Cytological nuclear atypia classification can predict prognosis in endometrial cancer patients

Tomohiko Yamaguchi CT[1], Akihiko Kawahara PhD[1], Satoshi Hattori PhD[2], Tomoki Taira CT[1], Hideyuki Abe CT[1], Sakiko Sanada MD[3], Jun Akiba MD[3], Shin Nishio MD[4], Kimio Ushijima MD[4], Toshiharu Kamura MD[4], and Masayoshi Kage MD[1]

1) Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Japan
2) Biostatistics Center, Kurume University, Kurume, Japan
3) Department of Pathology, Kurume University School of Medicine, Kurume, Japan
4) Department of Obstetrics and Gynecology, Kurume University School of Medicine, Japan

Corresponding author:

Tomohiko Yamaguchi.
Department of Diagnostic Pathology,
Kurume University Hospital
67 Asahi-machi, Kurume
Fukuoka 830-0011, Japan
Tel.: +81-942-31-7651
Fax: +81-942-31-7651
E-mail: yamaguchi_tomohiko@kurume-u.ac.jp

Objective: Endometrial cancer is one of the leading causes of malignancy in females. Nuclear findings are important to patients with cancer, and can provide valuable information to treating oncologists. We investigated whether nuclear findings are a useful prognostic factor in endometrial cancer patients.

Method: We investigated 71 cases of endometrial carcinoma with paired histology and cytology at Kurume University Hospital. We classified endometrial endometrioid carcinoma (EEC) G1 and G2 as type I carcinomas, and classified uterine papillary serous carcinoma (UPSC) and clear cell carcinoma (CC) and EEC G3 as type II carcinomas. For establishment of cytological nuclear atypia classification, we examined the following nuclear factors: mitotic figures, prominent nucleoli, nuclear area and anisonucleosis on the cytological smears.

Results: There was a significant difference in mitotic figures ($P<0.001$) and anisonucleosis ($P=0.026$) in cytological smears between type I and type II carcinomas. Based on these findings we categorized cytological nuclear atypia into three groups: nuclear atypia-1 (57.7%), -2 (19.7%) and -3 (22.5%), and this classification system correlated well with prognosis in endometrial cancer patients ($P<0.001$). Furthermore, this classification system was able to extract patients with a good prognosis from among high-grade carcinomas such as UPSC+CC+EEC G3, or patients with a poor prognosis from EEC G1 patients.

Conclusions: Our system of cytological nuclear atypia classification based on endometrial cytology can predict patient prognosis. Cytological nuclear atypia classification and histological typing may be useful for treatment and follow up of endometrial cancer patients and should be routinely incorporated in cytological reports.

Running Head: nuclear atypia classification for endometrial carcinomas

KEY WORDS: cytological nuclear atypia classification, nuclear factors, endometrial cytology, endometrial carcinoma, cytological diagnosis

Introduction

Endometrial cancer is one of the leading causes of malignancy in females. Endometrial cancers have long been divided into two major subtypes (I and II), based on light microscopic appearance, clinical behavior, and epidemiology.[1,2] Type I endometrial carcinomas are mostly endometrial endometrioid adenocarcinomas (EEC), are associated with unopposed estrogen exposure and are often preceded by premalignant disease. In contrast, type II endometrial carcinomas have nonendometrioid histology, such as uterine papillary serous carcinoma (UPSC) and clear cell carcinoma (CC) with an aggressive clinical course, and are commonly described as estrogen independent.[3,4] It is important from a treatment perspective to distinguish between subtypes I and II. In recent years, several clinical and histological studies [5,6] have shown that high-grade EEC behave similarly to type II carcinomas, and that risk factors for such high-grade ECC are similar to type II carcinomas.[7]

In Japan, endometrial cytology and/or biopsy is generally performed in patients with abnormal uterine bleeding in order to rule out carcinoma.[8] It is known that nuclear findings of cancer cells are important predictive factors of patient prognosis in many cancers,[9-11] and nuclear grade of breast cancer is a powerful indicator of risk of cancer death.[12] In endometrial cytology, to our knowledge there have been no attempts to relate cytological nuclear atypia classification with prognosis prediction.

In the present study, we investigated retrospectively whether the nuclear findings in endometrial carcinomas are a useful prognostic factor in endometrial cancer patients with type I (EEC G1 and G2), or type II (UPSC and CC and EEC G3) carcinomas, and we also developed a cytological nuclear atypia classification for endometrial cancer patients.

Material and Methods

Case selection and cell sampling

We investigated 71 cases of endometrial carcinoma with paired histology and cytology at Kurume University Hospital, between 2000 and 2007. Cytological smears were obtained routinely during outpatient visits and resected tissue specimens were obtained from patients who underwent surgery. All specimens were examined for histology and cytology by different pathologists, and cytological diagnosis always preceded the histological diagnosis. Patient age ranged from 25 to 81 years (mean 57.1). Histological types were classified according to the WHO classification[13], such as EEC G1 to G3, UPSC and CC. We classified EEC G1 and G2 as type I carcinomas, and classified UPSC, CC and ECC G3 as type II carcinomas. Cancer stages included 17 (23.9 %) cases of stage I, 7 (9.9 %) stage II, 42 (59.2 %) stage III and 5 (7.0 %) stage IV. At the time of surgery, 44 (62.0 %), 24 (33.8 %), 40 (56.3 %), 15 (21.1 %) and 16 (22.5 %) patients had over 50% depth of myometrial invasion, endocervical invasion, vascular invasion, adnexal metastasis or lymph node metastasis, respectively (Table 1).

Endometrial cytology was performed using an Endocyte® (Laboratorie CCD, Paris, France). The gynecological doctor performed the cell sampling, and immediately immersed the Endocyte® (Laboratorie CCD, Paris, France) into the disposable tube containing physiologic saline. The cytological sample was centrifuged at 1,500 rpm for 5 minutes and the supernatant liquid was removed. The sediment was smeared onto two sandwiching glass slides, and slides were immediately fixed in 95% ethanol. After overnight fixation, slides were stained by conventional Pap staining.[14]

Evaluation of nuclear findings in endometrial cytology

1. Nucleoli and mitosis findings by microscopy

The endometrial cytological smears were microscopically examined for prominent nucleoli and mitosis figures (Figure 1). Endometrial cytological specimens were evaluated as positive for these features when carcinoma cells with large prominent nucleoli and at least one mitotic figure were observed.

2. Morphometric image analysis for nuclear area and anisonucleosis

We examined nuclear area and anisonucleosis in endometrial cytological specimens by morphometric image analysis[15] using 'Win ROOF' (version 5.7, Mitani Corporation, Osaka) computer software. Images of cancer cells were selected for clarity in each of 10 high-power fields from each cytological specimen, using a CCD digital camera (Nikon, DXM1200). The nuclear area of the cancer cell was measured in 50 cells, and the mean and range of areas (μm^2) recorded. Data of anisonucleosis used a standard deviation (SD) of nuclear area.

Statistical analysis

Association between clinicopathological factors and subtypes (type I and type II) were examined using Fisher's exact test or Wilcoxon rank-sum test depending on type of data. We looked for a combination of subtype-specific biomarkers. To do so, we began by investigating the association between nuclear factors and subtypes using Fisher's exact test or Wilcoxon's rank sum test. Further, we applied logistic regression with the nuclear factors as explanatory variables to examine whether each nuclear factor is associated with the subtypes independently of the other factors. From these analyses, we identified biomarkers that could be used as a basis

for constructing a cytological nuclear atypia classification. We further improved the nuclear atypia classification using logistic regression and ROC analysis. With the Youden index, which is defined as the maximum value of sensitivity+specificity-1 over cut-off values[16], we defined the nuclear atypia classification. Next, we asked whether or not the resulting nuclear atypia classification is useful for discriminating patients into good or poor prognosis groups with respect to overall survival (OS) and progression-free survival (PFS), where OS and PFS were defined as durations until date of death due to any cause and date of progressive disease, respectively, from date of diagnosis. To this end, we applied the Kaplan-Meier method and logrank test. All statistical analysis was performed using SAS version 9.13 (SAS Institute Inc., Cayley, NC).

Results

1. Clinicopathological features in type I and type II endometrial carcinomas

Seventy-one endometrial cancer patients were classified into two types, including 48 patients of type I, and 23 patients of type II (Table 1). Although there were no significant differences in clinicopathological factors between type I and type II carcinomas, PFS ($P<0.001$) and OS ($P<0.001$) were significantly longer in patients of type I than those of type II, suggesting that the differences in tumour characteristics are more closely related to the type of carcinoma than to the stage at diagnosis.

2. Establishment of cytological nuclear atypia classification for endometrial cancer patients.

2-1. Association of nuclear factors and type I and type II carcinomas

Frequencies of mitotic figures, prominent nucleoli, nuclear area and anisonucleosis are summarized for type I and type II carcinomas in Table 2. In endometrial cancer cells, mitotic figures and prominent nucleoli were observed in 16 (22.5%) and 32 (45.0%) patients, respectively. In Table 2, we present p-values from the logistic regression analysis applied to the subtypes with the four nuclear factors (log-transformed) to examine whether each nuclear factor was significantly associated with the subtypes independently of the other nuclear factors. Mitotic figures were significantly associated with subtypes ($P<0.001$) independently of the other nuclear factors, but prominent nucleoli were not ($P=0.256$). In morphometric image analysis, although an average increase in both nuclear area and anisonucleosis was observed in patients with type II carcinoma compared with those of type I, the difference was significant only for

anisonucleosis (P=0.026).

2-2. Cytological findings of nuclear atypia classification

From the results given in Table 2, we constructed a classification of cytological nuclear atypia by combining mitosis and anisonucleosis. We performed ROC analysis by applying logistic regression with anisonucleosis as an explanatory variable to mitosis-negative or positive patients (Figure 2). For mitosis-positive patients, anisonucleosis (log-transformed) was not statistically significant (P=0.503). For mitosis-negative patients, it was statistically significant (P=0.008) and the area under the ROC curve (AUC) was 0.800 (95% confidence interval: 0.667, 0.943), indicating that anisonucleosis had a clear diagnostic ability for mitosis-negative patients. These results suggested that nuclear atypia classification by mitosis could be improved by including anisonucleosis for mitosis-negative patients, but not for mitosis-positive patients. Using the Youden-index[16], we selected 14.5 as the cut-off value of anisonucleosis in mitosis-negative patients.

With the cut-off value, we proposed to classify patients into three categories of cytological nuclear atypia; (1) mitosis-negative and anisonucleosis less than 14.5 (Nuclear atypia-1), (2) mitosis-negative and anisonucleosis equal to or more than 14.5 (Nuclear atypia-2), and (3) mitosis-positive (Nuclear atypia-3) (Table 3). On cytological findings, endometrial carcinomas with nuclear atypia-1 show uniform, small round to oval nuclei, without mitotic figures. Endometrial carcinomas with nuclear atypia-2 show irregular or variation in nuclear size (anisonucleosis), without mitotic figures. Endometrial carcinomas with nuclear atypia-3 show moderate to large irregular nuclei with mitotic figures.

Table 4 shows the association between this cytological nuclear atypia classification and subtypes. For patients in nuclear atypia-1, only 7.3% (3/41) were type II carcinoma, whereas

50% (7/14) of patients in nuclear atypia-2 and 81.3% (13/16) in nuclear atypia-3 were type II carcinoma. This indicated that anisonucleosis was very useful for nuclear atypia classification in mitosis-negative patients.

3. Effect of cytological nuclear atypia classification on OS and PFS

Kaplan-Meier plots of subpopulations by the proposed nuclear atypia classification for PFS and OS are shown in Figure 3. Survival curves of the three categories of nuclear atypia were significantly different ($P<0.001$) (Figure 3A). Among patients with endometrial carcinomas, those who were mitosis-positive had significantly shorter survival than those who were mitosis-negative ($P=0.001$), and among mitosis-negative patients, those with anisonucleosis $\geqq$ 14.5 had significantly shorter survival than those with anisonucleosis <14.5 ($P=0.004$). Furthermore, both PFS and OS were significantly longer in patients with nuclear atypia-1 than in those with nuclear atypia-2 and -3 (Figure 3B).

As regards the nuclear atypia classification in UPSC, CC and EEC G3, nuclear atypia-1 was 15.4% (2/13) in UPSC, 25.0% (1/4) in CC and 0% (0/6) in EEC G3. The three patients with nuclear atypia-1 are alive (Figure 4), suggesting that nuclear atypia classification can extract patients with good prognosis from high-grade carcinomas. Similarly, endocervical invasion and lymph nodes metastasis in EEC G1 and G2 of nuclear atypia-3 were higher than those of nuclear atypia-1, with a poor prognosis (Figure 5), suggesting that this classification can extract patients with poor prognosis from EEC G1 and G2.

Discussion

The value of endometrial cytology in assessing endometrial abnormalities has been shown and is widely accepted for screening in Japan[17, 18] Endometrial cytology is an effective method of assessing benign endometrium and discovering premalignant and malignant endometrial states.[19] In the present study, we investigated whether nuclear findings, such as mitotic figures, prominent nucleoli, nuclear area and anisonucleosis in endometrial cancer are useful prognostic factors in type I and type II carcinomas, and we also tried to establish a cytological nuclear atypia classification that could be a predictor of prognosis in endometrial carcinoma. We observed that mitotic figures ($P<0.001$) and anisonucleosis ($P=0.026$) were significantly associated with the subtypes independently of the other nuclear factors. These findings are familiar to and commonly used by cytopathologists/technologists when describing malignant tumor cells. The cytological nuclear atypia classification we developed using a combination of mitotic figures and anisonucleosis consisted of nuclear atypia-1 (57.7%), -2 (19.7%) and -3 (22.5%), and these categories correlated well with prognosis for endometrial cancer patients ($P<0.001$).

In various cancers such as breast and lung cancer, nuclear findings, such as size variability, shape, nucleoli, nuclear inclusions, chromatin patterns, and mitosis have been used clinicopathologically to evaluate malignancy. On cytology grading, Sigel et al. reported that nuclear size, chromatin pattern, and nuclear contours showed a significant association with histological grade and DFS in lung cancers.[10] On histological grading, Nakazato et al. reported that nuclear area and nuclear major dimension are useful independent markers for evaluating the prognosis of lung adenocarcinoma.[11] Furthermore, Maezawa et al. reported that nuclear overlapping, in more than 3 layers and more than 3-fold variation in nuclear size can predict

invasion in small-sized peripheral lung adenocarcinoma with bronchioloalveolar carcinoma component.[20] Thus, the nuclei of cancer cells are considered as one of the factors which can predict patient prognosis or invasion. Although the nuclear atypia of cancer is well investigated based on this grading system, these effects may differ by organ, and it is important to establish effective nuclear findings for each organ.

In subtypes of endometrial cancers, Voss MA et al. reported that EEC G3 is better characterized as type II cancer, because EEC G3, UPSC and CC have similar clinical and immunohistochemistry profiles, and survival outcomes.[5] Therefore, in the present study we classified EEC G3 as a type II carcinoma, along with UPSC, CC. Almost all EEC G3 (83.3%, 5/6) was classified into nuclear atypia-3 (mitotic figures and poor prognosis), and EEC G3 was clearly distinguished from EEC G1 and G2 in cytology. This result supports our decision to categorize EEC G3 as a type II rather than type I carcinoma. EEC G1 is generally a low-grade tumor[20], and nuclei show cytologically uniform, round to oval nuclei, with inconspicuous nucleoli, and resemble the nuclei of functional endometrium.[19] In the present study, ten EEC G1 patients were classified as nuclear atypia-2 and -3, and these patients had a clearly poorer prognosis compared with nuclear atypia-1 (PFS; P=0.027, OS; P=0.008). This result suggests that cytological nuclear atypia classification can extract patients with poor prognosis from EEC G1 and G2, and may be useful for the follow-up of such patients. On the other hand, UPSC and CC are generally high-grade tumors[21], with large and pleomorphic nuclei and moderate-to large irregular nucleoli.[19] Hagiwara et al. reported that endometrial cytology findings accurately suggested the histological diagnosis of UPSC[22], however, UPSC and CC often overlap cytologically and these tumors may be difficult to subclassify, whereas they are easily recognized as highly malignant. Surprisingly, three patients in UPSC and CC were included in nuclear atypia-1, and these patients, which have stage IIb or IIIa tumors, showed a tendency

toward a better prognosis compared with those with nuclear atypia-2 and -3. Although patient numbers in this study are small, this is an attractive result with potentially important implications for treatment and follow up of endometrial cancer patients, and further study is needed.

Cytological diagnosis using the cytological nuclear atypia classification in endometrial carcinomas may provide valuable information to the treating oncologists to plan patient management. Therefore, we recommend using this classification in addition to histological typing in cytological diagnosis.

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