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Original article

Simple classifiers for molecular subtypes of colorectal cancer

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ABSTRACT

Background and study aim: Colorectal cancer (CRC) is a heterogeneous disease entity with a diverse biological pathogenesis. This study aims to validate the two studies published in 2013 which established a separate CRC molecular subtype classification by utilizing a rapidly accessible miniclassifier, and verify a simplified version thereof.

Patients and methods: Participants diagnosed with CRC (n = 568) were subtyped in three classifications for characteristic, and prognostic purposes. Colorectal cancer subtypes (CCS) were classified as: i) CCS1 (CDX2+, microsatellite stable (MSS)/microsatellite instability (MSI)-low), ii) CCS2 (MSI-high), and iii) CCS3 (FRMD6/ZEB1/HTR2B +, CDX2-, MSS/MSI-low]. Simplified CCS (SiCCS) subtypes were grouped as: i) CDX2 (CDX2+, MSS/MSI-low, ZEB1 $\leq$ 2), ii) MSI-H (MSI-high, CDX2/FRMD6/ZEB1/HTR2B +/-), and iii) ZEB1 (ZEB1 $\geq$ 2, CDX2-, MSS/MSI-low). New molecular classification (NMC) subtypes were defined as: i) enterocyte (E-C) (MUC2 +), ii) goblet-like (G-L) (MUC2 + and TFF3 +), iii) transit-amplifying (T-A) (CFTR +), and iv) stem-like (S-L) (ZEB1 +).

Results: In total, 53.5% (n = 304) CCS, 58.3% (n = 331) SiCCS, and 37.7% (n = 214) NMC tumours could be evaluated. CCS2 and MSI-H CRCs had the most favourable survival outcome, whereas the CCS3, ZEB1 and S-L subtypes showed the poorest prognosis. A significant overlap between CCS3, ZEB1, and S-L tumours was demonstrated.

Conclusion: There is still a need for a consensus gene expression-based subtyping classification system for CRCs, thereby allowing the categorization of most CRC tumours. This study reveals that a simple and rapidly accessible process could replace the complicated, costly and mostly inapproachable methods clinical practices that have been introduced in the majority of previous studies.

Introduction

Colorectal cancer (CRC) is a heterogeneous disease, emerging from biologically diverse pathways distinguished by various compositions of gene-based transitions within the tumour [1]. Each of these different pathways can produce a distinct subtype with specific clinicopathological tumour characteristics and patient survival, resulting in the need for different therapeutic methods. Many previous studies have contributed consistent efforts to identify a novel molecular classification of CRC in association with cellular and molecular features. Most of these studies have concentrated on and extensively investigated the following tumour markers: microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and BRAF and KRAS mutations [2–9]. However, to date, there has been no consensus on internationally standardized CRC molecular classification with reliable prognostic stratification [10].

In 2013, among the several groups that have reported on CRC taxonomy, two separate studies introduced comprehensive human CRC molecular subtype classification systems by gene expression profiling [11,12]. One study by De Sousa E. Melo, et al. [11] described three main colon cancer subtypes (CCSs) by deriving a 146-gene classifier to categorize 90 patients into CCS1, CCS2 and CCS3. In addition, they provided a rapidly accessible classification tool, namely a tissue microarray-based miniclassifier using immunohistochemistry for four epithelial gene encoding proteins (FRMD6, ZEB1, HTR2B and CDX2), in combination with microsatellite status to categorize CCS1 to CCS3. Cross-validation confirmed that the tissue microarray-based miniclassifier could achieve an accurate classification when compared to the microarray-based classifier, thus resulting in significant prediction of disease outcome. The second study by Sadanandam et al. [12] introduced a

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new molecular classification (NMC) of CRCs by defining five distinct high-consensus molecular subtypes by identifying subtype associated markers using significance and prediction analyses [12]. The five subtypes were named as (i) enterocyte (E-C), defined by high expression of enterocyte-specific genes (MUC2 only) (ii) goblet-like (G-L), high mRNA expression of goblet-specific MUC2 and TFF3; (iii) transit-amplifying (T-A), a heterogeneous collection of samples with variable expression of stem cells and Wnt-target genes; (iv) stem-like (S-L), with high expression of Wnt signaling targets plus stem cell, myoepithelial and mesenchymal genes, and low expression of differentiation markers; and (v) inflammatory (I), marked by comparatively high expression of chemokines and interferon-related genes.

The aim of this work was to search for a simple classifier of the molecular classification system of the CRCs with an easy and rapid accessibility. In the present study, the CRC molecular subtyping algorithms published in the above two studies were applied to patients who had previously undergone primary resection for CRCs in the present institute (n = 568), in order to validate these previously published two high-consensus molecular classification systems. A combination of microsatellite instability and immunohistochemical expressions of the following epithelial gene-coding proteins, FRMD6, HTR2B, ZEB1, MUC2, TFF3 and CDX2 were applied, to compare patient outcomes and establish a community-based cancer subtyping for clinical practice. CDX2 is a biomarker absent in colon cancers with high level of ALCAM, a molecule expressed on colon-cancer cells with enriched tumourigenic capacity and CDX2-negative expression is an independent adverse prognostic marker of colon cancers [13]. ZEB1 plays an important role in tumour invasion and metastasis by activating urokinase plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1) which forms the key protease cascade of tumour invasiveness in the plasminogen activation system. ZEB1 and uPA are expressed in tumour cells at the invasive front of primary CRCs, setting ZEB1 as a potential prognostic biomarker and potential therapeutic target in CRCs [14]. Therefore, an additional classification method using only CDX2, MSI and ZEB1 was analyzed to verify whether it could represent the simplified the version of CCS.

Patients and methods

Patients and sample collection

A retrospective study for CRC classifications recruited a total of 568 CRC patients from January 2004 to December 2008. The eligibility criteria for inclusion were surgically resected CRC at the Pusan National University Hospital (PNUH) (Busan, Korea) with histological diagnosis of adenocarcinoma. All patients were chemotherapy-naive and underwent R0 resections for primary CRCs independently, before receiving any chemotherapy. Haematoxylin and eosin (H&E)-stained slides of all surgically resected tumour samples were reviewed by two pathologists. Clinicopathological variables of each patient and tumour were retrieved, including age, sex, tumour location (right or left), tumour size, histologic differentiation, lymph node (LN) status, pathologic T- and N stages, lymphovascular tumour invasion (LVI), perineural invasion (PNI), survival status, metastasis, recurrence, and response to a fluorouracil-based chemotherapy regimen (5-FU). Vital status, date of death, recurrence, and metastasis were determined through clinical records of the patients.

The study was approved by the university ethics committee, and all participating patients were informed about the study and had to provide signed, written consent before enrollment.

Tissue microarray and immunohistochemistry

A tissue microarray (TMA) composed of 568 tumour samples was constructed from standard formalin-fixed and paraffin-embedded sections that were obtained from the Department of Pathology, and the National Biobank of Korea, PNUH. All samples from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols.

An appropriate H&E-stained section from each tumour was selected and a representative area was identified, and the congruent area in the paraffin block was collected for the TMA. A semi-automated tissue arrayer (Beecher Instruments, WI, USA) was used to construct the tissue microarrays. In each tumour, two cores with diameter of 2.0 mm were obtained using the tissue microarray instrument and inserted in a recipient block. Recipient blocks were sectioned at 3 μm, dried for 20–30 min at 60 °C, deparaffinized in xylene and rehydrated in graded alcohol as per routine practice. The sections were subsequently submerged in citrate antigen retrieval buffer, microwaved for antigen retrieval (pH 9.0), treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, and then incubated with 1% bovine serum albumin overnight at 4 °C to block non-specific binding. Slides were stained manually with anti-ZEB1 (1:100, Sigma, HPA027524), anti-CFTR (1:100, Abcam, ab59394), anti-FRMD6 (1:100, Sigma, HPA001297), anti-HTR2B (1:100, Sigma, HPA012867), anti-TFF3 (1:100, R&D systems, MAB4407), and anti-MUC2 (1:100, Novocastra, NCL-MUC-2). After a secondary incubation, staining was developed using DAB + Chromogen (Dako, K5007), and slides were counterstained with hematoxylin. Only anti-CDX2 (free dilution, 0.32 mg/L, Leica, PA0535) was stained with Bond-Max (Leica) autostainer. All immunohistochemical (IHC) stainings were scored for each antibody expression in a blinded fashion by two pathologists. The two cores from each tumour were scored independently and paired at the end. If scores for the two samples were discordant, the final score for the tumour was upgraded to the higher score. ZEB1 and CDX2 were scored in the nucleus, MUC2 and TFF3 in cytoplasm whereas CTFR, FRMD6, HTR2B showed cytoplasmic and membranous expressions. Normal tissues utilized for positive control for ZEB1, CFTR, FRMD6, HTR2B and TFF3 were kidney glomeruli, epithelial cells of the lung, hepatocytes, endometrial glands and goblet cells of colon, respectively. For MUC2 and CDX2, normal colonic tissue was tested for positive control. IHC findings except CDX2 were scored from 1 to 4 according to intensity; 1 for negative, 2 for weak, 3 for moderate, and 4 for strong expression and were considered positive for tumour cells with any intensity expressions. For CDX2, all tumours in which the malignant epithelial component showed widespread nuclear expression, either in all or a majority of cancer cells, were scored as CDX2-positive. All tumours in which the malignant epithelial component either completely lacked CDX2 expression or showed faint nuclear expression in a minority of malignant epithelial cells (<10%) were scored as CDX2-negative.

The concordance between the two pathologists was analyzed by using contingency tables to calculating the Cohen's kappa Index which showed an excellent agreement (k = 0.89).

Microsatellite instability (MSI)

MSI status of the samples was determined using the MSI Analysis System, and GeneMarker version 2.6 (SoftGenetics, LLC State College, PA, USA), according to the manufacturer's instructions. Samples were considered MSI-high (MSI-H) when two or more markers were instable, and MSI-low (MSI-L) or Microsatellite Stable (MSS) were defined as one or zero out of five markers were instable, respectively. The genomic DNA from the tumour samples was isolated using the QIAamp DNA FFPE Tissue Kit (Cat. No.

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