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Objective analysis of cancer stem cell marker expression using immunohistochemistry

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Summary

Analysis of immunohistochemical expression is often a subjective and semiquantitative process that can lead to the inconsistent reporting of results. To assess the effect that region selection and quantification method have on results, five different cancer stem cell markers were used in this study to compare tissue scoring with digital analysis methods that used three different tissue annotation methods. Samples of tumour and normal mucosa were used from 10 consecutive stage II colon cancer patients and stained for the putative cancer stem cell markers ALDH1, CD44v6, CD133, Lgr5 and SOX2. Tissue scoring was found to have considerably different results to digital analysis with the three different digital methods harbouring concordant results overall. However, SOX2 on normal tissue and CD133 on tumour and normal tissue produced discordant results which could be attributed to the different regions of tissue that were analysed. It is important that quantification method and selection of analysis areas are considered as part of study design to ensure that reproducible and consistent results are reported in the literature.

Key words: Colorectal cancer; cancer stem cell; immunohistochemistry; ALDH1; CD44v6; CD133; Lgr5; SOX2.

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INTRODUCTION

The existence of cancer stem cells (CSC) in colorectal cancer (CRC) is currently an active area of investigation. Following the initial descriptions of CSC,[1,2] several studies have investigated their role in human CRC by using immunohistochemical (IHC) analysis to quantify the expression of CSC biomarkers in tumour samples.

The method of analysis varies between studies but semiquantitative scoring of biomarker expression is the most widely used. However, this is somewhat subjective and can result in inter-observer variability.[3,4] Different interpretations of staining intensity, or variations in scoring methods, may also contribute to contrasting findings being reported in the literature.[5–9] For example, whereas one study counting the number of glands expressing the glycosylated transmembrane protein CD133 found that high expression was independently prognostic for poor cancer-specific survival,[6] another study using a scale of 0–3 for positive tumour staining found that CD133 was not a significant prognostic factor in CRC.[7] These studies analysed different areas of the tumour samples and used different cut-off scores, possibly impacting the final result.[6,7] Studies using tissue microarrays (TMAs) have been reported to result in more false-negative associations between CD133 expression and CRC patient survival,[10] implying that tissue selection is an important factor. More objective and defined methods for analysis and tissue selection are likely to improve the reproducibility of published results.

Automated digital platforms now offer the possibility for objective analysis of protein expression in tissue samples. Algorithms supplied with the software packages allow quantification of the percentage of positivity by tissue area, or by cell count. In this study we aimed to compare different tissue annotation methods and to assess the expression of five CSC biomarkers using the Aperio Imagescope software platform.

MATERIALS AND METHODS

Patients and samples

Formalin fixed, paraffin embedded samples of tumour and adjacent normal mucosa were retrieved from 10 consecutive patients who underwent potentially curative resection in 2014 for stage II colon cancer at the St John of God Hospital, Subiaco, Western Australia. The study was approved by the St John of God Healthcare and University of Western Australia Human Research Ethics Committees. All patients used in this study consented to the use of their biological samples.

Immunohistochemistry

Six serial sections were cut from each block and stained with haematoxylin and eosin (H&E) and for five different CSC markers associated with colorectal cancer: CD133, aldehyde dehydrogenase-1 (ALDH1), CD44-variant -6 (CD44v6), leucine-rich repeating G-protein coupled receptor-5 (Lgr5) and sex-determing region-Y homeobox-2 (SOX2). Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 6 min at 121°C using the DakoCytomation Pressure Cooker (Dako, Denmark). Incubation with primary antibody was performed at room temperature for 30 min for CD44v6 (clone VFF-18; 1:1000 dilution; Abcam, Australia), CD133 (clone AC133; 1:50 dilution; Miltenyi Biotec, Australia) and Lgr5 (clone MC-1235; 1:200 dilution; MBL International, USA) and for 1 hour for ALDH1 (clone 44; 1:1000 dilution; BD Biosciences, USA) and SOX2 (clone EPR3131; 1:50

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dilution; Abcam). Endogenous peroxide was blocked using Peroxidazed 1 (Biocare Medical, USA) and non-specific IgG interactions were blocked using Background Sniper (Biocare Medical). Immunostaining was then completed using the EnVision-HRP/DAB System (Dako). SOX2 antibody required use of the EnVision Rabbit Link reagent (Dako) for amplification. All sections were lightly counterstained in Mayers haematoxylin (Hurst Scientific, Australia) before dehydration and mounting. Slides were scanned using a high resolution digital scanner (Aperio Scanscope XT; Leica Biosystems, Australia).

Quantification of marker expression

Three regions of interest (ROI) for each of tumour and normal epithelium were selected at medium power (20×) for analysis. To avoid being influenced by the CSC marker staining pattern, regions for analysis were first selected using H&E images and these areas were then selected on the corresponding IHC-stained images (Fig. 1). For each stain, five different quantification methods were performed.

1. Scoring: The three ROIs were scored semi-quantitatively for staining intensity and proportions of cells stained on a scale of 0–3 by one observer (TM). Separate scores were given for epithelium and stroma.
2. Grid-plot: A 2 × 2 mm grid was overlayed on each ROI and each intersecting point was scored using the same scoring system, noting the tissue type (epithelium/stroma/lamina propria).
3. Digital ROI: The Aperio colour deconvolution algorithm v9 (Leica Biosystems) was used to objectively quantify the percentage area of positive staining for CD133, CD44v6, ALDH1 and Lgr5 on each ROI. The nuclear quantification algorithm v9 was used to calculate the percentage of cells positive for SOX2. Luminal areas that contained cell debris were excluded from this analysis.
4. Digital full-face: Tumour or normal area of tissue was traced at full-face with the same algorithms used to quantify expression.
5. Digital whole section: Algorithms were used on the whole of each section without any tracing or annotation.

Calculation of H-scores

To compare the manual ROI scoring method to the three digital analysis methods, H-scores were calculated to give a score from 0 to 300 based on intensity and proportions of expression for each marker and method. For the manual 'Scoring' method, and for SOX2 expression quantified using any of the digital analysis methods, H-scores were calculated using the following formula:

H-score = (3× percentage of 3+ cells) + (2× percentage of 2+ cells) + (1× percentage of +1 cells).

For ALDH1, CD44v6, CD133 and Lgr5 analysed using the digital analysis methods, H-scores were calculated using the following formula:

H-score = (3× percentage area of strong staining) + (2× percentage area of moderate staining) + (1× percentage area of weak staining).

Statistical analysis

Kendall's W statistic and Kendall's tau-b concordance coefficients were calculated to determine the level of agreement between the analysis methods used in this study. Overall p values of <0.05 were considered significant where the Bonferroni adjustment ($p < 0.017$) was made for pairwise comparisons. Bland–Altman plots were constructed for pairwise comparisons as previously described.[11] SAS v9.4, STATA v14 and Graphpad Prism v6 were used for statistical analysis.

RESULTS

Representative IHC staining patterns for all markers are presented in Fig. 2.

The results of manually scoring ROIs for expression of each marker are reported in Supplementary Table 1

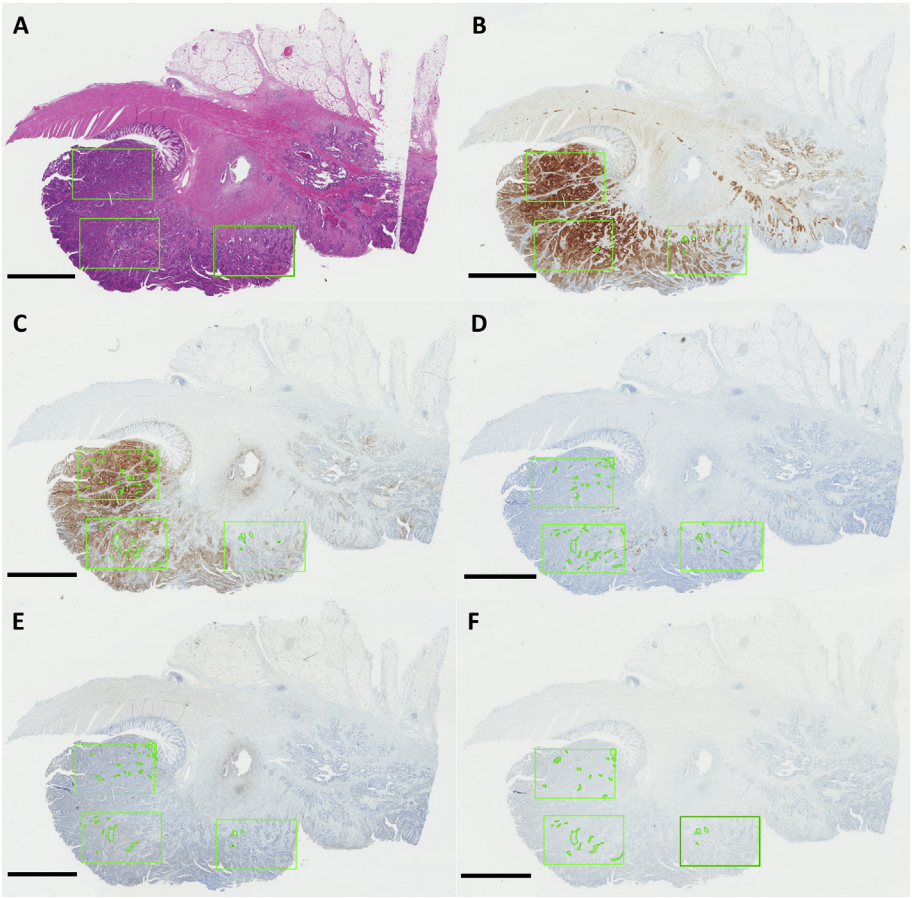

Fig. 1 Region of interest (ROI) selection for manual scoring and digital ROI methods. (A) Three regions of tumour or normal tissue were selected on the H&E section and copied to all IHC images (B–F). Bar = 5 mm.