Automated Evaluation of Tumor-Associated Macrophages (TAMs) Local Density in Colorectal Cancer

Rogojanu R[1,2], Mesteri I[1], Ellinger I[1], Thalhammer T[1], Kallay E[1], Heindl A[1,3], Seewald A[3], Bises G[1]

1) Medical University Vienna, 2) TissueGnostics GmbH, Vienna, 3) Seewald Solutions

Introduction

To study cellular systems (cytomics), microscopic detection of molecules in tissues is combined with quantitative analysis of images using dedicated software.

This cytomics technology provides new qualitative and quantitative information on tissues at a cellular and subcellular level.

In the last years studies on tumor progression have pointed out the importance of stromal factors in the development and the faith of solid tumors. The tumor mass is composed of different cell types that produce inflammatory mediators. TAMs represent a high percentage of the tumor mass.

Recently it was reported that in colorectal cancer a low proportion of epithelial cells in relation to stromal cell influences the tumor progression.

Aims

Because the macrophages secrete cytokines to their surroundings, as well as colon tumor cells secrete chemokines, the density of macrophages (CD68+) and their distance to the epithelial cell area (K8+) could be of importance for tumor prognosis.

To be able to measure and statistically evaluate these parameters in a larger set of samples, the aim of this project is to establish automatic assessment procedures for these characteristics of TAMs.

Methods

Paraffin sections from 8 colorectal G2 tumors with adjacent mucosae were available for immunofluorescent double staining. Four patients had already developed liver metastasis.

Macrophages were stained by antibodies against CD68 (clone KP1) and epithelial cells by antibodies against Keratin 8 (clone EP1628Y) from Thermo Fisher Scientific.

AlexaFluor 647 and AlexaFluor 546 were used as secondary antibodies (Invitrogen Molecular Probes). Nuclei were labeled with DAPI (Roche).

Images were recorded by the automatic slide scanner TissueFaxs (TissueGnostics) using a 20x objective. The images in Fig. 1 and 2 will be used to exemplify our approach.

Automated analysis of image data was done using classical image processing algorithms optimized for large images.

Results

1. Epithelial area detection

Assessment of macrophages (white cells in Fig. 2) in the stromal area required also the detection of the epithelial area (recognized by the K8+ staining, see Fig.1 and red in Fig. 2) followed by exclusion of the detected area.

The detection approach was watershed with imposed seeds, due to the morphological structure of epithelial area and was performed on a virtual channel combined from both DAPI and the K8 images (see results in Fig. 3). We calculated then the distance transform (see Fig. 5) using a fast marching algorithm. Stitching of images from the whole sample is required for a proper estimation of the distance.

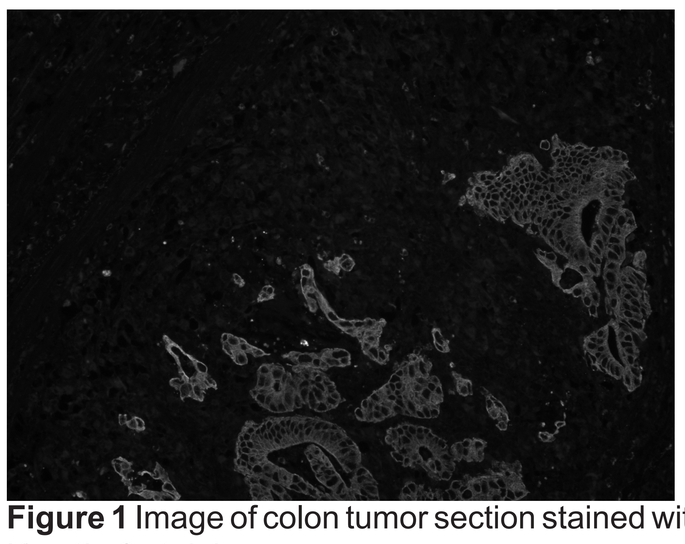


Figure 1 Image of colon tumor section stained with Keratin 8 staining

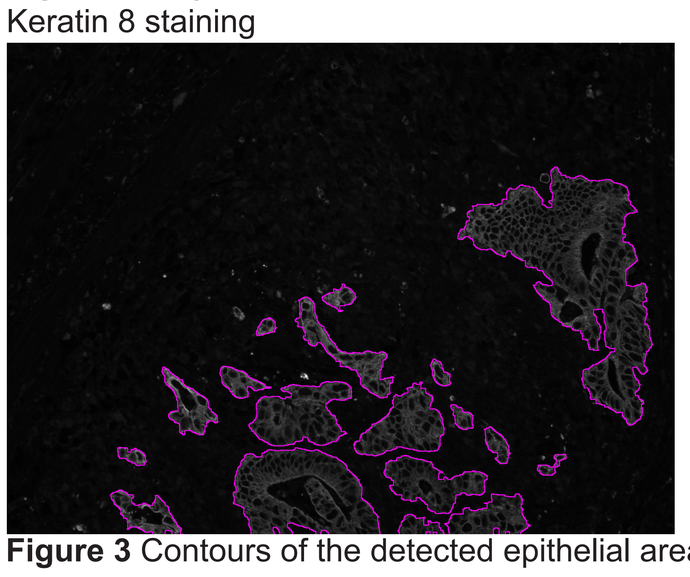


Figure 3 Contours of the detected epithelial area

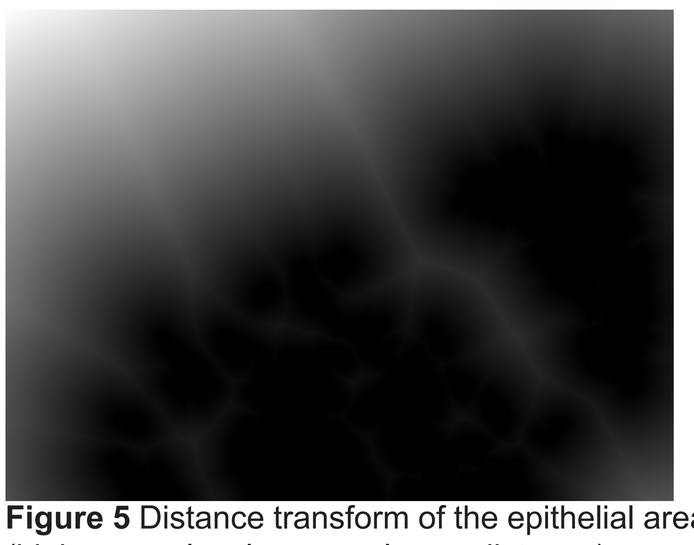


Figure 5 Distance transform of the epithelial area (higher gray levels means larger distance)

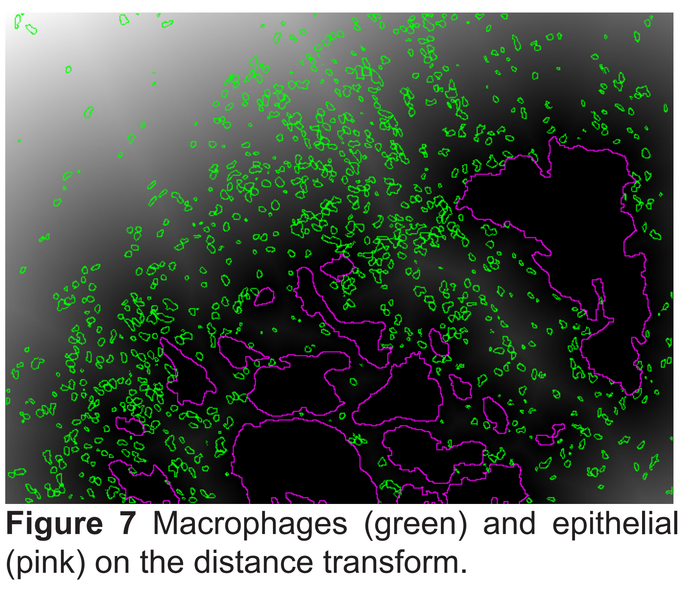


Figure 7 Macrophages (green) and epithelial area (pink) on the distance transform.

2. Calculation of the macrophage density

Next, the number of macrophages was determined by gating the population in the Cy5 vs DAPI scattergram (see Fig. 4). The TAM density per stromal area was measured as being 21% higher than when considering the total area of the image in Fig. 2.

3. Calculation of the distance of TAMs to epithelial tissue

Finally, by using the distance transform (Fig. 5) and the location of the detected macrophages (green contours in Fig. 6) we determined the distance of each macrophage to the closest epithelium (see Fig. 7 and 8).

Conclusion

We established methods that allow TAMs scoring in stromal area only and calculation of the distance between TAMs and epithelial area. This is important for tumor prognosis because TAM produce their effect through the secretion of soluble mediators that have to reach certain local tissue concentrations for proper function.

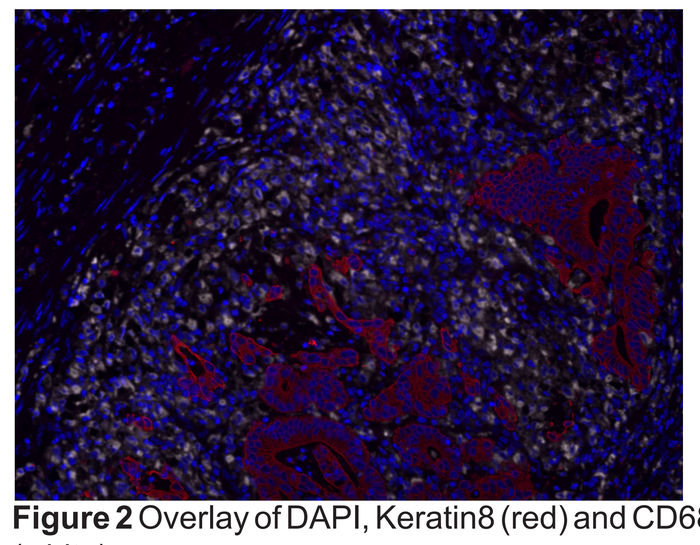
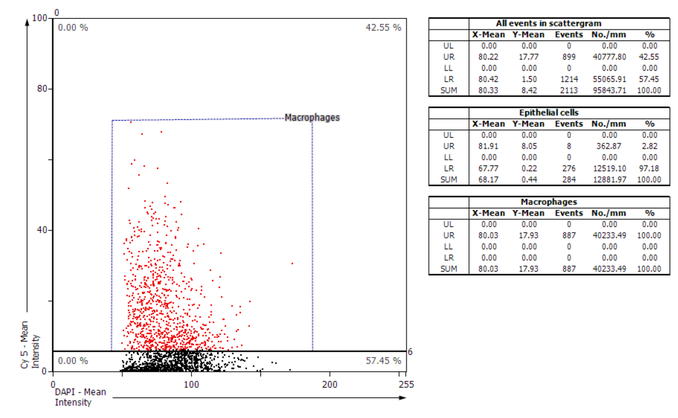


Figure 2 Overlay of DAPI, Keratin8 (red) and CD68 (white)



All events in scattergram	X-Mean	Y-Mean	Events	No./mm	%
UL	0.00	0.00	0	0.00	0.00
UR	80.22	17.77	899	40777.80	42.55
LL	0.00	0.00	0	0.00	0.00
LR	80.42	1.50	1214	55065.91	57.45
SUM	80.33	8.42	2113	95843.71	100.00

Epithelial cells	X-Mean	Y-Mean	Events	No./mm	%
UL	0.00	0.00	0	0.00	0.00
UR	81.91	8.05	8	362.87	2.82
LL	0.00	0.00	0	0.00	0.00
LR	67.77	0.22	276	12519.10	97.18
SUM	68.17	0.44	284	12881.97	100.00

Macrophages	X-Mean	Y-Mean	Events	No./mm	%
UL	0.00	0.00	0	0.00	0.00
UR	80.03	17.93	887	40233.49	100.00
LL	0.00	0.00	0	0.00	0.00
LR	0.00	0.00	0	0.00	0.00
SUM	80.03	17.93	887	40233.49	100.00

Figure 4 Gating the macrophages in TissueQuest

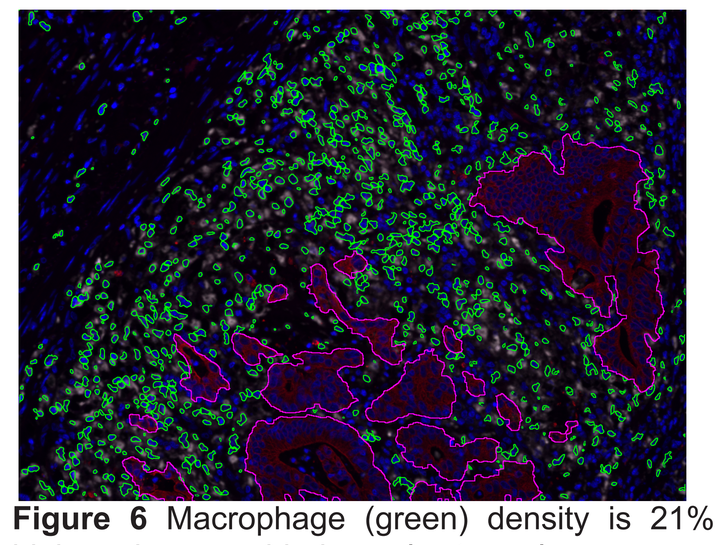
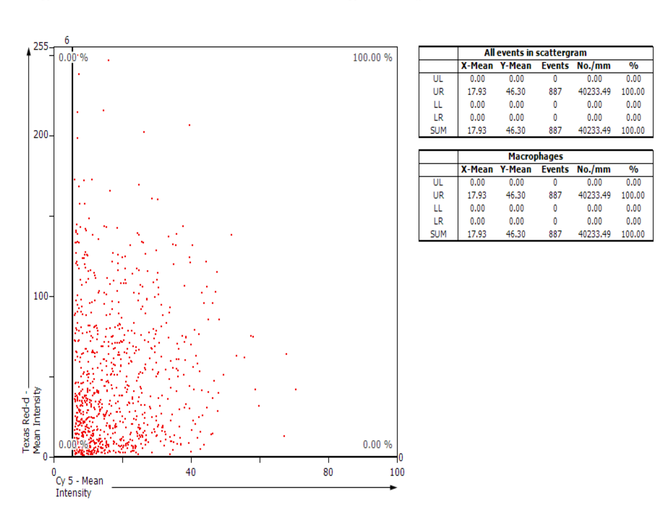


Figure 6 Macrophage (green) density is 21% higher when considering only stromal area



All events in scattergram	X-Mean	Y-Mean	Events	No./mm	%
UL	0.00	0.00	0	0.00	0.00
UR	17.93	46.30	887	40233.49	100.00
LL	0.00	0.00	0	0.00	0.00
LR	0.00	0.00	0	0.00	0.00
SUM	17.93	46.30	887	40233.49	100.00

Macrophages	X-Mean	Y-Mean	Events	No./mm	%
UL	0.00	0.00	0	0.00	0.00
UR	17.93	46.30	887	40233.49	100.00
LL	0.00	0.00	0	0.00	0.00
LR	0.00	0.00	0	0.00	0.00
SUM	17.93	46.30	887	40233.49	100.00

Figure 8 Scattergram of macrophage CD68 expression versus distance to closest epithelium

Outlook

The final algorithm(s) will be applied in all sections from patients with CRC. Because macrophages have the potential to express pro- and anti-tumor activity in relation of their different activation, TAMs will be characterized by several markers and the results will be correlated with the diagnosis and the patient history (e.g. liver metastasis).

Acknowledgements

This project is funded by FFG (Bridge 818094)