cores in case multiple tumour cores are evaluated per patient. In this study, we evaluated a single tumour core per patient and therefore, we did not compensate for variation in the amount of tissue in different cores.

The mean percentage HLA class-I positive tumour epithelium area scored with the HCA2/HC10 antibody mix was 96.7%± 5.3 (Table 2). In total, 220/284 (77.5%) of the TMA cores were scored with 95-100% HLA class I-positive tumour epithelium (Table 2). Additionally, 56/284 (19.7%) and 8/284 (2.8%) tissue cores were scored with 80-95% and 60-80% HLA class I-positive tumour epithelium respectively (Table 2). No TMA cores were scored with <60% HLA class I-positive tumour epithelium (Table 2). These results indicate that the double staining method and subsequent semi-automated image analysis can be used to score HLA class I expression in TMA cores of rectal cancer, enabling use of objective and consequent scoring criteria.

Semi-automated scoring of HLA class I expression using EMR8-5 antibodies

Next, we assessed HLA class I expression in TMA cores of rectal cancer with

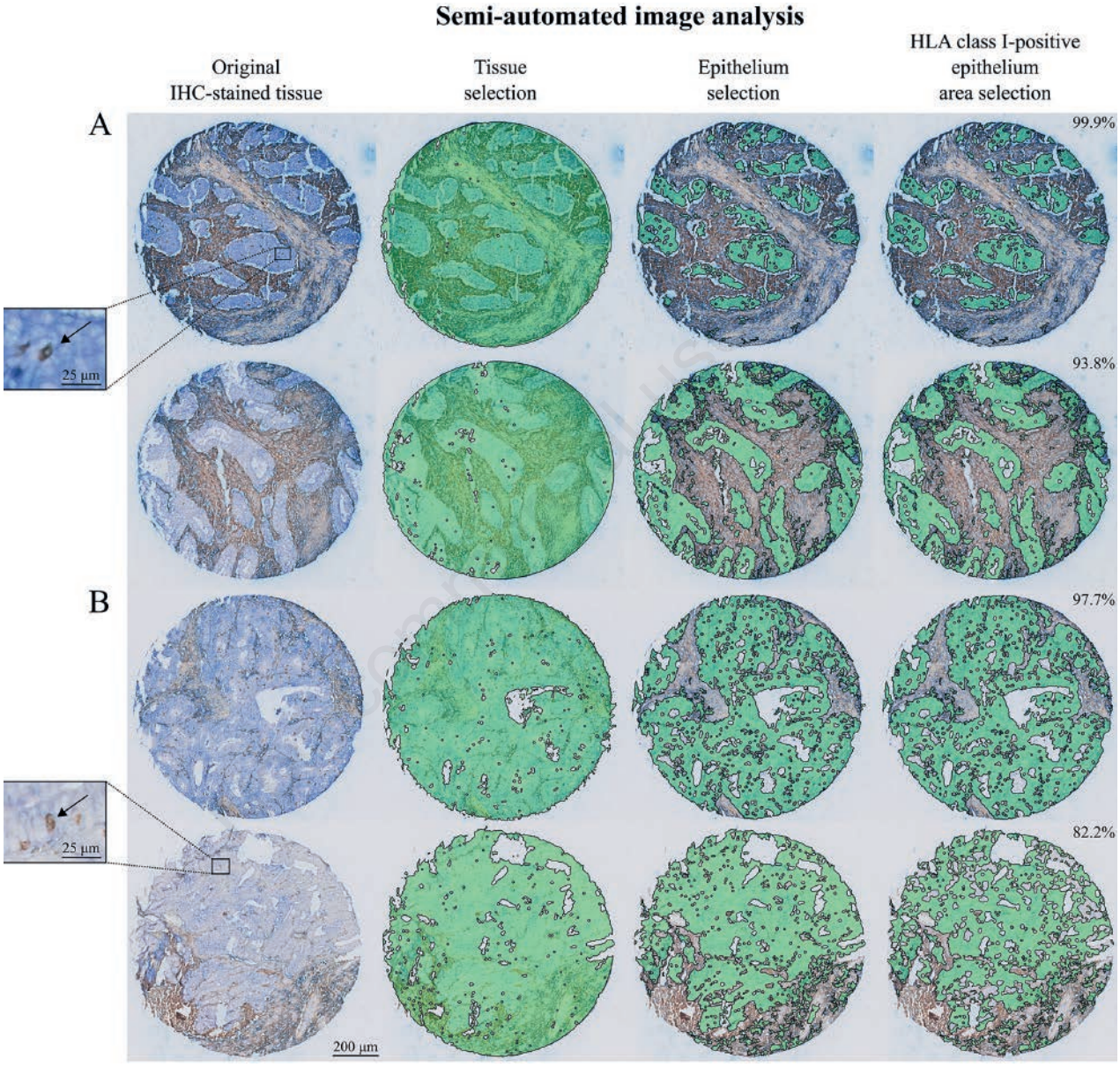

Figure 1. IHC double staining with subsequent semi-automated image analysis for HLA class I expression in four different TMA cores of rectal tumours with HCA2/HC10 or EMR8-5 antibodies. An IHC double staining was set up to analyse HLA class I expression in rectal cancer. Stromal tissue, blood vessels, and immune cells were stained brown whereas HLA class I expression (HCA2/HC10 antibody mix or EMR8-5) was stained blue. Representative images are presented of two TMA cores stained with the HCA2/HC10 antibody mix (A) and two TMA cores stained with EMR8-5 antibodies (B). The arrows in the high magnification inserts indicate (brown-stained) tumour-infiltrating immune cells. The images illustrate the different steps of the semi-automated image analysis of HLA class I expression in rectal cancer. Tissue selected in each step of the analysis is indicated in green. First, all tissue in the core was selected. Second, tumour epithelium was identified within the tissue selection by subtraction of the brown stroma. Third, the percentage of HLA class I-positive epithelium area was scored within the epithelium selection as displayed in the upper right corner of each TMA core. IHC, immunohistochemistry; HLA, human leukocyte antigen; TMA, tissue microarray.

EMR8-5 antibodies using the same image analysis workflow as described above. The threshold for HLA class I-positive staining was used as determined for the HCA2/HC10 stained TMA cores. Figure 1B shows two representative TMA cores stained with EMR8-5 with the sequential steps in the semi-automated image analysis. More variation was observed in the percentages of HLA class I-positive tumour epithelium in TMA cores with EMR8-5 compared to HCA2/HC10 (Table 2). The mean percentage HLA class-I positive tumour epithelium area scored with the EMR8-5 antibodies was 92.9%±9.5 (Table 2). In contrast with the HCA2/HC10 antibody mix, almost no deposition of blue chromogen outside the tissue area was observed

Table 2. Overview of semi-automated scoring of HLA class I expression in a TMA of rectal cancer using HCA2/HC10 or EMR8-5 antibodies. Tumour epithelium expression of HLA class I was scored in TMA tumour cores of 284 rectal cancer patients with HCA2/HC10 antibodies, and in 298 patients with EMR8-5 antibodies. The mean percentage of HLA class I-positive tumour epithelium area is shown in the table. In addition, HLA class I scores were categorized as follows: <60%, 60-80%, 80-95%, or 95-100% HLA class I-positive tumour epithelium.

	HCA2/HC10 N=284 (%)	EMR8-5 N=298 (%)
HLA class I expression		
Mean±SD	96.7±5.3	92.9±9.5
Range	69.1-100.0	18.9-100.0
HLA class I expression		
<60%	0 (0.0)	3 (1.0)
60-80%	8 (2.8)	16 (5.4)
80-95%	56 (19.7)	113 (37.9)
95-100%	220 (77.5)	166 (55.7)

HLA, human leukocyte antigen; SD, standard deviation.

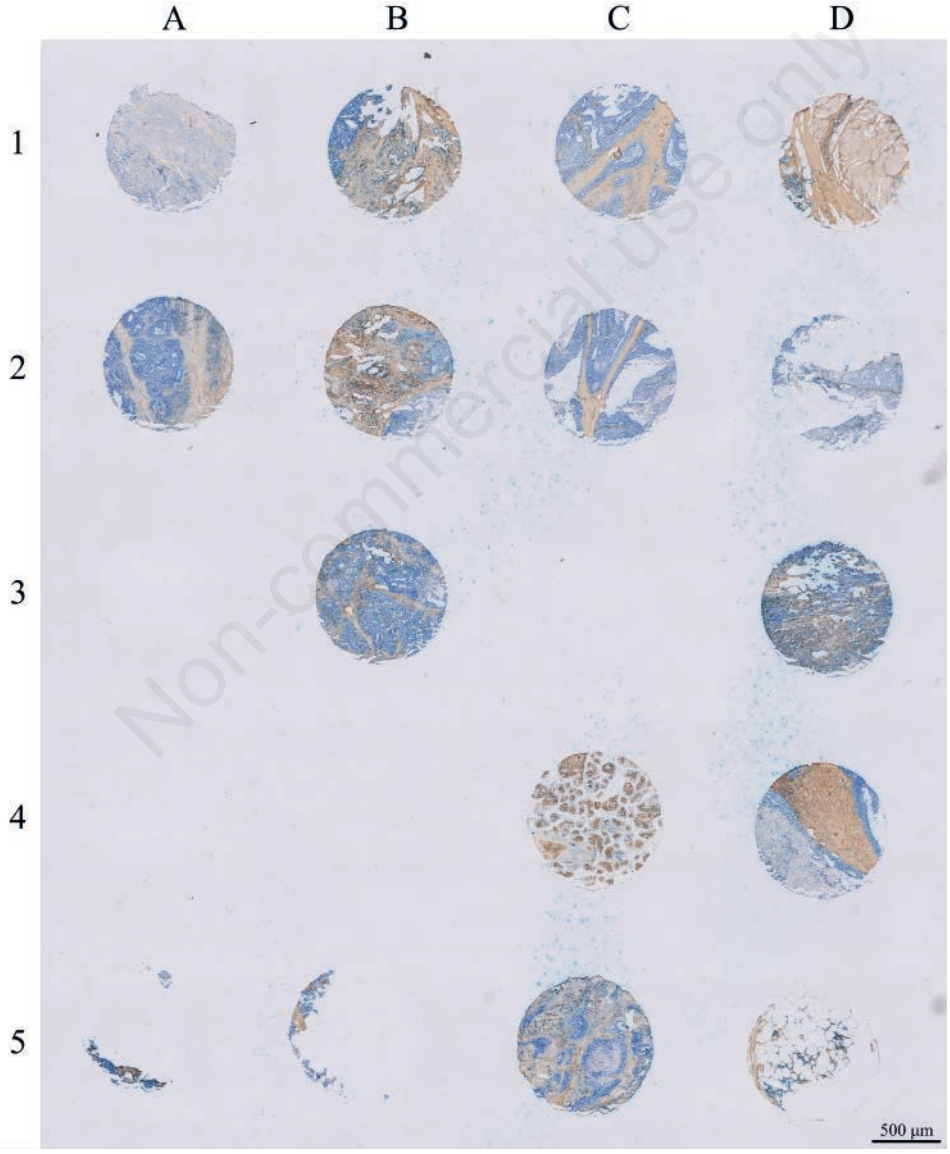

Figure 2. Example of TMA cores with rectal tumour tissue stained for HLA class I expression with EMR8-5 antibodies. An IHC double staining was set up to analyse HLA class I expression in rectal cancer. Stromal tissue, blood vessels, and immune cells were stained brown whereas HLA class I expression (EMR8-5) was stained blue. A representative area of a TMA stained for HLA class I expression, containing 20 TMA cores, is shown. TMA, tissue microarray; IHC, immunohistochemistry; HLA, human leukocyte antigen.

in the EMR8-5 staining, suggesting that EMR8-5 did not bind to remnants of the used tape. As a result, the total tissue area was not overestimated using EMR8-5. In conclusion, these results indicate that our IHC double staining technique and subsequent semi-automated image analysis can be used to score HLA class I expression on tumour epithelium using antibodies that recognize different HLA class I epitopes.

Comparison of HCA2/HC10 and EMR8-5 antibodies for the assessment of HLA class I expression

Finally, we compared the percentage of HLA class I-positive tumour epithelium in TMA cores of rectal cancer as assessed by HCA2/HC10 and EMR8-5 antibodies, respectively. In total, 280 tissue cores were successfully analysed for both IHC staining. Scorings of HLA class I expression on tumour epithelium with HCA2/HC10 and EMR8-5 antibodies significantly correlated (ρ=0.136, P=0.022). Hence, tumour cores scored with a high area percentage of HLA class-I positive tumour epithelium assessed by HCA2/HC10 antibodies were also scored with a high area percentage of HLA class-I positive tumour epithelium using EMR8.5 antibodies and the other way around. However, scoring of HLA class I expression in categories did not correlate when assessed with HCA2/HC10 and EMR8-5 antibodies (P=0.101). We hypothesized that tumour epithelium would be scored equal or higher for HLA class I expression with HCA2/HC10 compared to EMR8-5 antibodies due to known cross reactivity of HCA2 with non-classical HLA molecules.[12,13] In the majority of the TMA cores (82.5%), the percentage of HLA class I-positive tumour epithelium was scored equal (±10%) with HCA2/HC10 and EMR8-5 antibodies. In a fraction of the tissue cores (4.3%), the percentage of HLA class I-positive epithelium was scored >10% higher with EMR8-5 compared to HCA2/HC10. When examined in further detail, the scored differences in these tissue cores were due to inaccurate selection of the tumour epithelium as a result of tissue damage and a relatively high amount of blue chromogen deposition outside the tissue area in the HCA2/HC10 staining. In contrast, 4 times as many tissue cores (13.2%) were observed with >10% higher HLA class I-positive percentage tumour epithelium area when assessed by HCA2/HC10 antibodies compared to EMR8-5. A representative tumour that was scored >10% higher with HCA2/HC10 compared to EMR8-5 is shown in Figure 3. TMA cores with this staining pattern might express non-classical HLA class I molecules such as HLA-E, HLA-F and/or HLA-G that are recognized by HCA2/HC10 antibodies, but not by EMR8-5. In conclusion, the staining patterns of HCA2/HC10 and EMR8-5 antibodies are different, characterized by recognition of additional epitopes by HCA2/HC10, most likely non-classical HLA class I molecules.

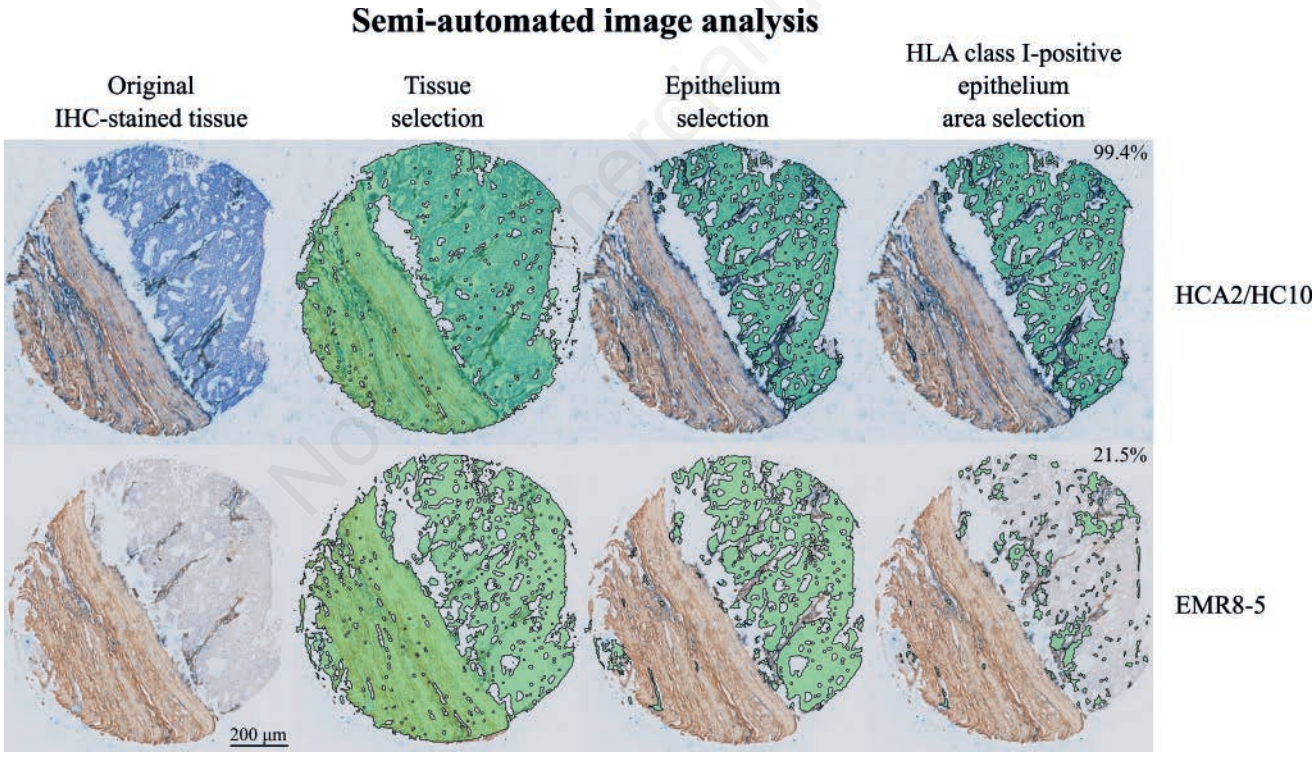

Figure 3. Comparison of HCA2/HC10 and EMR8-5 antibodies for the evaluation of HLA class I expression in a TMA core of rectal cancer. An IHC double staining was set up to analyse HLA class I expression with HCA2/HC10 and EMR8-5 antibodies in a TMA of rectal cancer. Stromal tissue, blood vessels, and immune cells were stained brown whereas HLA class I expression was stained blue. The percentage of HLA class I-positive tumour epithelium area (indicated in the figure) was evaluated using semi-automated image analysis. Tissue selected by the software in each step of the analysis is indicated in green. First, all tissue in the TMA cores was selected. Second, tumour epithelium was identified within the tissue selection. Third, the percentage of HLA class I-positive epithelium area was scored within the epithelium selection. The example shows a TMA core that was observed to be positively stained with the HCA2/HC10 antibody mix, while being mostly negative regarding EMR8-5. These tissue cores might express non-classical HLA class I molecules such as HLA-E, HLA-F and/or HLA-G that are recognized by the HCA2/HC10 antibody mix but not by EMR8-5. HLA, human leukocyte antigen; TMA, tissue microarray; IHC, immunohistochemistry.

Discussion

In order to study the expression of clinical predictive and prognostic biomarkers in tumour tissue using IHC, it is essential that scoring is standardized. Here, we presented an IHC double staining and subsequent semi-automated image analysis method that can be used to score the tumour epithelium expression of HLA class I in rectal cancer. Importantly, our developed technique has a major advantage by allowing discrimination between epithelium and non-epithelial tissue, which enabled simple semi-automated tissue selection based on colour. This method therefore enabled quantification of a biomarker (*i.e.* HLA class I) on tumour epithelium, even when also expressed by stromal tissue and/or immune cells. Using our relatively simple IHC double staining technique, a straight forward slide scanner is sufficient in order to obtain images that can be analysed at digital platforms that support JPEG files and that may be chosen based on availability and/or user experience. With the described image analysis method using *AxioVision* software, it was possible to calculate the percentage of HLA class I-positive tumour epithelium with a set threshold, thereby acquiring objective data. Since this scoring method is relatively fast and simple, it is highly suitable for analysis of biomarker expression on tumour tissue in both research and clinical settings. To the best of our knowledge, we are the first to describe a double staining method for semi-automated image analysis of biomarkers expressed by cancer tissue using a TMA.

Many different techniques have been reported to evaluate biomarkers on tumour tissue.[17-22] Some studies, using *MATLAB* software, could give insights in the relocation of tumour biomarkers between a cell's membrane, cytoplasm and nucleus using RGB unmixing of images of conventional IHC-stained sections (DAB and haematoxylin signals).[20,21] Additionally, *MATLAB* software (*MIAQuant* code) was recently reported as novel computational method for the quantification of IHC stained tissue sections.[23,24] This software is already used in clinical cancer research for determination of the expression of microRNAs in melanomas, which might have value in prediction of poor immunotherapy outcome.[25] As no discrimination could be made between tumour epithelium and non-epithelial tissue, this method is not suitable for quantification of tumour biomarkers that are also expressed by non-epithelial cells. Other studies reported that the software platform *ImageJ* can be used to evaluate the expression of biomarkers based on its immunohistochemical staining intensity on a TMA in a high throughput manner.[17-19] Using this method, the tumour-epithelium specific expression of biomarkers could be analysed since the biomarker expression signal on epithelium was higher compared to non-epithelial tissue. Hence, the threshold for positive staining was set in a way that the relatively low signal in stromal tissue was scored as negative. Unfortunately, this method cannot be used for tumour epithelium-specific evaluation of biomarkers like HLA class I, that are also expressed at high levels on non-tumour cells. Recent studies using digital image analysis software *QuPath* or *ImageJ* showed that it is in principle possible to score biomarkers such as p53, Ki67, PR, HER2, and CD3 on tumour epithelium cells with standard IHC and haematoxylin counterstaining without a need for tumour or stromal identification markers.[26-28] However, it should be considered that the deep learning algorithms used in these analyses have disadvantages as a large number of training samples are required that need to be manually annotated by experts, thereby making it a time-consuming method to set up.[29,30] Additionally, deep learning algorithms often require the use of expensive microscopes and complicated software. For the future, it is likely that deep-learning algorithms in combination with the presented IHC double staining are even better for accurate scoring of biomarker expression on tumour epithelium. Importantly, deep learning algorithms might be less complicated to set up based on our IHC double staining since we used staining of non-epithelial cells instead of morphological features to distinguish tumour cells from other cells.

Although our semi-automated image analysis method has many advantages compared to other automated image analysis techniques, it also presents with some limitations. For instance, it provides information regarding the percentage of HLA class I-positive tumour epithelial area instead of percentage of tumour cells. Inclusion of a nuclear stain to the presented IHC staining would be required to estimate the latter. For this purpose, it is necessary to discriminate between more than two colours on one tissue slide for which the current software like we used is not suitable. Deep learning software, for instance in combination with spectral imaging, may solve this problem. Additionally, although infiltrating immune cells could be visualised and excluded from the tumour epithelium selection, it was not possible to quantify them in TMA tumour cores. This was due to the use of one chromogen for both immune cells and other non-epithelial cells, thereby complicating discrimination of these cell types. For detailed analyses of infiltrating immune cells, multiplex immunofluorescence is advised.[31,32] Furthermore, our IHC double staining does not discriminate between normal epithelium and tumour epithelium. This is not an issue when analysing TMA tumour cores since the location of these tumour tissue cores has been annotated and checked by a pathologist. When analysing whole tissue sections, however, it will be necessary to visually verify which areas contain tumour epithelium and should therefore be analysed. Finally, as described in the Materials and Methods section, the presented IHC double staining uses species-specific antibodies, meaning that in combination with the rabbit-derived ECM/CD45 antibody mix, biomarkers can be evaluated using any non-rabbit-derived antibodies. In case only rabbit antibodies are available for the evaluation of a specific biomarker, a new ECM/CD45 mix has to be prepared with antibodies derived from another species.

HCA2 and HC10 antibodies are often used to study HLA class I expression in cancer.[4-6,15] Importantly, the combination of HCA2 and HC10 antibodies overestimates the total HLA class I expression due to the cross reaction of HCA2 with non-classical HLA molecules[12,13] that are known to be present in rectal tumours.[5,15] In this study, we showed that some TMA cores of rectal cancer positively stained with HCA2/HC10 antibodies while being negative regarding EMR8-5, thereby suggesting that EMR8-5 indeed does not cross react with additional non-classical HLA molecules.[16] Therefore, in our opinion, EMR8-5 is a better monoclonal antibody to assess the expression of HLA class I specifically compared to a mix of HCA2/HC10 antibodies.

In conclusion, the presented IHC double staining and subsequent semi-automated image analysis can be used for the scoring of HLA class I expression on tumour epithelium, thereby acquiring quantitative and objective data. This staining method and automated analysis can be expanded to any other tissue biomarker and also to different (epithelial) cancer types, and is therefore widely applicable for tumour biomarker analysis.

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