Quantitative image analysis

A whole slide scanner (NanoZoomer 2.0-HT, Hamamatsu) equipped with a 40X objective lens was used to digitize H&E and FAP stained sections of tumor and control tissue slides at 40x magnification (Figure 2). Quantitative analysis of IHC stained slides was then performed using an automated histomorphometry software Visiopharm® (Hoersholm, Denmark). Annotation of regions of interest (ROI) was performed roughly manually (P.B.) on whole-slide images. The ROIs were set to delineate tumor, peritumoral tissue and control tissues separately. For peritumoral and control to tissue, three different ROI were chosen - the epidermis and dermis (collagen), adipose and muscle tissue. In the inflammatory controls, the inflamed areas were included in the ROI (inflammation) and distinguished from adjacent non-inflammatory tissue. Prominent and large artefacts arising from tissue processing were manually cropped-out during ROI annotation.

Digital image analysis consisted of two steps. The first application was designed to detect and exclude artefacts from the analysis and delineated the ROI as accurate as possible. The software was trained on multiple slides for tissue detection to identify the areas that are to be included for analysis (e.g., tumor tissue) and those to be excluded (e.g. background and artefacts like tissue folds). This application detects tissue based on a thresholding technique for pixels and includes several post processing steps to in- or exclude elements based on size, surroundings or shape. The quantitative output of the tissue segmentation and classification is an optimized ROI for each tissue type. The output was visually controlled for each slide and the ROI manually adjusted in cases of wrong tissue detection (P.B.).

Within the second step, for each tissue type one application was designed to measure the FAP labeled area and the mean intensity (MI) of staining (Figure 3). The FAP labeled area was stratified by the intensity of staining defined by pixel thresholds. These thresholds were visually determined and predefined on a set of heterogeneous tumor samples. Staining intensity was classified in 5 levels. Pixel values above 176 were considered as background staining intensity (negative). For positively stained pixel values, four staining intensity levels were defined: level 1, low (131-175 pixel); level 2, low intermediate (101-130 pixel); 3, high intermediate (71-100 pixel); level 4, high staining intensity (<71 pixel). The area of FAP labeled tissue was measured within these 4 levels and the total FAP positively stained area was calculated as sum of level 1-4 for each slide. The different measured areas (ROIs) were normalized to a standardized area of ten high-power (x400) fields (HPF) for all further comparisons (2.37 mm^2) [41]. The second output measure was defined as the overall mean staining intensity (MI) of the ROI expressed as pixel value with lower values representing a stronger brown staining for FAP. Measurements were performed similarly for all tissue types and species using the same pixel thresholds.