



REVIEW ARTICLE

Keratins in colorectal epithelial function and disease

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SUMMARY

Keratins are the largest subgroup of intermediate filament proteins, which are an important constituent of the cellular cytoskeleton. The principally expressed keratins (K) of the intestinal epithelium are K8, K18 and K19. The specific keratin profile of a particular epithelium provides it with strength and integrity. In the colon, keratins have been shown to regulate electrolyte transport, likely by targeting ion transporters to their correct location in the colonocytes. Keratins are highly dynamic and are subject to post-translational modifications including phosphorylation, acetylation and glycosylation. These affect the filament dynamics and hence solubility of keratins and may contribute to protection against degradation. Keratin null mice (K8^{-/-}) develop colitis, and abnormal keratin mutations have been shown to be associated with inflammatory bowel disease (IBD). Abnormal expression of K7 and K20 has been noted in colitis-associated dysplasia and cancers. In sporadic colorectal cancers (CRCs) may be useful in predicting tumour prognosis; a low K20 expression is noted in CRCs with high microsatellite instability; and keratins have been noted as dysregulated in peri-adenomatous fields. Caspase-cleaved fragment of K18 (M30) in the serum of patients with CRC has been used as a marker of cancer load and to assess response to therapy. These data suggest an emerging importance of keratins in maintaining normal function of the gastrointestinal epithelium as well as being a marker of various colorectal diseases. This review will primarily focus on the biology of these proteins, physiological functions and alterations in IBD and CRCs.

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Keratins and intermediate filaments

Intermediate filaments (IFs), along with microfilaments (MF) and microtubules (MT), constitute the key components of the cellular cytoskeleton, a scaffolding within the cytoplasm of animal cells (Fuchs & Cleveland 1998; Ku *et al.* 1999). The IFs are subdivided into five major types based on amino acid sequences and protein structure. Types I–IV form cytoplasmic IFs, whereas type V IF proteins, the lamins, form the nucleoskeleton (Schweizer *et al.* 2006; Kim & Coulombe 2007). Keratins are the largest subgroup of IF proteins and

are classified as type I and type II proteins. They are composed of polymerized dimers of a type I (acidic) and type II (basic) keratin. Dimerization is facilitated by the interaction of coiled-coil domains in the protein pairs, generating coiled structures with free globular domains at the amino (N)- and carboxy(C)-termini (Omary *et al.* 2009).

The IF cytoskeleton radiates throughout the cell (shown in Figure 1a) and is interlinked mechanically and functionally to MF and MT cytoskeletons (Svitkina *et al.* 1996; Goldman *et al.* 1999). There is a strong similarity between the members of the class I and class II keratins (Figure 2),

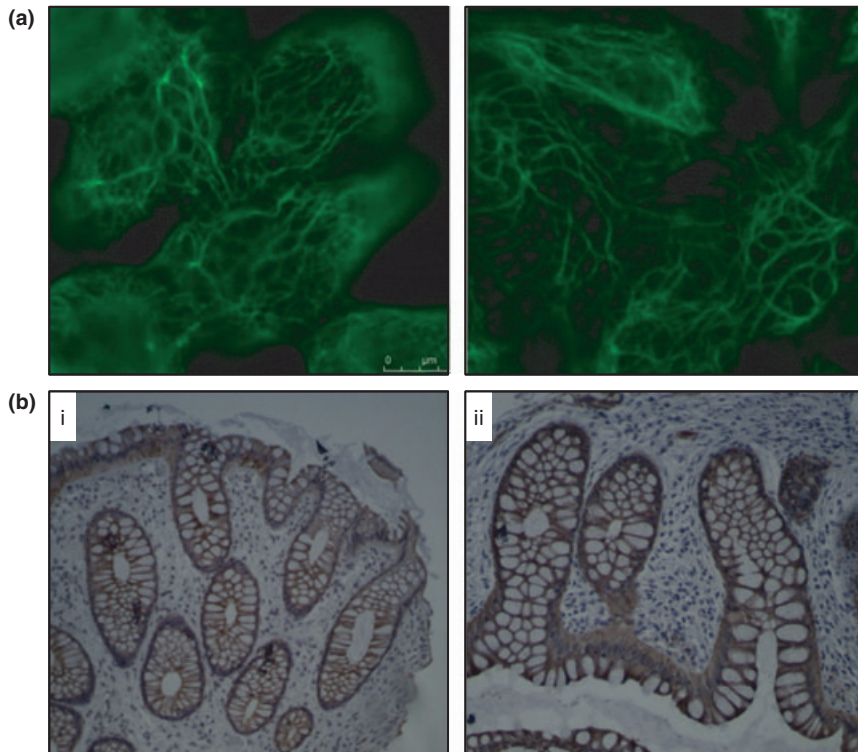


Figure 1 Organization and distribution of keratin 8 in colorectal cells. (Panel a) Representative images of immunocytochemical staining of keratin 8 in HCT116 cells. K8 (green) forms filaments, which radiate throughout the cell. (Panel b) Immunohistochemical staining of keratin 8 (i) and 18 (ii) staining in normal colon crypt.

implying a similarity in function; however, the profile of keratin expression is specific to the particular epithelium, and the close association of IFs with desmosomes and hemidesmosomes is thought to reflect a role for IFs in providing the epithelium with strength and integrity (Herrmann & Aebi 2004).

Keratins are expressed in different epithelial cells (Moll *et al.* 1982). The keratins that are primarily expressed in simple (single-layered) epithelia are known as simple epithelial keratins and comprise K7, K8, K18, K19, K20 and K23 (Omary *et al.* 2009); the principal keratins in the intestinal epithelia being K8, K18 and K19 (Ku *et al.* 2004). In stratified epithelia, the main keratins include K5, K14 as well as K6 and K16 (Moll *et al.* 1982).

Isolation and solubilization of keratins

An important feature of keratins by which they differ from other cytoskeletal filament proteins such as actin, myosin and tubulin is that they are resistant to buffers containing non-denaturing detergents and high concentrations of ions, including chaotropic salts such as potassium chloride or iodide (Franke *et al.* 1982; Steinert *et al.* 1982; Achtstaetter *et al.* 1986). This is critically important while attempting to study the expression of keratins because they exist in a soluble form as well as an insoluble filamentous state. Usually, 5% of the keratin pool exists in a soluble state (Chou *et al.* 1993), although the state of solubility is determined by various factors including cellular stress (Liao *et al.* 1995) and the presence of post-translational modifications (PTM) (described below). The insoluble fraction can be solubilized

with high concentrations of urea (9–10 M) or guanidine hydrochloride (4–6 M) to produce soluble tetrameric or oligomeric subunits to result in solubilization of the denatured monomers (Achtstaetter *et al.* 1986; Ku *et al.* 2004).

Isolation of keratins from mammalian cell culture monolayers has usually been described using a sequential fractionation technique (Herrmann *et al.* 2004; Ku *et al.* 2004). This technique has generally been modified for human tissue samples (Herrmann *et al.* 2004) although this has not been studied specifically for colorectal biopsies. We have described a further modification of the technique for the isolation of IF from colorectal biopsies and their effective solubilization in 4 M guanidine hydrochloride to facilitate parallel analysis of IFs by gel-based and gel-free liquid chromatography MS/MS (LC-MS/MS) approaches (Majumdar *et al.* in press).

PTM of keratin

Following translation, most proteins undergo changes through PTM (Cho 2007). Such modifications modulate the function of proteins in a manner which is not directly coded for by genes (Cho 2007). Keratins are highly dynamic and reorganize in response to many stimuli particularly to cellular stress as well as during apoptosis and mitosis (Ku *et al.* 1999; Toivola *et al.* 2010). Characteristic PTMs of keratins include serine phosphorylation (Oshima 1982; Omary *et al.* 1998, 2006) threonine phosphorylation (Steinert 1988), tyrosine phosphorylation (Feng *et al.* 1999), lysine acetylation (Leech *et al.* 2008), serine/threonine glycosylation (Chou *et al.* 1992), cleavage (Makowski & Ramsby 1998; Ditzel *et al.* 2002) and sumoylation (Snider *et al.* 2011).

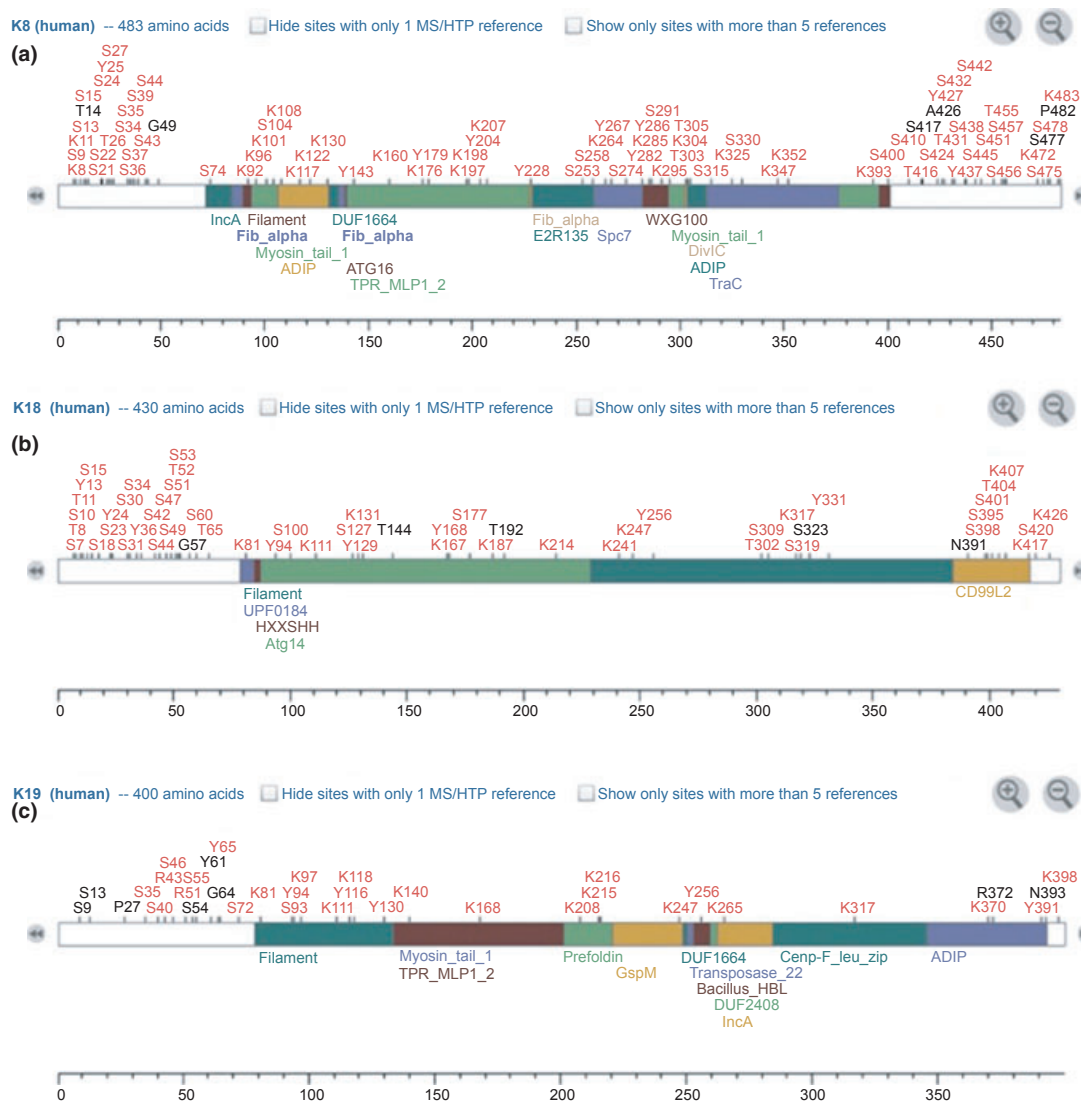


Figure 3 Summary of post-translational modifications of keratin 8 (Panel a), keratin 18 (Panel b) and keratin 19 (Panel c) (accessed from phosphosite.org, 21 February 2012). p, Phosphorylation; a, acetylation; m, methylation; m1, mono-methylation; m2, di-methylation; m3, tri-methylation; u, ubiquitination; s, sumoylation; n, neddylation; g, O-GlcNAc; h, palmitoylation; ad, adenylation; sn, S-nitrosylation; ca, caspase cleavage.

of filament formation/depolymerization although movement of the K8/K18 heterodimer pairs in and out of the filament (Ku & Omary 1994; Mizuuchi *et al.* 2009). Different protein kinases have been shown to phosphorylate keratins. Keratin-8 residue, Ser 73, has been identified as a substrate for phosphorylation by p38 kinase and c-Jun N-terminal kinase (both of which are involved in cellular responses to stresses, cytokines and apoptosis) (Kyriakis & Avruch 1996; Ip & Davis 1998; Davis 2000) and this has likewise been shown to regulate filament organization (He *et al.* 2002; Ku *et al.* 2002). The serine residue Ser 431, on the other hand, has been shown to be phosphorylated by the kinase ERK1 (MAP kinase) mediated by stimulation of epidermal growth factor (Omary *et al.* 1998). These changes not only affect the

solubility of keratins, but hyperphosphorylation may protect K18 from degradation by caspases (Ku & Omary 2001).

Dephosphorylation of K8 in colorectal cancer (CRC) tumour cells has been shown to be associated with tumour progression (Mizuuchi *et al.* 2009). In this study, K8 has been demonstrated as a physiological substrate of phosphatase of regenerating liver-3 (PRL-3) a subclass of tyrosine phosphatases (Stephens *et al.* 2005) that play a role in cancer migration and metastasis including in CRCs (Bardelli *et al.* 2003; Kato *et al.* 2004). CRC tumour cells in metastasis and at the invasive front were shown to have high PRL-3 associated with reduced or absent K8 phosphorylation thus demonstrating a role of K8 dephosphorylation in increased tumour aggressiveness.

Acetylation

Acetylation as a form of post-translational modifier of protein functions is being increasingly recognized as being as important as phosphorylation (Kouzarides 2000). Acetylation levels are the product of the balance of lysine acetyl transferase (KAT) (previously called histone acetyl transferase) and histone deacetylases (HDAC) (Sadoul *et al.* 2011). It is being increasingly recognized that HDACs regulate acetylation and function of proteins other than histones (Sterner *et al.* 1979; L'Hernault & Rosenbaum 1985). Acetylation may contribute to the regulation of protein–protein interaction (Chen *et al.* 1999) and protein stabilization (Martinez-Balbas *et al.* 2000). Two studies have recently reported a large number of acetylation sites in K8 (Leech *et al.* 2008; Choudhary *et al.* 2009). We have demonstrated five acetylated lysine residues: lys10, lys100, lys392, lys471 and lys482 in K8 in the insoluble IFs isolated from cultured HCT116 colon cancer cell lines using a multiple reaction monitoring-initiated detection and sequencing (MIDAS) workflow (Leech *et al.* 2008), which uses existing knowledge to predict the potential masses of acetylated peptides, then searches for such masses and sequences them to confirm the presence; three of these five (K10, K471 and K482) occur predominantly outside the coiled-coil domains with K482 at the C-terminus. Two other acetylation sites were noted to lie near the ends of the coiled-coil domains. Additionally, an acetylation site was also noted in K18. In contrast, an analysis of the global acetylome (presumably primarily using soluble material) identified six acetylation sites within the coiled-coil domain (Choudhary *et al.* 2009). We have demonstrated that acetylation of K8 is associated with depolymerization of filaments, and furthermore that this occurs at a similar concentration and time to the acetylation of lysine 482 (Drake *et al.* 2009). It has not been possible to distinguish cause from effect but this is supportive of a potential role of acetylation in the regulation of filament stability.

Glycosylation

Glycosylation of keratins have also been studied. Using human colon adenocarcinoma cell lines (HT29), Chou *et al.* (1992) demonstrated dynamic glycosylation of K8 and 18 at multiple sites with a single O-linked N-acetylglucosamine (O-GlcNAc). A subsequent study identified three sites of glycosylation on K18 at Ser 29, 30 and 48 (Ku & Omary 1995). Ku *et al.* (2010) investigated the role of K18 glycosylation by generating mice overexpressing human K18 S30/31/49A substitution mutants – K18-Gly⁻ mice that are not glycosylated and compared them to wild type mice. K18-Gly⁻ mice were also noted to be susceptible to apoptosis and liver and pancreatic injury by streptozocin, thus suggesting a role for K18 glycosylation in protecting epithelial cells from injury. Similar to phosphorylation, glycosylation too has been shown to play an important role in modulating equilibrium of keratin between soluble and filamentous insoluble state (Srikanth *et al.* 2010). In this study,

malignant HepG2 hepatoma cell lines (which have decreased O-GlcNAcylation of cellular proteins because of an increased activity of the enzyme O-GlcNAcase), when compared to hepatocyte cell lines, demonstrate decreased solubility of K8 and K18.

Sumoylation

Sumoylation, a reversible PTM by a process of addition and removal of small ubiquitin-like modifier (SUMO) polypeptides, has emerged as an important mechanism of regulating protein activity (Seeler & Dejean 2003; Geiss-Friedlander & Melchior 2007). This process creates an isopeptide bond between the C-terminal Gly residue of the modifier protein and the ε-amino group of a Lys residue of the target protein (Geiss-Friedlander & Melchior 2007). The role of Ubc9, which covalently joins SUMOs to proteins in the gastrointestinal tract, was studied using inducible knockout mouse line for the SUMO E2 enzyme Ubc9 (Ubc9^{fl/-}/ROSA26Cre-ERT2) (by intra-peritoneal injection of 4-hydroxytamoxifen) (Demarque *et al.* 2011). Decreased sumoylation, which primarily affected the small intestine, resulted in severe diarrhoea along with the depletion of the intestinal proliferative compartment. K8 as a SUMO substrate was demonstrated by the presence of SUMO-modified K8 on Western blotting; this coupled with enterocyte fragility (at the top of the villi with detachment of enterocytes from the underlying basal lamina) suggests a putative role of sumoylation in tissue fragility disorders. In addition, an *in vitro* study (Snider *et al.* 2011) has shown that keratin sumoylation is augmented during apoptosis, oxidative stress and by phosphatase inhibition. In this study, human mutations in K8 that predispose their carriers to liver disease exhibit hypersumoylation and decreased solubility, thus demonstrating an effect of sumoylation on filament dynamics.

Cleavage

Keratins may be modified by cleavage *in vivo*. Degradation products of keratins have been identified in cancers (Ditzel *et al.* 2002), and several new neo-epitopes have been identified including the widely used M30 (Leers *et al.* 1999). Calpain cleavage of K8 has been identified as a para-apoptotic event (Makowski & Ramsby 1998).

Keratin distribution and function in the normal colonic epithelium

We recently reported the distribution of K8 expression in the morphologically normal colon mucosa in subjects with cancer (Khan *et al.* 2011). K8 is predominantly expressed in the flat mucosa, with expression decreasing from the top towards the base of the crypt axis. We noted that there is considerable variation between subjects, in the extent of expression along the crypt axis (Figure 1b).

The functional roles of keratins vary according to the organ. The major function of K8/K18 in the liver is protection

from mechanical and non-mechanical forms of stress (Omary *et al.* 2002). Cytoprotective, non-mechanical functions of keratins also include protection from apoptosis (Eriksson *et al.* 2009). There is increasing evidence that IFs also have a role in cell-death signalling pathways, in particular apoptosis mediated by tumour necrosis factor- α (TNF- α) and Fas (Paramio & Jorcano 2002). Epithelial cells lacking K8 and K18 are significantly more sensitive to TNF-mediated apoptosis (Caulin *et al.* 2000).

The function of keratins in the intestine is poorly understood, although the K8-null mouse phenotype suggests they may have a potential role in cell growth, differentiation or targeting of proteins to the apical compartment (Ameen *et al.* 2001; Toivola *et al.* 2004). K8-null (K8^{-/-} and K8^{+/-}) mice in comparison with K8^{+/+} mice have been shown to have abnormal colonic active ion transport (Na⁺ and Cl⁻). Similarly, the short circuit current (I_{sc}) was significantly decreased in K8^{-/-} and K8^{+/-} mice compared to K8^{+/+} mice. Conductance of colonic tissues and paracellular transport were, however, noted to be normal (Toivola *et al.* 2004).

These findings suggest a putative role of colonic keratins in regulating electrolyte transport, likely by targeting ion transporters in the colonocytes (Toivola *et al.* 2004).

Network analyses of the interactomes of the three major colorectal keratins are shown in Figure 4. It is notable that K19 does not directly or indirectly occur in the interactomes of K8 and K18 (which holds when the networks are expanded). The networks show close interaction with both MAP Kinase and p53 pathways, highlighting the importance of keratins in linking physical function into cell signalling and cell fate determination.

Keratins and colorectal disease

Inflammatory bowel disease

Ulcerative colitis (UC) along with Crohn's disease comprises the chronic, inflammatory bowel diseases (IBD), both conditions characterized by a relapsing and remitting clinical course (Silverberg *et al.* 2005). The main symptoms of the

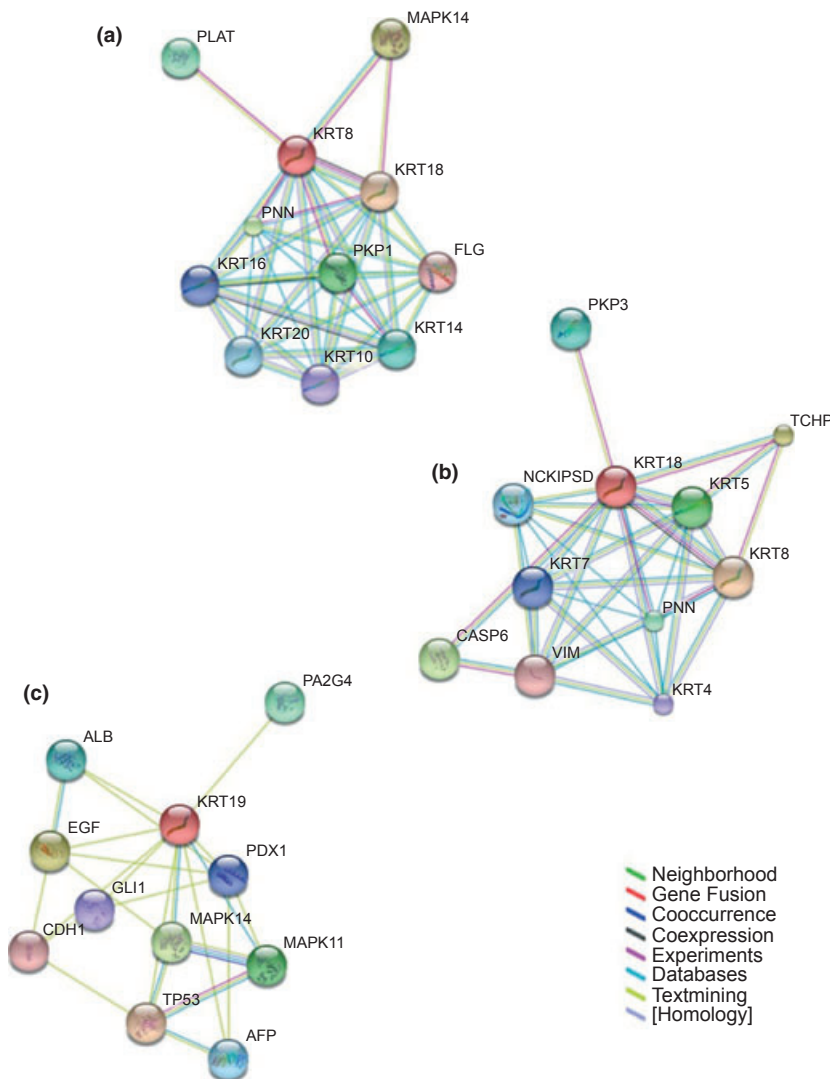


Figure 4 Protein–protein interaction network view summarizing the network of predicted associations for keratins 8, 18 and 19 (data accessed from STRING 9.0 (<http://string-db.org/>)). The network nodes represent proteins, and edges demonstrate predicted functional associations between them. The colour of the node for the protein analysed is indicated in red; other nodes are coloured to indicate a direct link with the protein. Seven different coloured lines indicate the edges representing the seven types of evidence used in predicting the associations. The lines indicate the following evidences: red line – fusion; green line – neighbourhood; blue line – co-occurrence; purple line – experimental; yellow line – text-mining; light blue line – database; black line – co-expression.

conditions include diarrhoea, often bloody diarrhoea with passage of mucus. Other symptoms that can be variably present include abdominal pain, tenesmus or fever (Carter *et al.* 2004). The aetiology of UC remains unknown, and no single agent or distinct single mechanism has been implicated in the pathogenesis (Fiocchi 1998); rather it is believed that several factors viz. genetic susceptibility, environmental triggers and an altered immune response (Kucharzik *et al.* 2006; Xavier & Podolsky 2007) to luminal microbial antigens interact for the condition to become clinically apparent (Sartor 2006). Perturbations in the intestinal epithelial barrier, which provide a protective barrier against the luminal microflora, are now considered to play an important roles in the pathogenesis of the condition (Dignass *et al.* 2004).

Keratins and IBD

Several lines of evidence point towards an association of keratins with IBD, although this linkage is not fully clear. TNF- α is a pro-inflammatory cytokine, the levels of which are increased in blood, colonic tissue and stools of patients with UC (Murch *et al.* 1991, 1993; Braegger *et al.* 1992). K8 and K18 have been noted to co-localize with cytoplasmic domain of TNF receptor 2 (TNFR2) and moderate TNF-induced, Jun NH2-terminal kinase intracellular signalling and NF κ B activation (Caulin *et al.* 2000). In addition, normal and malignant epithelial cells lacking in K8 and K18 were found to be sensitive to TNF-mediated cell death. It is suggested that attenuating effects on TNF function by K18 may be mediated by TNFR1-associated death domain protein (Inada *et al.* 2001).

Effects of interleukin 6 (IL-6) an immunoregulatory cytokine on keratin expression and its implications on the intestinal mucosal barrier function have been studied (Wang *et al.* 2007). Intestinal disorders including IBD are often linked to perturbations in the barrier function of the epithelial cells. It is believed that such loss of barrier function results in sensitization of the immune system to antigens previously localized to the intestinal lumen (Dignass *et al.* 2004; Shen & Turner 2006). Using a combination of Caco2-BBE cell line and IL-6 null mice, Wang *et al.* (2007) demonstrated that IL-6 significantly induces not only the expression of insoluble cytoskeletal fraction of K8 and K18, but also serine phosphorylation of K8 at phosphoserine 431 and 73, suggesting PTM of K8 in response to IL-6. IL-6 null mice also demonstrated significantly increased intestinal permeability with dextran sodium sulphate administration. Taken together, these findings demonstrate regulation of colonic expression of K8 and K18 by IL-6 as well as a potential role of K8 in mediating effects of IL-6 on the barrier function.

Two animal studies have supported a role of keratins in the pathogenesis of IBD. In one study (Baribault *et al.* 1994), homozygous mK8^{-/-} FVB/N mice were shown to develop colonic hyperplasia, colitis and rectal prolapse. Histology revealed hyperplasia without any abnormalities in the crypt cells or mucin-producing goblet cells, suggesting that

the inflammatory component of mK8⁻ bowel disease may be secondary to colorectal hyperplasia. This colonic inflammation in K8-null mice may thus represent a unique model for IBD resulting from a primary epithelial rather than an immune cell defect (Baribault *et al.* 1994).

K8 deficient mice (K8^{-/-}) have also been shown to develop spontaneous chronic T helper type 2 colitis (Th2 colitis) (Habtezion *et al.* 2005). In this study, a significant increase in T-cell receptor beta (TCR β)-positive CD4⁺ T cells infiltrating the colon lamina propria in association with enhanced Th2 cytokine (IL-4, IL-5 and IL-13) production have been noted in K8^{-/-} mice by immunohistochemistry and flow cytometry. Colonic epithelial cells in the K8^{-/-} mice expressed MHC class II antigens; such antigen expression in K8^{+/+} mice was limited to the lamina propria. The colonic inflammation in the K8^{-/-} mice was, however, prevented by administering antibiotics (vancomycin and imipenem) suggesting a possible role of luminal bacteria in triggering colitis in mice with a primary epithelial defect (Habtezion *et al.* 2005).

The role of keratins in the pathogenesis of colitis and the mechanisms by which K8^{-/-} mice develop colonic inflammation and hyperplasia are poorly understood. To investigate further the colonic mucosal changes in K8^{-/-} mice and their response to antibiotics, a genome-wide microarray was undertaken using isolated mouse colonocytes (Habtezion *et al.* 2011). Microarray analysis demonstrated differential regulation of several genes in K8^{+/+} and K8^{-/-}-isolated mouse colonocytes, with apoptosis being the major altered pathway. These differentially altered genes in K8^{-/-} colon were almost normalized with antibiotic pretreatment. Similar to results demonstrated with isolated colonocytes, K8^{-/-} compared to K8^{+/+} colons were also noted to be resistant to the induction of apoptosis by both staurosporine and Fas. This suggests that lack of K8 confers resistance to apoptosis [unlike in hepatocytes where absence or mutation in K8 or K18 renders them susceptible to apoptosis (Omary *et al.* 2009)]. The authors hypothesized that difference between the hepatocytes and colonocytes probably reflects the influence of the luminal microflora on the colonocytes (Habtezion *et al.* 2011).

Lack of keratin filaments in the colon has been noted to cause abnormalities in transport of ions, protein mistargeting and diarrhoea even before occurrence of hyperproliferation and inflammation (Toivola *et al.* 2004). In this study, K8^{-/-} mice were noted to have diarrhoea and significantly higher stool water content compared with K8^{+/+} mice. Colonic tissue conductance, a measure of the function of tight junctions, was not significantly different, suggesting that the diarrhoea was unrelated to an alteration of the paracellular transport or increased tight junction permeability. Colonic ion transport studies revealed marked reduction in short circuit current (I_{SC}) with markedly decreased net Na⁺ absorption associated with reversal of net Cl⁻ movement to net Cl⁻ secretion in K8^{-/-} mice. In comparison with wild type mice, K8-null mice distal colon revealed an altered distribution of epithelial markers H,K-ATPase and F-actin to apical/

lateral locations in the enterocytes. Colonic expression of anion exchanger AE1/2 was significantly higher in K8^{-/-} mice, whereas the Na-transporter ENaC showed an altered distribution.

Mistargeting in K8^{-/-} colons, which normalizes after antibiotic treatment, suggests that luminal bacteria and/or their consequent inflammatory response may promote the observed protein mistargeting (Habtezion *et al.* 2005). The mistargeted ion transporters may be responsible in creating an environment for pathogenic bacteria to thrive, which in turn stimulate the colitis and maintenance of the mislocalized transport proteins. These studies may lend credence to the role of luminal bacteria in the pathogenesis of colonic inflammation and colitis. Although the aetiology of IBD is multifactorial, luminal microflora particularly an altered host tolerance to them is believed to play an important role (Fava & Danese 2011).

Genomic DNA from patients with UC and Crohn's disease, 50 with sporadic and 47 with familial IBD, has been studied (Owens *et al.* 2004). Heterozygous missense mutations in K8 were identified in five (5.2%) unrelated IBD patients in this study. Three separate KRT8 mutations (G62C) resulting in amino acid substitutions at K8 were identified: two were in unrelated UC patients with a family history of IBD and one was in a Crohn's disease patient with no family history. Patient with sporadic Crohn's disease was found to have a mutation in K8 (I63V), whereas another mutation resulting in amino acid substitution in the tail domain of K8 (K464N) was found in an UC patient with family history of IBD. Four patients were noted to have heterozygous sequence variation in KRT18 resulting to K18 (S230T) in the L12 linker region. All these changes were noted to lie within the non-helical domains, which are usually associated with milder forms of disease. The K8 (K464N) mutation was, however, not found in 194 controls, whereas the K8 (G62C) and K8 (I63V) sequences were each identified once. However, none of these changes noted were compared to identify statistical difference from controls; and morphological changes in keratins in tissue samples were not investigated.

Such mutations were also noted to be responsible for incompetent polymerization of keratins particularly on assembly of K8/K18 filaments *in vitro* and in cultured cells (Owens *et al.* 2004). Another German study investigated 217 patients with Crohn's disease, 131 patients with UC and 560 healthy controls (Buning *et al.* 2004). Heterozygous G62C mutation in K8 was detected in five (2.3%) and three (2.3%) patients with Crohn's disease and UC, respectively, whereas 9 (1.6%) control subjects were noted to carry such mutation. None of the patients or controls was homozygous for this mutation and they did not show the Y54H mutation. Patients with IBD demonstrating the heterozygous mutation did not show any phenotypic difference from those without such mutation.

A more recent study investigated the association of mutations in KRT8 and KRT19 in patients with IBD (Tao *et al.* 2007). In this study, 16 of the 184 patients with IBD dem-

onstrated heterozygous variants in KRT8 (G62C, R341H); 4 of 70 unaffected/unrelated volunteers also carried the KRT8 (R341H) mutation. A novel KRT8 (R341C) mutation correlating with extensive UC was identified. KRT19 variant frequencies were not different between the groups. KRT8 variants (G62C, R341H and R341C) were tested in 682 independent nuclear families (with both parents and at least one IBD affected offspring) and 273 controls. They failed to show any significant departure from random transmission. Thus, overall, the genetic studies fail to provide any conclusive evidence of keratin mutations in the pathogenesis of IBD.

Sporadic CRC

CRC is among the most common malignancies worldwide. Single-gene abnormalities account for a minority of CRCs commonly in the form of either familial adenomatous polyposis or hereditary non-polyposis CRC; the vast majority of CRCs being of sporadic origin arising from adenomas (Hisamuddin & Yang 2004). The traditional adenoma-carcinoma sequence in the pathogenesis of CRC involves a stepwise progression of normal colonic epithelium to malignant neoplasm involving progressive sequential accumulation of molecular and genetic abnormalities in each step leading to the development of malignancy via adenomatous polyps (Arnold *et al.* 2005). The pathways involve either the classical chromosomal instability pathway or a microsatellite instability pathway (Lengauer *et al.* 1998). A small number arise from either long-standing colitis (Choi & Zelig 1994) or sessile serrated adenomas, a subset of hyperplastic-like polyps that arise mainly in the right colon of middle-aged women (Jass 2003).

The bulk of data on keratins in colorectal diseases come from studies on patients with sporadic CRCs although there have been some studies from patients with colitis-associated cancers and cancers arising from serrated adenomas.

Keratin expression in sporadic adenomas and CRC

Field change or changes in the surrounding uninvolved colonic mucosa in patients with CRCs have been described previously (Shen *et al.* 2005). To date, there remain relatively few reports on keratin expression profile in the normal colon and in early neoplasia, indicating an unmet need for understanding its potential roles.

Polley *et al.* (2006) used a two dimensional gel electrophoresis and mass spectrometry (MALDI/TOF) approach to investigate proteomic changes in CRC tissue and compared them with morphologically normal colonic mucosa of patients with CRC (i.e. cancer field) as well as colonic adenomatous polyps and colonic mucosa of controls. In this study, differential expression of seven forms of K8, four of K19 and one each of K9 and K20 was observed. In comparison with healthy mucosa (of controls, cancer and polyp patients), all four forms of K19 were under expressed in

tumour tissue and one spot identified as K9 was absent. In comparison with the colonic mucosa of controls, field biopsies in cancer patients showed overexpression of seven K8 forms, one of K9 and one of K20; field mucosa in polyp patients too revealed overexpression of K8 and K9. There were multiple forms of keratins differing slightly in mass and charge although the data did not allow determination of what the novel form of keratins were. The findings of this study support the hypothesis of changes in uninvolved field mucosa both in patients with cancer and in those with polyps. Taken together with the data from K8-null mice (mK8^{-/-} FVB/N) indicating a risk of colonic hyperplasia (Baribault *et al.* 1994) and data from hepatocytes implicating keratin in regulation of cell cycle and apoptosis (processes which are deranged in cancer) (Ku *et al.* 2007) may imply a role for alteration in keratin expression and function at the earliest stages of colorectal carcinogenesis with increased expression of K8 in the morphologically normal mucosa as the adenoma-carcinoma sequence progresses.

Similar to pathogenesis of IBD, mutations in K8 too have not yet been directly implicated in the development of CRCs. A direct link between keratin mutation and development of cancer has only been demonstrated in cutaneous basal cell cancers (BCC) (Stacey *et al.* 2009). In this study, a G138E substitution in the keratin 5 (*KRT5*) gene was noted to confer an increased risk of BCC.

Serrated adenoma-associated CRC

Serrated adenomas have now been recognized as a distinct neoplastic lesion separate from sporadic adenomas with a serrated architecture that containing areas of epithelial dysplasia (Longacre & Fenoglio-Preiser 1990). It is now known that a subset of such adenomas can undergo malignant transformation (Jass 2003). Using an immunohistochemical approach, Tatsumi *et al.* (2005) investigated K7 and K20 expression in a variety of colorectal lesions including serrated adenomas, hyperplastic polyps, traditional adenomas and colorectal carcinomas. Majority of serrated adenomas (71%) demonstrated a K7⁺/K20⁺ pattern; in contrast, most sporadic adenomas and adenocarcinomas showed a K⁻/K20⁺ pattern.

Colitis-associated CRC

Ulcerative colitis as a risk factor for CRC was first described by Crohn & Rosenberg (1925). Patients with long-standing UC have an increased risk of developing CRC. Although CRC in IBD constitutes 1% of all cases of CRCs, patients with IBD represent one of the highest risk groups for developing this complication (Choi & Zelig 1994).

Dysplasia (defined as unequivocal neoplasia of the epithelium confined to the basement membrane without invasion into the lamina propria) represents the premalignant phase of the condition (Riddell *et al.* 1983). Colitis-associated CRC is usually preceded by and co-exists with dysplastic changes elsewhere in the bowel. Neoplastic changes in

colitis have been shown to be associated with genomic abnormalities throughout the whole colon, including in the non-dysplastic mucosa. This is referred to as molecular field defect or genomic field defect (Rabinovitch *et al.* 1999; Issa *et al.* 2001; O'Sullivan *et al.* 2002; Chen *et al.* 2003).

Various factors are associated with an increased risk of CRC in UC; the most important factor identified across most studies is the duration of the disease (Eaden *et al.* 2001). Others include anatomical extent of the disease in the colon (Ekobom *et al.* 1990), presence of family history of CRC (Askling *et al.* 2001), co-existent primary sclerosing cholangitis – a rare chronic inflammatory and fibrotic disease of the biliary tract of unknown aetiology (Soetikno *et al.* 2002) – and severity of inflammation in the colon (Rutter *et al.* 2004).

Keratins in colitis-associated CRC

Similar to sporadic CRC, K7 and K20 expression in dysplastic and neoplastic changes in UC has been investigated (Tatsumi *et al.* 2006; Stenling *et al.* 2007). Immunohistochemical staining of paraffin-embedded tissue sections has shown that in contrast to sporadic adenocarcinomas, vast majority of UC-associated neoplasm expressed K7, including those with low- and high-grade dysplasia (LGD and HGD), K20 expression was not significantly different between the two groups (Tatsumi *et al.* 2006). In a study from northern Sweden in subjects undergoing colonoscopic surveillance, colonic biopsies were analysed for immunohistochemical expression of K7 and K20 in different groups of patients with UC. Active disease was present in 10 patients; LGD, HGD and cancer were present in 10, 6 and 5 patients respectively. K20 expression was noted increased in the lower part of the crypts in neoplasia-associated lesions, while 2 of 5 patients with CRC, 3 of 6 patients with HGD and 7 of 10 patients with LGD were positive for K7 (Stenling *et al.* 2007).

The significance of such changes in keratin expression in the colorectal mucosa in patients with colitis is still not clear; it is possible that they may represent markers of neoplastic changes in patients with UC.

Keratins as tumour markers of CRC

One important characteristic of keratins and other IFs is that they remain relatively stable even after transformation to pathological states including transformation of normal cells into malignant cells. This property has been exploited with keratins being used as tumour markers (Omary *et al.* 2009). Keratins are not organ specific, which limits their diagnostic utility. However, their role as markers of tumour prognosis and for monitoring treatment response is relatively well established (Barak *et al.* 2004). Several studies have investigated keratin expression in sporadic CRC. Moll *et al.* (1983) found K8 and K19 to be the most abundant, while Chu *et al.* reported that while gastrointestinal carcinomas uniformly express K8, 18 and 19, most colorectal

adenocarcinomas also express K20 (Chu *et al.* 2000; Chu & Weiss 2002). Unlike K8, 18 and 19, K20 expression is limited to gastric and intestinal epithelium, urothelium and Merkel cells (Moll *et al.* 1992). Moll *et al.* (1992) proposed K20 as a diagnostic marker to distinguish types of carcinoma when disease is present but the primary site is unknown, or in cases where accurate diagnosis is difficult because of poor differentiation or invasion into adjacent anatomical structures. Yun *et al.* (2000) sought to determine K20 specificity in detecting submicroscopic lymph node metastases by correlating its expression with mutant K-RAS expression (the K-RAS gene is mutated in 30–40% of colorectal tumours) (Sidransky *et al.* 1992; Pretlow *et al.* 1993) to show that K20 expression in lymph nodes in patients with CRC genuinely reflects metastatic disease. By using reverse transcription polymerase chain reaction (RT-PCR) assays for K20, they calculated a specificity of 97.6% in 42 patients with tumour and 41 controls (Yun *et al.* 2000).

Likewise, K7 immunostaining in the majority of colorectal adenocarcinomas tends to be weak or negative compared to other adenocarcinomas (for example, ovary, pancreas and lung), leading to its use in diagnosing the primary tumour in case of adenocarcinoma metastases (Moll *et al.* 1992; Chu *et al.* 2000). When combined with K20, for example a K7⁻/K20⁺ phenotype, a metastasis is very likely to be of colorectal origin (Moll *et al.* 1992; Chu *et al.* 2000).

Paraffin-embedded tissue sections of 196 colonic adenocarcinomas were investigated by immunohistochemistry for K7 and K20 (Bayrak *et al.* 2011). K7 and K20 expression was noted in 17.3% and 81.1% of patients; K7⁻/K20⁺ had the greatest proportion (65.8%) while K7⁺/K20⁺ immunophenotype was identified in 15.3%. K20 positivity was greater in low-grade than in high-grade carcinomas (85.1% *vs.* 47.6%) and in rectal and sigmoid carcinomas than in proximal colon carcinomas (88.2% *vs.* 63.2% and 88.9% *vs.* 63.2%, respectively). Tumours with lymph node metastasis showed increased K7 expression than non-metastatic tumours (25.3% *vs.* 11%).

RT-PCR assays for K20 have also been used to detect disseminated tumour cells in the peripheral and central blood of patients undergoing resection of the primary tumour (Weitz *et al.* 1998) in an effort to predict metastasis and add weight to the 'no-touch isolation technique' of surgical resection with lymphovascular ligation prior to resection of the tumour (Barnes 1952; Turnbull *et al.* 1967).

Keratins as prognostic markers of CRC

Tumour prognosis could be predicted using the pattern of expression of keratins. In case of CRCs, a low K20 expression is noted in CRCs with high microsatellite instability (McGregor *et al.* 2004). This group constitutes a distinct 15% of CRCs and has been noted to have improved survival compared with CRCs that have low microsatellite instability (McGregor *et al.* 2004). Similarly, a reduced expression of K8 and K20 has been noted to be associated with some CRC cell epithelial to mesenchymal transition

(Knosel *et al.* 2006). Such changes are usually associated with more aggressive tumours and decreased survival (Knosel *et al.* 2006). K8 dephosphorylation by phosphatase of regenerating liver-3 (PRL-3) has also been shown to be a factor in contributing towards metastasis of CRC cells (Miz-uuchi *et al.* 2009).

PTM and cleavage of keratins in colorectal neoplasia

Data from our group (Arasaradnam, Riley, Shaw & Corfe, unpublished) show increase in lysine acetylation in fields and in adenoma, which we have associated with depolymerization *in vitro* (Drake *et al.* 2009). These data imply that not only the level, but also the PTM (and by implication the enzymes regulating them) of keratin may be important in contributing to the development and progression of neoplasia.

Finally, majority of pathways leading to apoptosis involve caspase activation and initiation of a caspase cascade (Petak & Houghton 2001). The caspases cleave specific cellular substrates, and most type 1 keratins are thought to be substrates for caspases (Ku & Omary 2001). In the case of K18, this leads to caspase-cleaved fragments in the serum that can be analysed to assess the cancer load, cancer progression and response to chemotherapy by distinguishing between necrosis and apoptosis (Linder 2007). Ausch *et al.* (2009) analysed a caspase-cleaved fragment of K18 (M30) in the serum samples of patients with CRC pre- and postoperatively and found that M30 correlated significantly with tumour recurrence where it remained raised postoperatively. M30 neo-epitope in K18 on circulating tumour cells (CTCs) has been investigated to identify proportion of apoptotic and viable cells in peripheral blood of patients with epithelial cancers (Rossi *et al.* 2010). In this study, it was noted that a change in balance of M30-negative/positive CTC might indicate active disease.

Concluding comments

Keratins, key constituents of the IF proteins, are now established as having a significant role in maintaining epithelial integrity, in structuring and organizing cells, and in cell signalling pathways. Expression of keratins in colorectal epithelium has been shown to vary in health and in disease states. Keratins undergo PTM in response to various stimuli; the full significance of these still remains to be understood but has been implicated as having functional consequences. Likewise, the regulatory enzymes governing keratin modification are largely uncharacterized yet represent valuable potential targets in the search for new strategies to halt epithelial-mesenchymal transition. In sporadic CRCs, keratins have already been used as a tool for characterization of tumours, as well as in identifying their prognoses. Human studies in well-defined IBD patients categorized into disease type and extent or severity of disease are, however, lacking. Despite the increasing interest in keratins in both CRCs and

IBD, further studies are needed to define their role in the pathogenesis of these conditions, identify expression states and solubility (including occurrence of PTM) in different disease states and to evaluate their secondary potential as diagnostic and therapeutic targets.

Declaration of interest

The authors declare no competing interest. This work was funded by the Bardhan Research and Education Trust.

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