New algorithms based on the Voronoi Diagram applied in a pilot study on normal mucosa and carcinomas

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An adequate reproducibility in the description of tissue architecture is still a challenge to diagnostic pathology, sometimes with unfortunate prognostic implications. To assess a possible diagnostic and prognostic value of quantitative tissue architecture analysis, structural features based on the Voronoi Diagram (VD) and its subgraphs were developed and tested.

A series of 27 structural features were developed and tested in a pilot study of 30 cases of prostate cancer, 10 cases of cervical carcinomas, 8 cases of tongue cancer and 8 cases of normal oral mucosa. Grey level images were acquired from hematoxyline-eosine (HE) stained sections by a charge coupled device (CCD) camera mounted on a microscope connected to a personal computer (PC) with an image array processor. From the grey level images obtained, cell nuclei were automatically segmented and the geometrical centres of cell nuclei were computed. The resulting 2-dimensional (2D) swarm of pointlike seeds distributed in a flat plane was the basis for construction of the VD and its subgraphs. From the polygons, triangulations and arborizations thus obtained, 27 structural features were computed as numerical values. Comparison of groups (normal vs. cancerous oral mucosa, cervical and prostate carcinomas with good and poor prognosis) with regard to distribution in the values of the structural features was performed with Student’s t-test.

We demonstrate that some of the structural features developed are able to distinguish structurally between normal and cancerous oral mucosa ($P = 0.001$), and between good and poor outcome groups in prostatic ($P = 0.001$) and cervical carcinomas ($P = 0.001$).

We present results confirming previous findings that graph theory based algorithms are useful tools for describing tissue architecture (e.g., normal versus malignant). The present study also indicates that these methods have a potential for prognostication in malignant epithelial lesions.

Keywords: Graph theory, Voronoi Diagram, diagnostic pathology, prognostication tissue architecture, reproducibility, oral mucosa, oral carcinoma, carcinoma of the cervix, carcinoma of the prostate

Abbreviations: CCD, Charged Coupled Device; DT, Delaunay Triangulation; GG, Gabriel Graph; HE, Hematoxylin-Eosine; IOD, Integrated optical density; MST, Minimum Spanning Tree; sd, Standard deviation (used in the Appendix); UT, Ulam Tree; VD, Voronoi Diagram; 2D, Two-dimensional.

1. Introduction

Several authors have demonstrated that subjective grading of malignant lesions is associated with poor reproducibility and accordingly with reduced prognostic power [1–5], although some indicate the opposite [6]. Recently, semiquantitative studies of tissue architecture have shown promising diagnostic and prognostic results [2,4,7–9]. Nevertheless, the problem of intra- and inter-observer variability persists. Over the last decades, several studies have quantitatively studied the relation of structure and function in biological systems, including pathologically altered tissues [10–15]. In vitro transformation studies have demonstrated that the addition of carcinogens to contact inhibited ordered fibroblast monolayer cultures results in loss of contact inhibition, with cells displaying striking criss-crossing growth patterns, where the degree of criss-crossing pattern may reflect the extent of oncogenic transformation [16–19]. These in vitro findings are an indication that the biological status of cells also is expressed in the tis-

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**For a complete list describing the structural features, see Appendix.
sue architecture. Hence, it is biologically meaningful to extract structural features from tissues for diagnosti
c and prognostic purposes, and to do this in a quanti-
tative manner might improve the prognostic value in
some tissues [20–32]. Previous findings in transitional
carcinomas of the bladder indicate that graph theory
based methods are useful tools in grading of malignant
lesions [29], but a prognostic value was not demon-
strated.

We have undertaken the present study in order to
develop tools for fast, strictly quantitative and repro-
ducible tissue architecture analysis in epithelial tissues
(squamous cell carcinomas from the prostate, cervix
and oral cavity and normal oral mucosa) and to eval-
uate the diagnostic and prognostic potential of these
methods in such tissues. By employing graphs such as
the Voronoi Diagram (VD [Figs 1–4]) [33] and its sub-
graphs, the Delaunay Triangulation (DT [Fig. 2]), Min-
imum Spanning Tree (MST [Fig. 5]) [34–38], Ulam
Tree (UT [Fig. 6]) [39] and the Gabriel Graph (GG
[Fig. 7]) [40], the structural manifestations of cellu-
lar interactions in tissues may be quantified [41–47].
A total of 27 structural features were developed, tak-
ing into consideration the shape of individual structural
entities (polygons, triangulations, arborizations), par-
ticularly derived from the VD; clusterings, particularly
from the GG, and studying the order or randomness in
the distribution of pointlike seeds, particularly derived
from the UT and MST.

2. Materials and methods

2.1. Material

The biological material investigated consisted of
8 cases of normal oral mucosa obtained from sur-
plus tissue after plastic surgery on the gingiva in re-
lation to implant surgery, 8 cases of carcinomas of
the tongue, 10 cases of cervical carcinomas and 30
cases of prostate carcinomas. HE stained sections
were made from paraffin embedded tissue blocks fixed in
4% formaldehyde.

2.2. Data acquisition

Grey level images from 5–7 μm thick HE stained
sections were digitised using a charged coupled device
(CCD) camera (Philips® LDH 0670/00 equipped with a
Hamamatsu® AC Adaptor, type A3472) mounted on a
Zeiss Axioplan 2 microscope using a Plan-Neofluar
40×0.75 lens in addition to a Prior H152V2 micro-
scope stage. The final magnification was 400× at a res-
olution of 876 nm (0.9 micrometers) per pixel.

2.3. Segmentation

Local segmentation was used, and developed from
an algorithm based on the size of the elements to be
detected and their contrast to the background. Thresh-
olding was based on the pixel darkness measured as in-
tegrated optical density (IOD). Any pixel with an IOD
within a given range is turned ON, otherwise OFF. The
algorithms for construction of a continuous area of in-
terest (e.g., cell nucleus) were further based on math-
ematical morphology [48]. From the nuclear profiles,
the geometrical center of gravity was computed. The
resulting data were stored as files of coordinates, where
the coordinates represented a center of gravity. Further
analysis of these raw data with computation of struc-
tural features was done on a Pentium® based PC run-
ning Windows 98®. Among the software facilities de-
veloped was the possibility to define digitally a sub-
set of pointlike seeds in order to run the analyses in a
limited window of analysis (Fig. 4).

2.4. Building a composite picture

A composite picture consisting of up to 50 fields
of view was constructed by aligning each field of
view according to a simple algorithm developed by
the authors. For composite pictures generated by man-
ual movement of the microscope stage, an algorithm
using the binary mask of segmented nuclei was em-
ployed. One field of vision is composed of a matrix of
512 × 512 pixels, i.e., 512 separate columns and rows.
When moving to the left in the visual field, the 128
left columns of the binary image are copied from the
left to the right margin of the screen. The microscope
stage is then moved until the binary mask is congru-
et to the grey level image. The offset of the move-
ment thus is 384 columns (75% of the field of view),
giving an overlap from one field to another of 25%.
No further correction of the image alignment was per-
formed. For automatic movement of the microscope
stage a predetermined pattern of movement (spiralling)
using the same offset as default was employed. Hereby,
the stage was first moved one offset (384 columns) to
the left, then one offset (384 rows) up, then two off-
sets (768 columns) to the right. Thereafter two offsets
down, then three offsets to the left, then three offsets
up, three followed by four offsets to the right and so
on. The typical number of fields of view to be included
was 25, 36 or 49, which makes up a square compos-
tive picture. For the automatic image acquisition, align-
ment of visual fields relied on the mechanical accuracy
of the microscope stage.
Fig. 1. A single Voronoi polygon, $V_x$ (Panel A) is defined by the halfplanes $H(p_x, p_y)$ that perpendicularly bisect the lines between a centre point and its neighbouring pointlike seeds. When we use this rule for every point considered, the area of interest is completely covered by adjacent polygons, constituting the Voronoi Diagram (VD). Panel B shows the VD for a set of randomly distributed pointlike seeds in a flat plane.

Fig. 2. The Delaunay Triangulation (DT) represents the dual of the VD and is constructed by drawing lines between pointlike seeds in adjacent Voronoi polygons. The completed construction is a triangular network that covers the whole area. A Delaunay network in two dimensions consists of non-overlapping triangles where no pointlike seeds in the network are enclosed by the circumscribing circles of any neighbouring triangle. The VD is shown in blue and the DT in red.

2.5. Space partitioning

Space partitioning in our context is based on the geometrical center of segmented cell nuclei. Computing the geometrical centers for each nucleus within a considered area creates a 2D swarm of pointlike seeds, from which the VD (Fig. 1) is constructed. All other graphs employed (DT, MST, GG and UT) are subgraphs of the main graph, the VD (Fig. 8). This graph was chosen as the principle tool for exploring the tissue structure, as it is considered to be the most informative [33]. The algorithms for generating these graphs
Fig. 3. An epithelial island from the part of an oral squamous cell carcinoma (E, Panel C) bordering onto the underlying connective tissue (S, Panel C). Panel A is a detail from a HE stained section from the invasive front. The corresponding grey level image is shown in Panel B. Panel C shows Voronoi polygons superimposed on the enlarged grey level image shown in Panel B. The pointlike seed are given as black dots superimposed on the cell nuclei, and represent the geometrical centre of gravity of each nucleus. Each Voronoi polygon represents the area of influence of one pointlike seed, which in epithelial tissues roughly correspond to the extension of a cell. Some cell nuclei are missed (white arrows), but the overall precision in the segmentation is acceptable. The dotted line denotes the basal membrane, which in this case also comprises a part of the border of the window of analysis. The scale bar is 0.10 mm (400× magnification).

have been presented elsewhere [9,33,39,49] and are only briefly commented here. The schematic relationships between the VD versus DT and VD versus GG are shown in Figs 2 and 5, respectively. Figure 3 shows relationship of the VD to tissue structures. In Panel A (HE stained section) an epithelial island (E) can be seen bordering onto the underlying stroma (S). Panel B is the corresponding grey level images. To the right the detail is shown in larger magnification, where the pointlike seeds are superimposed on the cell nuclei, and Voronoi polygons are constructed to make up the VD for the considered area. A program for eliminating border effects in marginal polygons (Fig. 8) was also developed. The window of analysis was defined digitally, by defining a closed contour with a digitizing pad and storing the coordinates of the contour. Only pointlike seeds within the contour were included in the analysis. The coordinates of the part of the contour crossing a marginal polygon were defined as the new edge in the polygon.

VD: The VD for a set of random distributed pointlike seeds is shown in Fig. 1. When two points, \( p_x \) and \( p_y \) are in a plane \( \pi \), a half-plane, denoted \( H(p_x,p_y) \), is defined by the perpendicular bisector of \( p_x,p_y \). The locus of points closer to \( p_x \) than to any other point is the intersector of \( N-1 \) perpendicularly oriented halfplanes, where \( N \) is the number of points in the considered space. Hence,

\[
V(x) = \bigcap_{x \neq y} H(p_x,p_y) \quad \text{(Panel A, Fig. 1)}.
\]

A single Voronoi polygon is defined by the intersection \( \cap \) of \( N-1 \) halfplanes in the considered space, i.e., the center point and its surrounding pointlike seeds (Panel A, Fig. 1). When applied to every point in the considered area, this rules gives the VD (Panel B, Fig. 1).

DT: The DT represents the dual of the VD and is constructed by drawing the lines between the pointlike
Fig. 4. VD with Voronoi polygons shown in pseudocolours, where the areas selected for analysis represent an epithelial island in the underlying stroma. Note in particular that border effects of marginal polygons (the polygons in the periphery of the analysis windows) have been eliminated. The sample is from the invasive front of an oral squamous cell carcinoma of the tongue. The scale bar is 0.5 mm.

Fig. 5. The minimum spanning tree (MST) schematically drawn for the basal cell layers in normal oral mucosa. The MST represents the length of the shortest paths (or tours) through each point exactly once [37,38,50].

seeds in adjacent Voronoi polygons (Fig. 2). The completed construction is a triangular network that covers the considered area. A Delaunay network in two dimensions consists of non-overlapping triangles where no pointlike seeds in the network are enclosed by the circumscribing circles of any neighbouring triangle.

**MST:** Considering $n$ distinct points in a $d$-dimensional space allows for $(n-1)!/2$ closed paths (or tours) through the space. Determining $L(n,d)$, the minimum tour length is possible by defining the smallest constant $\alpha(d)$ such that

\[
\limsup_{n \to \infty} \frac{L(n,d)}{n^{d/\sqrt{d}}} \leq \alpha(d).
\]

Additionally, $\beta(d)$ given by
Fig. 6. The Ulam Tree represents a mathematical object growing in space and time according to specified rules [39]. The UT is generated from the VD, in such a manner that the “branches” of the tree only traverses polygons that are not traversed by any other branch of the tree. The structural feature ELH$_{av}$ (average Edge Length Heterogeneity) is derived from the UT.

Fig. 7. The neighbourhood relation of two points ($p_x$ and $p_y$) according to the Gabriel graph. Two points, $p_x$ and $p_y$, are considered as neighbours if the circle on which they are placed, is empty. $p_k$ on the other hand, is not a neighbour to either $p_x$ or $p_y$ (Panel A). The Gabriel graph (Panel B) is similar to the Delaunay Triangulation but contains polygons in addition to triangles.

\[
\limsup_{n \to \infty} \frac{L(n,d)}{d^{d-1} \cdot \sqrt{d}} = \beta(d),
\]

applies to almost all optimal tours in the considered $d$-dimensional space. The above limit fails only for a negligible subset of tours [37]. Furthermore, the above approximation applies to any dimension [50]. The solution to the problem can be reached by several different computations. For our purpose, the MST was derived by a decimation of the DT [51].

**GG:** In a Gabriel graph (Fig. 7, Panel B), pointlike seeds are connected by an edge only if the circle defined by the diameter connecting the nodes contains no other pointlike seeds (Panel A, Fig. 7). The two points ($p_x$ and $p_y$) are neighbours if and only if the circle (with a center O) on which they are placed is empty.
Accordingly, $p_x$ and $p_y$ (and not $p_k$) are neighbours (Panel A, Fig. 7). A graph similar to the DT can be constructed. However, it differs from the DT in that it contains polygons in addition to triangles (Panel B, Fig. 7). Cases are matched by comparing the lengths and orientations of the edges associated with each pointlike seed in one graph with those of every pointlike seed in a second graph. The Gabriel graph is particularly sensitive to subtle differences in the number or relative positions of pointlike seeds, making it a suitable tool for detecting changes in cellular organization within tissues.

**UT**: The Ulam Tree represents a mathematical object growing in space and time according to specified rules [39]. The UT is generated from the VD, in such a manner that the “branches” of the tree only traverse polygons that are not traversed by any other branch of the tree (Fig. 6).

**2.6. Topographical analysis**

Applying the 27 algorithms we have developed on the polygons obtained when constructing the VD and its subgraphs (space partitioning) we have performed topographical analysis on tissue specimens from normal and cancerous epithelium. The number of epithelial cells included in the analysis varied from 1500–5000. For a more detailed description of the VD-based algorithms, see Appendix.

**2.7. Border effects**

Marginal seeds represent a source of error as they yield polygons with a morphology that deviates from the population as a whole (Fig. 8). Structural features derived from marginal polygons are irrelevant, as they represent a non-representative population, and thus are a source of errors. Accordingly, we developed software to eliminate these aberrations (Fig. 4).

**2.8. Temporal aspects**

Scanning 30–50 fields of view is done in 8–12 minutes, depending on whether it is done manually or automatically. Segmentation requires another 5–10 minutes, depending on the number of cells in the specimen. For computation of structural features, 30–90 seconds are required, giving a total time expenditure of approximately 14–25 minutes.

**2.9. Statistical analysis**

Student’s $t$-test was used for comparison of groups. All $P$-values were two-tailed, and values less than 0.05 were considered to indicate statistical significance.
Table 1
Diagnostic value of 10 structural features in normal and malignantly changed oral mucosa

| Structural feature | Mean values with ranges given in parentheses | Normal oral mucosa | Carcinoma of the tongue (n = 8) | P-value* |
|-------------------|----------------------------------------------|--------------------|---------------------------------|----------|
| RF_{av}           |                                              | 0.81 (0.73–0.87)   | 0.67 (0.57–0.76)               | 0.01     |
| RF_{dis}          |                                              | 0.74 (0.66–0.78)   | 0.67 (0.56–0.79)               | 0.05     |
| A_{dis}           |                                              | 0.45 (0.37–0.54)   | 0.39 (0.33–0.45)               | 0.09     |
| MSTEL_{av}        |                                              |                    | 5.9† (4.3–6.8)                 | 0.05     |
| PTS               |                                              | 0.74 (0.66–0.87)   | 0.69 (0.54–0.74)               | 0.10     |
| DEL_{av}          |                                              | 26.8† (25.9–27.3)  | 25.8 (4.9–26.7)                | 0.001    |
| ELH_{av}          |                                              | 0.41 (0.33–0.58)   | 0.32 (0.23–0.48)               | 0.02     |
| DKNN_{av}         |                                              | 728† (703–738)     | 693 (641–723)                  | 0.03     |
| NNRR              |                                              | 32.8 (29.1–33.6)   | 31.9 (28.4–33.2)               | 0.16     |
| RMPB              |                                              | 15.7† (13.6–17.1)  | 12.9 (11.6–14.6)               | 0.03     |

* Two-tailed.
† Measured in pixels.
‡ Structural features for which the differences between groups reaches statistical significance.
§ See Appendix for an explanation of the separate structural features.

Results from running 10 different form parameters on altogether 16 samples of oral mucosa, 8 cases from normal mucosa and 8 cases with carcinomas of the tongue. Numbers in parentheses denote the range. 5000 cells were included in the analysis. For a further description of the form features used, see Appendix. P-value for the best structural feature was 0.001 (DEL_{av}).

3. Results

A total of 10 of the 27 structural features we investigated were able to distinguish between normal and malignantly altered tissue and/or were shown to have a possible prognostic value (Tables 1–3).

3.1. Normal oral mucosa versus carcinoma of the tongue (Table 1)

Eight biopsies from assumptively normal oral mucosa (acquired during gingivoplastic procedures in relation to serial extraction of teeth) and 8 cases of oral carcinomas of the tongue were compared with regard to values of 10 structural features. Of these, 6 features made it possible to distinguish between normal oral mucosa and carcinoma of the tongue, usually situated at the lateral border of tongue, bordering onto the floor of the mouth. This pertains to the features RF (roundness factor, from the VD, \( P = 0.01 \)), RF_{dis} (disorder of the roundness factor disorder derived from the VD, \( P = 0.05 \)), DKNN_{av} (average distance to the K-nearest neighbours, \( P = 0.03 \)), DEL_{av} (average Delaunay Edge Length, \( P = 0.001 \)), ELH_{av} (average edge length heterogeneity of the Ulam Tree, \( P = 0.02 \)) and RMPB (radius of the maximum percolating ball, percolating the Delaunay network, \( P = 0.03 \)). The ability of these methods to discern normal and cancerous oral epithelium points to a diagnostic potential as they obviously detect structural differences between normal and malignantly changed mucosa.
Table 2
Prognostic values of 10 structural features in carcinomas of the cervix

| Structural feature | Mean values with ranges given in parentheses | \(P\)-value* |
|-------------------|-----------------------------------------------|--------------|
|                   | Cervical carcinomas with good prognosis \((n = 4)\) | Cervical carcinomas with poor prognosis \((n = 6)\) |
| RF\textsubscript{av} | 0.74 (0.55–0.86) | 0.69 (0.54–0.81) | 0.31 |
| RF\textsubscript{dis} | 0.69 (0.56–0.78) | 0.69 (0.56–0.78) | 0.24 |
| A\textsubscript{dis}\textsuperscript{†} | 0.45 (0.37–0.54) | 0.35 (0.27–0.41) | 0.02 |
| MSTEL\textsubscript{av} | 4.9\textsuperscript{†} (4.3–5.8) | 5.2 (3.3–6.8) | 0.33 |
| PTS | 0.78 (0.66–0.87) | 0.65 (0.54–0.74) | 0.14 |
| DEL\textsubscript{av} | 26.8\textsuperscript{†} (25.9–27.3) | 26.3 (24.9–27.7) | 0.31 |
| ELH\textsubscript{av}\textsuperscript{†} | 0.57 (0.39–0.68) | 0.38 (0.29–0.43) | 0.02 |
| DKNN\textsubscript{av} | 725\textsuperscript{†} (650–795) | 695 (630–740) | 0.09 |
| NNRR\textsuperscript{†} | 36.0 (30.6–41.2) | 30.1 (28.4–31.9) | 0.03 |
| RMPB\textsuperscript{†} | 16.1\textsuperscript{†} (14.6–17.1) | 13.9 (11.6–15.1) | 0.04 |

* Two-tailed.
† Measured in pixels.
‡ Structural features for which the differences between groups reaches statistical significance.
§ See Appendix for an explanation of the separate structural features.

Results from running 10 different form parameters on altogether 10 cases of carcinomas of the cervix, 4 with good (relapse-free survival more than 12 years) and 6 with poor (relapse-free survival less than 5 years) prognosis when 5000 cells are include in the analysis. The featured DEL\textsubscript{av} and ELH\textsubscript{av} display significant differences in the two prognosis groups in this test set. \(P\)-values for the best structural feature are 0.001 (DEL\textsubscript{av} and ELH\textsubscript{av}).

3.2. Carcinomas of the cervix (Table 2)
Altogether 10 biopsies; 4 with a good (relapse-free survival more than 12 years) and 6 cases with a poor (relapse-free survival less than 5 years) prognosis were examined. A total of 4 structural features made it possible to distinguish between the two outcome groups. These were A\textsubscript{dis} \textit{(area disorder, from the VD, \(P = 0.02\))}, ELH\textsubscript{av} \((P = 0.02)\), NNRR \textit{(number of nearest neighbours within a restricted radius of 75 pixels, \(P = 0.03\))} and RMPB \((P = 0.04)\).

3.3. Carcinomas of the prostate (Table 3) The values of the same 10 form features as above when applied to 30 cases of carcinomas of the prostate \(\left(15 \text{ cases with good and 15 cases with poor prognosis}\right)\) are shown. Altogether 5 structural features made it possible to distinguish between carcinomas of the prostate with a good and poor prognosis. These structural features were RF\textsubscript{dis} \((P = 0.01)\), A\textsubscript{dis} \((P = 0.02)\), DEL\textsubscript{av} \((P = 0.001)\), ELH\textsubscript{av} \((P = 0.02)\), DKNN \textit{(average distance to the K nearest neighbour, from the DT, \(P = 0.01\))} and RMPB \((P = 0.03)\). Note in particular that DEL\textsubscript{av} and/or ELH\textsubscript{av} as significant descriptors are common to all three sets of tissues.

4. Discussion
We present data from several tissues that demonstrate the possible diagnostic and prognostic value of
Table 3

Prognostic value of 10 structural features in carcinomas of the prostate

| Structural feature | Mean values with ranges given in parentheses | Prostate carcinomas (n = 15) | Prostate carcinomas (n = 15) | \( P \)-value* | Student’s \( t \)-test |
|--------------------|-----------------------------------------------|------------------------------|------------------------------|----------------|---------------------|
| RF\(_{av}\)         | 0.65 (0.52–0.78)                               | 0.71 (0.54–0.81)             | 0.31                         |
| RF\(_{dis}\)†       | 0.61 (0.53–0.68)                               | 0.74 (0.63–0.81)             | 0.01                         |
| A\(_{dis}\)†       | 0.55 (0.37–0.64)                               | 0.35 (0.27–0.41)             | 0.02                         |
| MSTEL\(_{av}\)     | 5.5† (4.3–6.8)                                 | 5.4 (4.1–6.2)                | 0.33                         |
| PTS\(_{dis}\)       | 0.73 (0.63–0.87)                               | 0.65 (0.54–0.74)             | 0.24                         |
| DEL\(_{av}\)†      | 27.1† (25.9–28.3)                              | 25.8 (24.9–26.7)             | 0.001                        |
| ELH\(_{av}\)†      | 0.41 (0.33–0.58)                               | 0.32 (0.23–0.48)             | 0.001                        |
| DKNN\(_{av}\)      | 705† (650–740)                                 | 680 (640–715)                | 0.11                         |
| NNRR\(_{av}\)      | 29.8 (29.1–30.6)                               | 33.8 (29.4–35.2)             | 0.01                         |
| RMPB\(_{av}\)      | 13.4† (11.3–14.4)                              | 16.2 (13.4–17.4)             | 0.03                         |

* Two-tailed.
† Measured in pixels.
‡ Structural features for which the differences between groups reaches statistical significance.
§ See Appendix for an explanation of the separate structural features.

Results from running 10 different form parameters on altogether 30 cases of prostate carcinomas, 15 with good (relapse-free survival more than 10 years) and 15 with poor (relapse-free survival less than 3 years) prognosis. 5000 cells were included in the analysis. \( P \)-value for the best structural feature is 0.02 (A\(_{dis}\) and ELH\(_{av}\)).

Over-fitting typically occurs when the number of features analyzed is high in relation to the number of samples considered [52]. The main graph in our context (VD) encompasses a number of subgraphs, such as the DT, MST, UT and GG, and is generally regarded as the most informative graph [33]. The VD is generated from the point-like seeds representing the centres of gravity within cell nuclei of the considered tissues. The Voronoi polygon represents the region of influence of each seed. A priori, this does not have any direct biological correlate. However, in epithelial tissues, with only sparse intercellular substance, it roughly corresponds to the somata of the epithelial cells. The shape and size of cells is a structural feature of considerable interest in tradi-
tional histological assessment of pathologically altered tissue, e.g., carcinomas. This relationship breaks down when we consider the stromal tissue, with abundance of intercellular substance (Panel C, Fig. 3). Thus, the algorithms we have developed directly derived from the VD we believe are best suited for tissues with a minimum of intercellular substance, as in epithelial tissues, although graph theory based methods could be applied to any tissue. Also, there is no reason that other features based on, e.g., the MST or UT should have such limitations.

The segmentation algorithms we developed performed with an acceptable level of precision (Panel C, Fig. 3). The algorithms represent a compromise of speed and precision. For the calculations, a minimum number of 1000–1500 cells were included. We have chosen such a fairly large number of objects to be included in the analysis, as preliminary runnings of computations indicated that the values of the structural features did not stabilise until at least 1000–1500 objects were included, the exact number depending on which tissue was analysed. The imaginary flat plane we consider in fact represents a 3-dimensional entity. Depending on the thickness of the sections considered some cell nuclei might be below or above the focal plane and therefore missed in the segmentation. However, for the sections thickness we have employed (5–7 μm), this has not been a major problem (Fig. 3). However, several observer note that in the most aggressive carcinoma of the oral cavity, the epithelial cells bordering onto the underlying stroma show a distinct blurring of their structural features, with a typically glossy appearance of the somata and nuclei (M. Bryne, personal communication). It is conceivable that because of this, a considerable number of nuclei in the area of interest could escape segmentation, with a resultant error in the estimation of the structural features. If this is abundant, a distinct prognostic group of lesions might be missed, at least with the HE staining procedures. In our study, however, this was not a prominent feature, and the precision in the segmentation is acceptable (Fig. 3).

A particular point of interest when investigating the invasive front of carcinomas, are the border effects, that tend to have a dominant effect when the epithelial islands become small and numerous (Fig. 8). In particularly aggressive lesions, the gross structures of the invasive front of carcinomas tend to disintegrate, with multiple small fingerlike projections into the underlying stroma. In sections, these projections will present as small epithelial islands, consisting of a very limited number of cells. This poses a possible problem with regard to border effects, as these will become dominant in small cluster of cells, perhaps eliminating them entirely. Again, these cases may be of particular prognostic interest.

Only structural features of the epithelial tissue have been investigated in this study. Most likely, the tumor-host response will result in structural alterations of diagnostic and prognostic value also in the underlying stroma. Such features could be the amount of inflammatory response, which can easily be assessed by our methods. However, current algorithms do not detect the specific nature of subepithelial lymphocyte infiltration. New methods for segmentation of immunohistochemically stained cell nuclei [53,54] might contribute to shedding more light on biological information contained in the pattern of inflammatory response.

Twenty-nine structural features based on algorithms derived from the Voronoi Diagram or its subgraphs on different sets of epithelial tissues, 10 of which were demonstrated to have a diagnostic or prognostic potential. The ultimate test for these methods will be to employ a limited number of structural features (e.g., DEL_av and ELH_av) on an independent test set [52].

Appendix

1) A_dis Area disorder. The Area denotes the area of a single Voronoi polygon, measured in pixels. Area disorder reflects the variation in the polygons associated with considered population of pointlike seeds:

\[ A_{\text{dis}} = 1 - \frac{1}{1 + \frac{\text{Area}_{sd}}{\text{Area}_{av}}} \]

The feature acquires the value of 0 if all polygons have the same area and tends towards 1 otherwise. The entire population except the marginal polygons is considered.

2) DEL_av Average Delaunay Edge Length. This feature sums up the edge length of the edges of the DT’s and divides it by the number of non-marginal seeds.

3) DEL_dis Delaunay Edge Length disorder. This feature considers the standard deviation (sd) in the lengths of the edges of the Delaunay triangles linking non-marginal seeds:

\[ \text{DEL}_{\text{dis}} = 1 - \frac{1}{1 + \frac{\text{DEL}_{sd}}{\text{DEL}_{av}}} \]
4) **DENS** This feature represents the density of the entire population, except for the marginal polygons, which are eliminated:

\[
DENS = \frac{\text{Polygon_nb}}{\text{Area_sum}}
\]

Polygon_nb denotes the number of polygons.

5) **DEP_av** Average Delaunay Edge Probability. One edge of the DT belongs to two triangles, each being associated with an overlapping circle.

\[
DEP_{av} = 1 - \frac{d1 + d2}{r1 + \frac{r2}{\sqrt{3}}}
\]

Here, \(d1\) denotes the distance of the first vertex to its nearest neighbour. \(d2\) denotes the distance of the second vertex to its nearest neighbour. \(r1\) is the radius of the first circle associated with the two triangles sharing the considered edge \(d1\). \(r2\) is the radius of the first circle associated with the two triangles sharing the considered edge \(d2\) (Panel B, Fig. 2). \(\sqrt{3}\) is a normalization factor with respect to the triangular lattice.

6) **DEP_dis** Delaunay Edge Probability disorder. This feature denotes the disorder of the abovementioned Delaunay edge probability, and is given by the following equation:

\[
DEP_{dis} = 1 - \frac{1}{1 + \frac{\text{DEP}_{sd}}{\text{DEP}_{av}}}
\]

7) **DFRAC_av** This feature denotes the average fractal dimension of the Ulam Trees, more precisely an application of the Hausdorff fractal dimension which express the properties of topological defects in the tree structure, e.g., as related to a highly regular tree. Consider a closed contour (e.g., Ulam Tree) within a 3-dimensional space, on which two points, \(x\) and \(y\), are placed. The Ulam Tree may be viewed as projected onto a 2-dimensional flat plane with a unit of length corresponding to the size of a pixel. The mean value of the area \(A\) covered by traversing in \(N\) steps from \(x\) to \(y\) along branches of the tree is given by \(A \sim r^2\) where \(r\) is given by the equation

\[
r = \sqrt{|x - y|^2}.
\]

The quantity \(r\) effectively expresses the radius of the circle or square that has the same area as the projection of the traversed part of the tree projected onto the 2D plane where \([\ldots]\) denotes the mean values for all the possible contours within the considered space. \(x\) and \(y\) are expressed as vectors.

The Hausdorff dimension of the Ulam Tree is given by the general equation

\[
D_H = \frac{1}{\Delta} \quad [56].
\]

A value of \(\Delta \neq 1\) indicates that the contour is fractal, i.e., how fuzzy the contour is.

The algorithm for computing \(\Delta\) is fairly simple. From the projection of the tree on a 2D plane (with pixels as the unit of length), one defines the upper, lower, left and right margins of the projected tree, which yields a rectangle with sides \(lx, ly\). The \(r\) accordingly is defined as

\[
r = \sqrt{lx \cdot ly},
\]

\[
\log N = \log k + \frac{1}{\Delta \cdot \log r}.
\]

\(N\) can be derived from the number of nearest neighbouring distances required to build the Ulam Tree within a square where \(px\) and \(py\) lies on two opposing margins of the square.

8) **DKNN_av** Average distance to the \(K\) nearest neighbours. \(K\) was set to 16 after preliminary simulations showed that normal oral mucosa was statistically different from carcinomas of the tongue with regard to this feature (Table 1). Considering the \(K\) nearest neighbours of a seed (according to the Delaunay neighbourhood), if all of them are non-marginal seeds, DKNN represents the sum of the distances to those neighbours. DKNN_{av} represents the average of DKNN over all the population where \(K\) neighbourhood does not contain any marginal seeds.

9) **DKNN_dis** Disorder of the DKNN.

\[
DKNN_{dis} = 1 - \frac{1}{1 + \frac{\text{DKNN}_{sd}}{\text{DKNN}_{av}}}
\]

10) **DRT** Divergence from the Regular Tree. Transposing the tree into a matrix, \(M\), quantitates the neighbourhood from the Ulam tree, composed of two orthogonal properties which are the integration and the topological properties of the tree. The integration property is the number of points
added to the tree at a given level of expansion, while the topological property is the number of simple, double, triple etc junctions in the tree.

\[ \text{DRT} = \sum_x \sum_y \frac{|M_{xy} - M_{ref,xy}|}{W_x}. \]

Hence, the DRT is the sum of the weighted absolute differences between all the elements of the actual tree matrix \((M_p)\) and a theoretical tree reference \((M_{ref})\) [51].

11) \text{ELH}_{av} This is the average edge length heterogeneity of the UT’s. ELH is representative of the intrinsic node to node distance variations in the current tree [39].

12) \text{HA}_{av} Average area of holes within the considered area. Considering the empty circles associated with each DT, a triangle is set to be a hole if a ball of the current radius is able to go through at least one edge of the triangle (i.e., the radius of the percolating ball is equal to or less than at least one of the edges of the triangle). The number of DTs that can be percolated by the ball is the average number of DT’s belonging to the defined holes within the architecture. Considering a Voronoi Edge (VE, denoted \(ls\)) and the Delaunay edge that it bisects (\(lg\)), let \(r_1\) and \(r_2\) be the radius of the circles associated with the vertices of VE. If the length of \(ls\) is smaller than or equal to both \(r_1\) and \(r_2\), then the two DT’s associated with the vertices of VE and accordingly belong to the same hole.

13) \text{HA}_{dis} Disorder of the whole area of the holes:

\[ \text{HA}_{dis} = 1 - \frac{1}{1 + \text{HA}_{sd}/\text{HA}_{av}}. \]

14) \text{MSPDG} Minimal step percolating the Delaunay network (PPDG). MSPDG is the length of the minimum step required to be able to move without interruption from neighbour to neighbour in a Delaunay network that comprises at least 50% of the entire network.

15) \text{MSTEL}_{av} Average Minimum Spanning Tree Edge Length. The MST is a tree that spans the entire population in such a way that the sum of the Euclidian edge length is minimal [49,55].

16) \text{MSTEL}_{dis} Edge length disorder of the minimum spanning tree:

\[ \text{MSTEL}_{dis} = 1 - \frac{1}{1 + \text{MSTEL}_{sd}/\text{MSTEL}_{av}}. \]

17) \text{NNRR}_{av} Average number of neighbours within a restricted radius. Considering a circle of which radius is set to 75 pixels around one seed, NNRR is the number of other seeds (neighbours) lying within the circle. \text{NNRR}_{av} is the average number of seeds over all the population located at least at 75 pixels from the border of the analysis window.

18) \text{NNRR}_{dis} Disorder in the Number of Neighbours within a Restricted Radius.

\[ \text{NNRR}_{dis} = 1 - \frac{1}{1 + \text{NNRR}_{sd}/\text{NNRR}_{av}}. \]

Considering a circle of which radius is set to 75 pixels around one seed, NNRR is the number of other seeds (neighbours) lying within the circle. \text{NNRR}_{dis} is the disorder in the number of seeds over all the population located at least at 75 pixels from the border of the analysis window.

19) \text{PTS} probability of topological stability. The center of gravity of the nuclei are stored as coordinates. For a small error tolerance (one pixel in diameter), we move at random all the pointlike seeds and rebuild the graph. As long as one seed keeps the same neighbourhood as without any disorder, the local PTS is computed to be 1, otherwise it is computed to be zero. At each step in the simulation (with one pointlike seed moved one pixel in any direction) the PTS represents the number of seeds that kept (after disorder is introduced) the same neighbourhood as initially. The more the PTS is close to 1, the more stable the graph is to a random alteration. The closer the PTS is to zero the less valid local topology is, only statistics can then be computed about local topology. The marginal polygons are not considered.

20) \text{PTS}_{av} Average of 10 runs of the PST computation.

21) \text{PTS}_{dis} is the PTS disorder, given by the equation

\[ \text{PTS}_{dis} = 1 - \frac{1}{1 + \text{PTS}_{sd}/\text{PTS}_{av}}. \]

22) \text{RMPB} Radius of the Maximum Percolating Ball. Considering the empty circles associated with each DT, a triangle is set to be a hole if the radius of its associated empty circle is below the current threshold. If a ball of the current radius is
able to go through one edge of the DT, then the
neighbouring triangle can be associated to the
same hole as the considered triangle. The RMBP
is the value of the radius of the biggest ball that is
able to percolate the Delaunay network. In other
terms, it is the maximum allowed radius to ob-
tain a hole that is larger in area than 50% of the
area of all the holes.

23) RF\_av Average Roundness Factor, given as

\[ RF_{av} = \frac{4\pi}{\text{perimeter}^2}. \]

This equals the roundness factor of one polygon
average RF is the average over the entire popu-
lation except for the marginal polygons.

24) RF\_dis Roundness Factor disorder.

\[ RF_{dis} = 1 - \frac{1}{1 + \frac{RF_{av}}{RF_{sd}}}. \]

This features acquires the value of 1 if all the
RF’s are the same and tends towards zero other-
wise. The entire population except the marginal
polygons were considered.

25) WGC Weighted global compacity. Consider one
Voronoi vertex and its associated three Delau-
nay seeds (Fig. 2). To each of these seeds corre-
sponds a set of nearest neighbours located at dis-
tance \( d_1, d_2, d_3 \) etc. If \( r \) is defined as the radius of the
considered Delaunay circle, the compacity is
defined as follows:

\[ \text{WGC} = \frac{d_1 + d_2 + d_3 \cdots + d_n}{9r^2}. \]

The weighted compacity (WC) is equal to C
multiplied by the area of the DT. By doing this,
one takes into account the possibility that two
different DT’s can have exactly the same propor-
tions, but different size and accordingly repre-
sent two different structural entities. Only con-
sidering the unweighted compacity does not take
this possibility into consideration. Only consid-
ering the WGC is the average WC over all non-
marginal polygons.

26) WGC\_av Average Comapcty.

\[ \text{WGC}_{av} = \frac{1}{n} \sum_{i=1}^{n} \frac{d_1 + d_2 + d_3}{9r^2 \cdot n}. \]

27) WGC\_dis This represents the disorder of the Com-
pacity, and is given by the equation

\[ \text{WGC}_{dis} = 1 - \frac{1}{1 + \frac{\text{WGC}_{av}}{\text{WGC}_{sd}}}. \]

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