curettage histology	359	41.71 (6.96 to 250.00)	<.001	98.7 (94.6 to 99.7)	75.0 (40.8 to 91.2)
High-risk curettage histology	44	6.38 (1.05 to 38.98)	.02	86.7 (63.0 to 95.7)	62.5 (14.2 to 89.3)
H + no AT + no L	120	5.67 (1.41 to 22.79)	.006	91.9 (81.7 to 96.6)	62.2 (21.3 to 86.4)
Low-risk curettage histology	105	3.96 (0.41 to 38.20)	.20	94.5 (82.8 to 98.3)	75.0 (12.8 to 96.1)
High-risk curettage histology	14	3.01 (0.48 to 18.93)	.21	70.0 (32.9 to 89.2)	33.3 (9.0 to 77.4)

*Curettage histology classified as low risk if benign, hyperplasia or endometrioid grade 1–2, and high risk if nonendometrioid or endometrioid grade 3. AT = adjuvant treatment; CI = confidence interval; CSS = cancer-specific survival; H = hysterectomy; HCE = high chromatin entropy; HR = hazard ratio; L = lymphadenectomy; LCE = low chromatin entropy.
†The *P* values were calculated using the two-sided Mantel-Cox log-rank test.

Based on training in the discovery set, we selected a threshold value on the proportion of high–chromatin entropy nuclei as high as 25%. Univariate and multivariable five-year overall survival analyses among sarcomas in the discovery set showed that the prognostic value of the marker was higher for this threshold value compared with threshold values in the interval defined by average patient values (of proportions of high–chromatin entropy nuclei) in the two prognostic groups. This observation indicates that there is a relation between prognosis and a critical mass in the proportion of high–chromatin entropy nuclei.

The biomarker had a high specificity and a low sensitivity. Although the marker identified only small subgroups of patients who had a poor prognosis, it added prognostic information within several subgroups of both low-risk and high-risk patients defined by established biomarkers and clinically relevant risk stratifications.

The Nucleotyping biomarker is based on the same high-resolution digital images used in the well-known DNA image cytometry method for measuring nuclear DNA content, which is a relatively simple, inexpensive, and robust methodology that can easily be automated (3). Both aneuploidy and chromatin entropy are markers for large-scale genomic instability, but while DNA ploidy measures the overall amount of nuclear DNA, Nucleotyping measures changes in the chromatin structure. The proportions of gynecological cancer patients classified as aneuploid (14–16) are relatively large compared with the small subgroups of patients classified as high chromatin entropy. Further analyses of the high–chromatin entropy nuclei are needed to gain more insight into the underlying mechanisms and the contribution of these cells to carcinogenesis and metastatic potential.

The main limitation with this study is that the benefit of different treatment strategies in the proposed risk groups based on chromatin entropy and established clinicopathological parameters are not evaluated in randomized clinical trials.

In conclusion, a novel method for detecting high–chromatin entropy nuclei was developed, and the proportion of such deviating nuclei per patient was a statistically significant prognostic marker in three gynecological cancer cohorts and could be used as a supplement in defining high-risk patients.

Funding

This work was supported by the Research Council of Norway through its IKTPLUSS Lighthouse program (259204) and Centers of Excellence funding scheme (179571), and the South-Eastern Norway Regional Health Authority (2012027 and 2015070).

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The study funders had no role in the design of the study; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

We thank Marian Seiergren for creating Figure 1, Dr. Wanja Kildal, Marna Lill Kjæreng, and the laboratory personnel at the Institute for Cancer Genetics and Informatics for assistance, the reviewers of the *Journal of the National Cancer Institute* for valuable suggestions; last, but not least, we thank the participating centers in the MoMaTEC trial, the staff at the Norwegian Radium Hospital, and all participating patients for making this study possible.

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