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Evaluation of Automated Estimation of Epithelial Volume and Its Prognostic Value in Ovarian Tumors

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The paper describes an improved segmentation method to measure the percentages of epithelium and stroma in ovarian (tumor) tissue with automated image analysis and evaluates its prognostic value. In the image processing method, a blue-yellow image pair is recorded from standard paraffin sections and stained with pararosanilin Feulgen and naphthol yellow. The blue image is used for automated determination of the total tissue area and the yellow image for the epithelial area. Results are obtained with 114 ovarian tumors of the common epithelial types (14 borderline tumors and 100 invasive carcinomas with varying degrees of differentiation). The fraction of epithelium in the total tissue shows a strong correlation with the epithelial percentage resulting from interactive morphometry ($r = 0.991$) for 15 tumors of varying histological grades. The prognostic value is evaluated on the 100 invasive carcinomas. Survival analysis implies that the epithelial percentage is of prognostic importance (Mantel-Cox 7.4, $p = 0.0064$). Multivariate analysis shows that the estimated fraction of epithelium is the strongest factor and that the FIGO stage has additional prognostic value (Mantel-Cox 12.5, $p = 0.0004$). It can be concluded that epithelial volume, as automatically estimated by image processing, seems useful in predicting the prognosis of patients with ovarian cancers.

Additional key words: Image analysis, Ovary, Tissue section, Epithelial volume, Prognostic evaluation.

The overall 5-year survival of patients with ovarian carcinomas is poor (30%) and varies depending on stage, grade, and other factors (16, 18). Within the ovarian epithelial tumors, grading methods allow for distinction of subsets with varying 5-year survival: borderline, well-differentiated, moderately differentiated, and poorly differentiated.

Grade has important prognostic implications (5), but unfortunately assignment is subjective and not always well reproducible. Considerable variability occurs among the diagnoses of different pathologists (2, 11). This variability is also reflected in an associated prognostic variation and may result in undertreatment or overtreatment of the patient (1). Quantitative, objective, and reproducible features are preferred to prevent undesired consequences of these falsely positive and falsely negative diagnoses and improve the consistency of the assessment of survival probability, and hence therapy.

The volume percentage of epithelium is such a feature because it has aided prediction of the outcome of patients with borderline tumors and invasive carcinomas (3, 4), in addition to cellular DNA content (8, 9) and mitotic activity index (5). The relative volume of epithelium can be estimated through measurement of the area percentage covered by epithelium. The common way to estimate the epithelial area is by interactive morphometry with the method of point counting (23). Practical application in diagnostic pathology requires many microscope fields to be counted and may therefore be tedious and time consuming. In this paper, instead, digital image processing is explored as a means of measuring the area of epithelium automatically.

In the image processing method, a blue-yellow image pair is recorded from the epithelium-rich areas of Feulgen-naphthol yellow stained specimens. The total area in a microscope field covered by tissue (epithelium plus stroma) is automatically determined from the blue image. The automated segmentation of the epithelial area is based on the difference in packing of the epithelial and stromal nuclei in the yellow image. The ratio of the number of pixels in the epithelium segment and the pixels in the total tissue segment gives an estimate of the relative volume of epithelium. A previously conducted pilot study indicated that these automatically obtained ratios are strongly correlated with the epithelial percentages, established by interactive morphometry (21). The relationship between the image processing results and the prognosis of patients is investigated in this paper on the basis of more patient material.

The paper describes the correlation between auto-

mated and interactive assessments of the epithelial percentage in borderline tumors and invasive carcinomas and evaluates the prognostic value of the image processing results in tissue sections of 100 ovarian cancers with varying degrees of malignancy.

EXPERIMENTAL DESIGN

Patient Material

In this study 114 ovarian tumors, including serous, mucinous, endometrioid, and mixed types of varying histological grades, were used. Of these tumors, 34 were obtained from one institution over 7 years and the remaining 80 tumors from nine institutions over 3 years. Based on well-defined histopathological criteria (4), the tumors were diagnosed as border line (BO, $n = 14$), well-differentiated carcinoma (WDC, $n = 23$), moderately differentiated (MDC, $n = 22$), or poorly differentiated carcinoma (PDC, $n = 55$). All patients were extensively staged: 29 FIGO I, 9 FIGO II, 64 FIGO III, and 12 FIGO IV patients. FIGO I patients did not receive additional treatment to surgery; FIGO II, III and IV patients were debulked when possible and received uniform chemotherapy containing cisplatin.

Tissue was fixed in 10% neutral Formalin for approximately 24 hours at room temperature, dehydrated in alcohol of increasing strength, and embedded in Paraplast. Standard 4-μm sections were prepared and stained with hematoxylin-eosin for visual inspection.

To discriminate epithelium from stroma, a stoichiometric stain or a component-specific stain is preferred to facilitate image segmentation. The standard hematoxylin-eosin staining method is not suited for application in image processing because of insufficient spectral specificity of the dyes and the absence of tissue component specificity. The Feulgen-naphthol yellow combination stain, however, gives good spectral separation of the dyes and was selected for this study.

In the standard Feulgen procedure, sections are hydrolyzed in 5 N HCl for 30 minutes and stained with a solution of 0.5 g pararosanilin in a mixture of 15 ml 1 N HCl and a solution of 0.5 gm K2S205 in 85 ml distilled water for 45 minutes. Hereafter, sections are stained for 5 seconds with a solution of 0.1 gm Naphthol Yellow in a mixture of 100 ml distilled water and 1 ml concentrated acetic acid.

Despite careful preparation of the tissue, the slides show some variability in staining intensity. Such a variation reflects a realistic situation, especially when tissue is preprocessed in more than one laboratory.

Equipment

The Feulgen staining method colors the nuclei reddish brown and the remaining tissue components yellow. A monochromatic blue filter ($\lambda = 420$ nm; $\Delta\lambda < 10$ nm, Schott, Tiel, The Netherlands) for which naphthol yellow shows maximum absorption was used to distinguish the total area of tissue (both nuclei and cytoplasm) from the background area. A monochromatic green filter ($\lambda = 552$ nm; $\Delta\lambda < 10$ nm) for which the Feulgen pararosanilin shows maximum absorption was used to distinguish the nuclei of both epithelium and stroma from the cytoplasm and the background area.

A blue-yellow image pair is recorded per field using the two filters sequentially on a Zeiss UEM microscope (Carl Zeiss, Oberkochen, West Germany) connected with a Chalnicon TV camera (Bosch, West Germany). A dry *6.3 objective with a N.A. of 0.16 (Zeiss) is used, corresponding to a pixel-to-pixel distance of 2.0 μm at specimen level. The images are analysed on a Kontron Image Processing System (Kontron Bildanalyse GmbH, Eching, West Germany).

Interactive Morphometry

To facilitate evaluation of the image processing results, the control percentages of the epithelial and stromal areas are measured via interactive morphometry. The point-counting method is performed on 15 blue-yellow image pairs recorded from 15 ovarian specimens with varying degrees of malignancy. For assessment of the control percentage of epithelium, a 168-point regular grid is randomly positioned on the images, and data points overlying epithelium, stroma (together forming the tissue), and lumen are accumulated separately. The control percentage of epithelium in the borderline and invasive specimens ranges from 39.8 to 93.5%.

Image Processing

The image processing method for the automated assessment of the epithelial percentage is detailed in Ref. 21. It consists of two parts: processing of the blue image to determine the area of tissue (epithelium and stroma) and processing of the yellow image to segment the epithelial area.

In quantitative microscopy, images must be corrected for shading, *i.e.*, uneven illumination and recording effects (22). For this purpose, at the beginning of each day, a blank-field image pair is recorded (13) and smoothed [by rank filtering (rank 80%, window 15 × 15 pixels) averaging, and again rank filtering (rank 50%, window 15 × 15 pixels)], see Ref. 12. This blank-field image pair, then freed from noise and small dust particles, is used as a reference image for the rest of the day.

For a blue image, recorded from a specimen, the image processing consists of the following steps.

Shading Correction. The image is corrected for shading by subtraction of the blue reference image.

Segmentation of Tissue. The image is segmented with a well-known global threshold technique (10, 20). The threshold is derived from the histogram. If the smoothed grey value histogram has only one peak, the image is assumed to contain tissue only. If the smoothed histogram has two peaks, the image is assumed to contain tissue and lumina. According to the Bayes rule, to minimize the probability of misclassifying an object point as background point and vice versa, the minimum between the two peaks is selected for the threshold level (19).

Elimination of Artifacts. After threshold, unresolved tissues are small artifacts in the background area and tears or holes in the tissue area. Small artifacts, such as dirt, mucus, or loose cells in the thresholded binary image, are eliminated by 10-fold erosion (4-connected), effective for objects with a cross-size smaller than 40 μm. Small tears or holes in the tissue area are removed if the

smallest diameter is <20 μm (through application of a five-fold erosion, 4- and 8-connected).

This results in the approximated area of tissue (see example of a blue image in Fig. 1). In Figure 1 the overlayed contours represent the segmented area of tissue. Simultaneously, the yellow image of the same microscope field is processed automatically to determine area of epithelium. The image segmentation of the yellow image is based on the observation that, in general, epithelial nuclei are more tightly packed than stromal nuclei. The processing of the yellow image consists of the following steps.

Shading Correction. The image is also corrected for shading by subtraction of the yellow reference image.

Gap Bridging. Segmentation of the epithelial area is facilitated by bridging the gaps between epithelial nuclei (by application of a 3 × 3 linear Gaussian filter), without bridging the gaps between less closely packed stromal nuclei.

Segmentation of Epithelial Area. In the yellow images the grey value distribution is found to be unimodal in all cases but varying strongly in shape for the different histopathological tumor types due to the various intensity values of both epithelial and stromal nuclei. Because the threshold selection algorithm has to be resistant against variability in staining intensity, the threshold selection is based on the image contrast [*i.e.*, log(min) − log(mod 1)] and location of the highest tissue peak value (mod c), see Ref. 21. The threshold level (lvl) is computed from log(lvl/mod c) = 0.235 × log(min/mod 1). The minimum pixel value is defined as the grey value skipping 0.5% of the extreme grey values and should represent the staining intensity of the epithelial nuclei. In case of diffusely infiltrating lymphocytes or a great variability in staining intensity of the nuclei, the minimum pixel value represents the staining intensity of these lymphocytes or most dark nuclei. This gives too low a minimum pixel value and thus too low a threshold level, which results in underestimation of the epithelial area. To overcome this problem, a second minimum is defined, skipping 5.0% of the extreme pixel values. When the slope between these two minimum values in the grey value histogram exceeds the critical value of 25.0, it is supposed that lymphocytes and/or a great variability in staining intensity of the nuclei is present in the image. Then, the 5.0% extreme minimum value, representing the epithelial nuclei, is chosen as minimum pixel value in the threshold selection method.

Elimination of Artifacts. Again unresolved issues remain at this point. They are holes, stromal nuclei, and gaps between epithelial nuclei and the threshold binary image. To prevent elimination of single epithelial nuclei, first all holes in the binary image are filled. Then small objects, mostly stromal nuclei, are eliminated (by application of the skeleton operation, followed by a closing (4-connected) with a step size of two pixels to fit small epithelial gaps and removal of skeleton parts not exceeding 50 pixels). Gaps between epithelial nuclei are closed [by application of a two-fold skeleton operation followed by a two-fold erosion operation (4- and 8-connected)], effective for spaces between the epithelial nuclei with a cross size smaller than 16 μm. The result of this procedure is the approximated area of epithelium, determined from the yellow image. An example of a yellow image is shown in Figure 2, in which the segmented area of epithelium is represented by the overlayed contours.

The ratio of the number of pixels in the approximated area of epithelium and the pixels in the approximated area of tissue gives an estimate of the relative volume of epithelium in the image.

With this two-step automated image processing method, four image-pairs per slide with a total area of

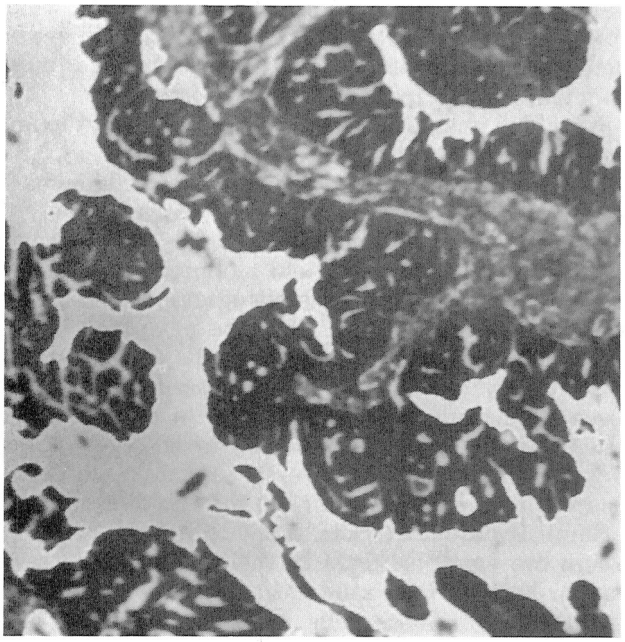

FIG. 1. Example of a blue image from an ovarian tumor. White two-pixel-thick lines give contours of segmented area of tissue.

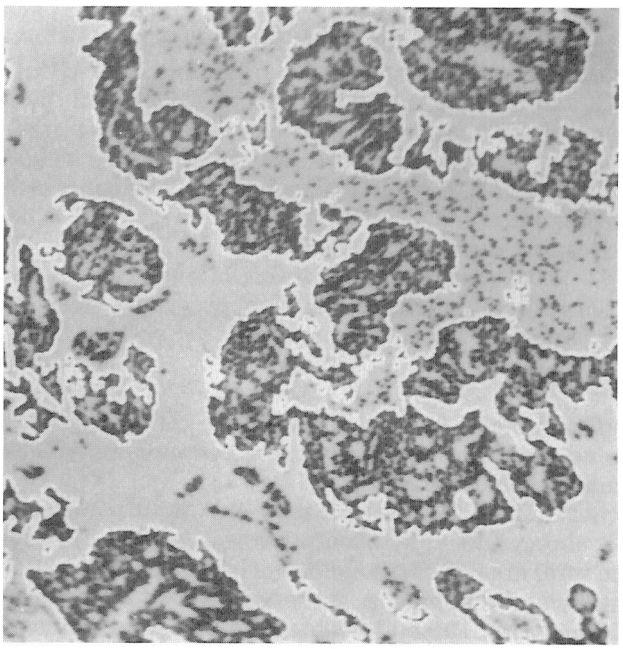

FIG. 2. Example of a yellow image from an ovarian tumor. White two-pixel-thick lines give contours of segmented area of epithelium.

4.2 mm^2 at specimen level are recorded from the most epithelium-rich areas of the specimen. The maximum over these four image-pairs is taken to represent the volume percentage of epithelium of the specimen.

Prognostic Evaluation

To study the prognostic value of the epithelial percentage, estimated by image processing, in ovarian cancers, univariate survival analysis according to Kaplan-Meier is performed (14). For this analysis, the epithelial percentage is divided into two categories in such a manner that differences between survival curves are at maximum. The number of borderline tumors in this study is too small to allow such an analysis. Differences between survival curves are analyzed using the Mantel-Cox test (17), with p values <0.05 considered significant.

For evaluation of additional prognostic value of the FIGO stage, multivariate survival analysis is performed using Cox's regression model (6). All analyses have been performed with the aid of the BMDP package, using the Life Tables (P1L) and Survival Analysis with Covariates programs (P2L), respectively (7).

RESULTS

For evaluation of the tissue segmentation in the blue image and the epithelium segmentation in the yellow image, a training set consisting of 15 ovarian specimens with varying degrees of malignancy (BO, $n = 4$; WDC, $n = 3$; MDC, $n = 2$; PDC, $n = 6$) was used. The estimates for the relative volume of epithelium, represented by the maximum over four image-pairs, are given in Table 1. From visual inspection of the resulting images for these specimens, it can be concluded that both the tissue and epithelium segmentations give good results. For one borderline tumor (OT02) the percentage of tissue is overestimated by erroneously classifying mucus and artifacts in the lumina as a part of the tissue. In general, however, mucus does not influence the segmentation results, due to the elimination of small artifacts after threshold. The epithelium segmentation for borderline tumors and well-differentiated carcinomas, small stromal areas with tightly packed dark nuclei are sometimes erroneously counted as epithelial areas. The differences in the estimated epithelial percentages, however, have appeared to be negligible for the overall prediction result.

Table 1. Estimated Percentages of Epithelium for 15 Ovarian Specimens with Varying Degrees of Malignancy

Specimen	Grade	VEPDIP
OT01	BO	39.3
OT02	WDC	34.4
OT03	BO	44.9
OT04	PDC	72.0
OT05	PDC	95.5
OT06	WDC	52.0
OT07	MDC	76.7
OT08	PDC	83.3
OT09	MDC	74.1
OT10	PDC	95.4
OT11	BO	31.0
OT12	PDC	91.0
OT13	WDC	35.5
OT14	PDC	89.2
OT15	BO	59.6

BO, borderline; WDC, well-differentiated carcinoma; MDC, moderately differentiated carcinoma; PDC, poorly differentiated carcinoma; VEPDIP, epithelial percentage.

The results of the image processing method, obtained with all 114 ovarian tumors, are satisfactory. The estimated epithelial percentage is increasing with histological grade. For the borderline tumors, the percentage varies from 20 to 65%, and the well-differentiated carcinomas have an epithelial percentage ranging from 22 to 95%. The measured percentages vary from 60 to 100% for the moderately and poorly differentiated carcinomas (Table 2). From Table 2 it can be seen that carcinomas with an epithelial percentage between 55 and 75% are almost equally distributed among the different grades. Especially the well-differentiated carcinomas show a great variability in the measured epithelial percentage.

The selection of microscope fields may affect the image processing result because the amount of epithelium is not uniformly distributed throughout the slide, not even throughout the tumor. To evaluate the influence on the estimated epithelial percentage, the image processing procedure has been applied a second time for 15 ovarian specimens with varying degrees of malignancy. The results are compared with the first analysis, as displayed in Figure 3. The correlation between the first and second assessment of the epithelial percentage is 0.97, and the slope of the best linear fit is 1.07 ($p < 0.001$). It can be concluded that the reproducibility due to field selection is satisfactory.

Comparison of the image processing results with the area percentages resulting from interactive morphometry, for 15 ovarian specimens with varying degrees of malignancy (BO, $n = 4$; WDC, $n = 3$; MDC, $n = 2$; PDC, $n = 6$), shows a strong correlation ($r = 0.991$). The slope of the best linear fit is 1.04.

The epithelial percentage, as established with digital image processing in ovarian carcinomas, appears as a strong prognosticator in univariate survival analysis. From the different cutpoints analyzed, a two-group separation with an epithelial percentage of 76% as cut-off point has been proven to be the strongest prognostic factor. When this percentage equals or exceeds the critical value of 76%, the prognosis is considerably worse than for patients with an estimated epithelial percentage of $<76\%$ (Fig. 4: Mantel-Cox = 7.426, $p = 0.0064$).

For ovarian carcinomas, the epithelial percentage is not the only (tissue) characteristic that may have prognostic value. Significant prognostic value may be expected from the FIGO stage. Stepwise selection, using Cox's regression, resulted in a linear combination (F) of

Table 2. Matrix of Epithelial Percentage, Subdivided in Three Categories, and Histological Grade

Grade	VEPDIP		
	<55	55–75	>75
BO	10	4	0
WDC	12	8	3
MDC	0	5	17
PDC	0	2	53

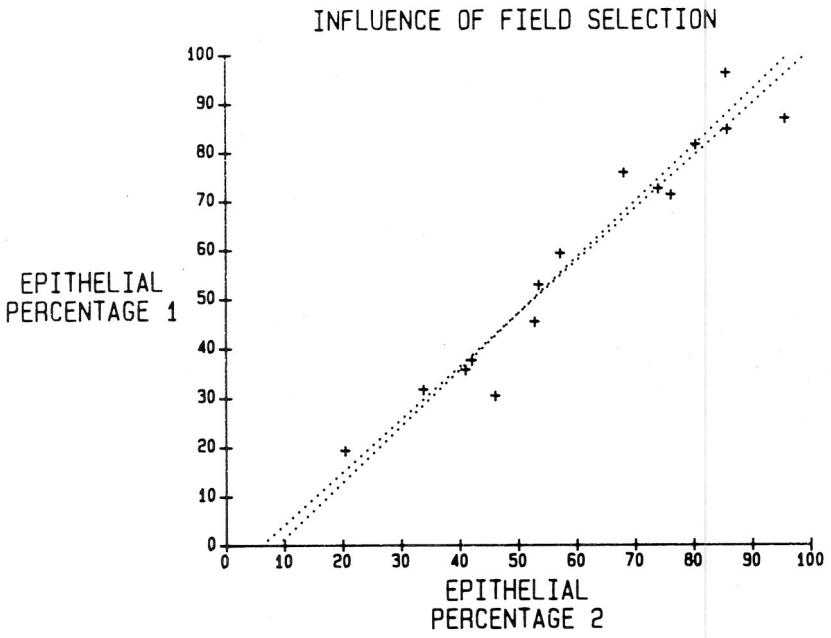

FIG. 3. First and second assessment of epithelial percentage with digital image processing for 15 ovarian specimens with varying degrees of malignancy; $r = 0.97$, and slope of best linear fit is 1.07.

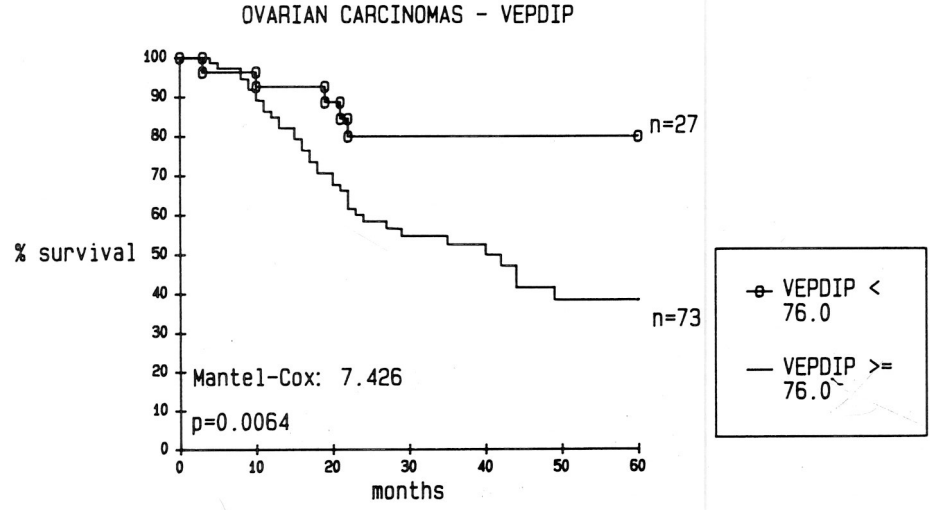

FIG. 4. Kaplan-Meier survival curves for patients with an estimated epithelial percentage (VERDIP) of <76% ($n = 27$) and patients with a percentage exceeding 76% ($n = 73$).

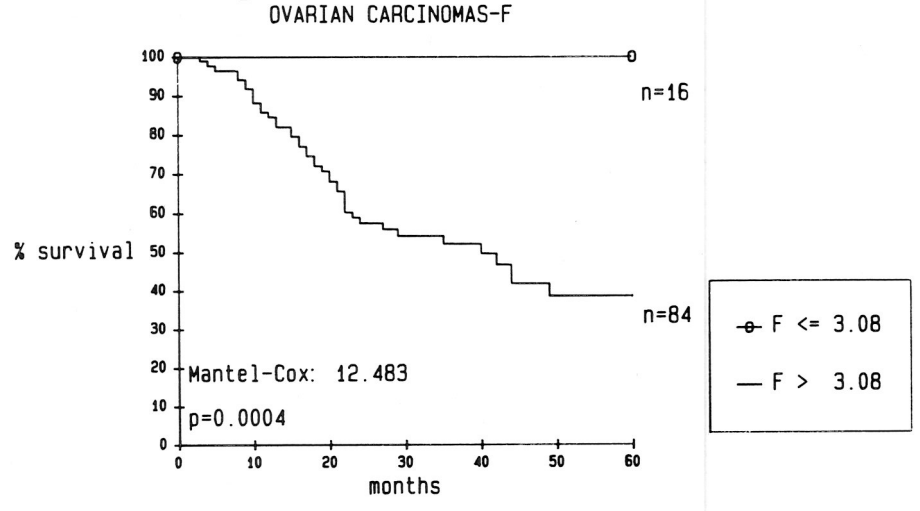

FIG. 5. Kaplan-Meier survival curves for patients with a low F score ($F <= 3.08$, $n = 16$) and high F score ($F1 > 3.08$, $n = 84$). $F = 0.026 \times$ VEPDIP + $0.980 \times$ GFIGO.

the epithelial percentage (VEPDIP) and the FIGO stage—divided into two categories (GFIGO: stage 1 and 2 versus stage 3 and 4). If the F score ($0.026 \times$ VEPDIP + $0.980 \times$ GFIGO) exceeds a critical value of 3.08, the prognosis is considerably worse than for patients with an F score of <3.08. Figure 5 gives the Kaplan-Meier curves for the low score ($F < 3.08$, $n = 16$) and high score ($F > 3.08$, $n = 84$) carcinoma patients.

DISCUSSION

The object of this study is to evaluate the image processing method, automatically estimating the relative volume of epithelium in (tumor) tissue and its prognostic value. Estimates of the epithelial percentage have been obtained for 14 borderline and 100 invasive ovarian tumors of the common epithelial types with varying degrees of malignancy.

The epithelial percentage of the specimen is based on the analysis of four microscope fields selected from the most epithelium-rich areas. The selection of these microscope fields may affect the results. In an experiment on 15 specimens with varying degrees of malignancy, however, no significant influence has been found. Therefore, it can be concluded that the effects on the image processing results due to field selection are negligible.

From visual inspection of the segmentation results in the blue and yellow images, it can be concluded that both tissue and epithelium segmentations are satisfactory. The image processing results are strongly correlated to control percentages, established by interactive morphometry ($r = 0.991$).

The epithelial percentage, as established with digital image processing, is shown to be a strong prognosticator. When the percentage equals or exceeds a critical value of 76%, the prognosis is considerably worse than for patients with an estimated percentage of <76%. Combination with FIGO stage adds to the prognostic power (Fig. 5).

Automated detection of all epithelium-rich areas in the specimen may help selecting the fields of interest (*i.e.*, areas in which quantitative microscopic features are assessed) objectively and may avoid potential influences, due to interactive field selection, on the results. However, such an analysis will be time-consuming and may introduce, apart from edge effects, the following complicating factors.

Areas of Tightly Packed Stromal Nuclei. Areas with compressed stroma may overestimate the epithelial percentage, especially when no epithelium is present in the image. Then, the threshold level to segment the epithelial area is erroneously computed, due to a high minimum pixel value. The potential overestimation, however, is limited by the presence of epithelial nuclei. For the specimens in this study the overestimation is found to be <5%. It can be concluded that overestimation of the epithelial percentage, due to compressed stroma, is neglectible when epithelium is present in the image.

Folds in Tissue. Tissue folds in the image may result in an incorrect estimation of the epithelial percentage. Folds in the epithelium may result in a lower threshold level to segment the nuclei in the yellow image, depending on the contrast between the fold(s) and the epithelial nuclei. A too low threshold level will underestimate the epithelial percentage. Folds in the stromal areas will result in an overestimation.

Areas of Necrosis. Necrosis in the epithelial areas will not influence the estimation of the epithelial percentage. However, necrosis in the stromal areas or in the lumina may cause an increase of the estimated epithelial percentage. In the specimens under study, this is the case for only one specimen (a moderately differentiated carcinoma). The overestimation, due to necrosis in the lumen, was found to be negligible for this specimen.

To overcome the potential problem of incorrect estimation and to correlate the Feulgen-naphthol yellow estimates of the epithelial percentage, an epithelium-specific stain can be used. Such a stain, for example the Cam 5.2 cytokeratin stain suitable for paraffin embedded tissue (15), provides a direct estimate of the epithelial volume. This staining procedure is currently tested for applicability in image processing of paraffin embedded tissue.

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