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Differential CD74 (major histocompatibility complex Class II invariant chain) expression in mouse and human intestinal adenomas

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ABSTRACT

CD74 (major histocompatibility complex (MHC) Class II invariant chain) has recently been identified as the cell-surface receptor for the pro-tumorigenic cytokine macrophage migration inhibitory factor (MIF). Therefore, we investigated *CD74* gene expression in intestinal adenomas in $Apc^{Min/+}$ mice and humans. *CD74* mRNA (p31 and p41 splice variants) and immunoreactive CD74 protein levels were significantly lower in small intestinal and colonic $Apc^{Min/+}$ mouse adenomas compared with histologically normal mucosa. These findings were mirrored by a reduction in MHC Class II expression and Class II *trans*-activator type IV transcripts. Conversely, CD74 protein levels were actually increased in dysplastic epithelial cells in 47/55 (85%) human colorectal adenomas, with CD74 and MIF protein levels together predicting increasing dysplasia in individual adenomas ($P = 0.003$). Down-regulation of *CD74* during $Apc^{Min/+}$ mouse intestinal tumorigenesis does not model increased CD74 expression at the early, benign stages of human colorectal carcinogenesis. Epithelial cell CD74 represents a valid target for anti-CRC therapy.

1. Introduction

CD74 (also known as major histocompatibility complex (MHC) Class II invariant chain or Ii) plays a major role in processing of MHC Class II molecules in antigen-presenting immune cells.[1] CD74 acts as a chaperone for MHC Class II molecules, directing hetero-trimeric CD74–HLA-DR complexes for endosomal degradation, subsequently leading to peptide binding and antigen presentation by Class II molecules at the cell surface.[1] At any one time, a significant number of CD74 molecules exist transiently as a type II trans-membrane glycoprotein at the cell surface of several cell types, including intestinal epithelial cells, prior to internalisation.[2]

Recently, a novel role for cell surface CD74 has been identified as part of a receptor complex (with CD44) for the cytokine macrophage migration inhibitory factor (MIF).[3,4] MIF has important roles in the innate and acquired immune response, as well as during carcinogenesis.[5] We had previously described up-regulation of MIF expression and pro-tumorigenic activity of MIF during the early stages of colorectal carcinogenesis (colorectal adenoma [or polyp] development and growth), including anti-apoptotic activity in human colorectal adenoma cells *in vitro* and pro-angiogenic properties in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis.[6]

Expression of CD74 at the early stages of intestinal tumorigenesis in mice and humans has not been adequately inves-

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tigated. To the best of our knowledge, there has been no published study of CD74 expression in murine intestinal tumours. However, CD74 protein localisation has been studied in a small number of human colorectal adenomas and in colorectal cancer (CRC) tissue, prior to identification of CD74 as a MIF receptor.[7–9]

Detailed investigation of CD74 expression in murine and human colorectal tumours is essential in order to further characterise CD74 as a target for anti-CRC therapy, either as part of an anti-MIF strategy[10] or as a mechanism for antibody-mediated internalisation of anti-cancer agents (as has been demonstrated for B-cell haematological malignancies[11]). Therefore, we examined CD74 mRNA and protein expression in $Apc^{Min/+}$ mouse intestinal adenomas and human colorectal adenomas.

2. Methods

2.1. Human colorectal tissue

Formalin-fixed paraffin-embedded (FFPE) sections were used from 55 human 'sporadic' colorectal adenomas, which were adjacent to sections from the same cohort of adenomas that had previously been subjected to immunohistochemistry (IHC) for MIF.[6] FFPE human CRC tissue and spleen were used as positive controls for CD74 IHC.[8] Approval for the use of all anonymised human tissue samples was obtained from The Leeds (East) Research Ethics Committee.

2.2. $Apc^{Min/+}$ mouse intestinal tissue

All mouse tissues were obtained from our established C57Bl/6 $Apc^{Min/+}$ colony, which are maintained under strict isolator conditions.[12] Small intestine (SI) and colon tissues were obtained from 120 d-old $Apc^{Min/+}$ mice and wild-type littermates immediately after sacrifice as described.[12] Tissue was either fixed for 24 h in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at 20 °C, prior to embedding in paraffin, or was snap frozen in OCT using liquid N_2 before storage at –70 °C, as described.[13]

C57Bl/6 $Ii^{-/-}$ mouse intestine already fixed in 4% (w/v) paraformaldehyde in PBS and stored in 70% (v/v) ethanol was a kind gift from Dr. Shachar, Weizmann Institute of Science, Israel.[14]

2.3. Laser-capture micro-dissection of mouse intestinal tissue

Frozen cryostat sections (thickness 7 μm) were fixed in 100% methanol at –20 °C for 20 min, and then stained with haematoxylin (Sigma) for 1 min under RNase-free conditions. Laser-capture micro-dissection (LCM) was performed using a Zeiss Axiovert 200 microscope with a PALM® Microlaser system and Zeiss LCM Caps (Carl Zeiss Ltd., Welwyn Garden City, United Kingdom (UK)). Total RNA was extracted from micro-dissected fragments using a PicoPure™ RNA isolation kit (Molecular Devices Ltd., Wokingham, UK). All samples were incubated with amplification-grade DNase I (Invitrogen, Paisley, UK) for 15 min at 20 °C, prior to inactivation by addition of 25 μM ethylenediamine tetra-acetic acid for 10 min at 65 °C.

2.4. Real-time RT-PCR

First strand cDNA was synthesised using Superscript III (Invitrogen) as per manufacturer's instructions. Real-time PCR was performed as described previously by our group[15] using an ABI 7700 or 7900 sequence detector and the SYBR Green® system (Applied Bioscience, Warrington, UK). Primer sequences are described in the Supplementary Table. PCRs were performed in duplicate for each cDNA sample, and the mean C_t value was used for further analysis. The mean of three 'housekeeping' gene mRNA C_t values (*β-actin*, *glyceraldehyde-3-phosphate dehydrogenase* [*Gapdh*] and *Psmb6* [encoding the 20S proteasome subunit]) for each cDNA sample was used for normalisation of transcript levels for *p31* and *p41 CD74* isoforms, Class II *trans*-activator (*CIITA*) isoforms *I*, *III* and *IV*, and *vimentin* using the ΔC_t method. This normalisation strategy had previously been demonstrated to produce accurate RT-PCR expression profiling.[16] All PCRs, including those for the 'housekeeping' gene transcripts, displayed similar reaction efficiencies over a range of cDNA template concentrations (Supplementary Fig. 1). Data are presented as the transcript level of interest normalised to the mean 'housekeeping' mRNA level in arbitrary units using the equation $2^{-\Delta C_t}$.

2.5. Immunohistochemistry for CD74 and MHC Class II

2.5.1. Mouse tissue

IHC for CD74 on 4% paraformaldehyde-fixed tissue sections was performed using goat polyclonal anti-CD74 antibody (C-16, Santa Cruz Biotechnology, Santa Cruz, CA, United States of America (USA)). Paraffin-embedded tissue sections (4 μm thickness) were de-waxed in xylene and rehydrated through a graded alcohol series into water. Endogenous peroxidase activity was quenched using 1.2% (v/v) hydrogen peroxide in methanol at 20 °C for 15 min followed by washing in running tap water for 10 min. Antigen retrieval was performed in a 900 W microwave oven for 10 min in 10 mM citrate buffer pH 6.0 followed by an endogenous avidin/biotin block (Vector Laboratories, Burlingame, CA, USA). Non-specific binding sites were blocked with 10% (v/v) rabbit serum (DakoCytomation Ltd., Ely, UK) in Tris-buffered saline (TBS) for 30 min at 20 °C. Sections were incubated with 2 μg/ml C-16 antibody in 10% (v/v) rabbit serum overnight at 4 °C before washing with TBS (5 min × 2) and addition of biotinylated rabbit anti-goat immunoglobulin (DakoCytomation Ltd.) at 1:200 dilution in 10% (v/v) rabbit serum for 30 min at 20 °C. Visualisation was obtained using StreptABComplex and DAB+ as per manufacturer's instructions (both DakoCytomation Ltd.). Sections were counterstained using Mayer's haematoxylin for 3 min, then Scott's tap water for 1 min, followed by dehydration and mounting in diphenylxylene.

Negative controls consisted of omission of the primary antibody, pre-absorption of the primary antibody with its cognate blocking peptide (10 μg/ml for 2 h at 20 °C; Santa Cruz) and use of $Ii^{-/-}$ tissue sections.

Immunofluorescence for CD74 was performed on 100% methanol-fixed (20 min at –20 °C) frozen tissue sections, blocked with 10% (v/v) goat serum in TBS for 30 min at 20 °C, using fluorescein isothiocyanate (FITC)-conjugated In-1 rat monoclonal anti-mouse CD74 antibody (1:400 dilution

for 60 min at 20 °C; BD Biosciences, Oxford). Sections were mounted in Prolong® Gold with DAPI.

IHC for MHC Class II molecules was also performed on 100% methanol-fixed frozen tissue sections using the same protocol as for CD74 IHC with C-16 antibody except that the primary antibody (rat monoclonal anti-mouse Class II ER-TR3; Abcam, Cambridge, UK) was incubated with sections at a 1:50 dilution in 10% (v/v) rabbit serum in TBS for 60 min at 20 °C, and biotinylated rabbit anti-rat immunoglobulin (1:200 for 30 min at 20 °C; DakoCytomation Ltd.) was used as the secondary antibody. Sections were mounted on MOWIOL.

2.5.2. *Human tissue*

IHC for CD74 was performed using two different anti-CD74 antibodies (C-16 and LN2 mouse monoclonal anti-human CD74 [BD Biosciences]) on adjacent FFPE sections as described above and previously,[6] except that slides were incubated with 10 μg/ml LN2 in 10% (v/v) goat serum in TBS for 1 h at 20 °C before incubation with biotinylated goat anti-mouse immunoglobulin (1:100 in TBS) from BD Biosciences for 30 min at 20 °C. Omission of the primary antibody was used as a negative control. CRC tissue and spleen were used as positive controls.

2.6. *Semi-quantitative scoring of CD74 immunoreactivity*

LN2 immunoreactivity was assessed by two independent observers blinded to the clinico-pathological characteristics and the MIF immunoreactivity score[6] of each adenoma section. A consensus CD74 protein expression score in both epithelial (EP) and stromal (STR) cells was produced for each section on a scale of 0–2 (0, no staining; 1, weak intensity, patchy staining; 2, moderate to strong intensity staining with a widespread distribution).

2.7. *Statistical analysis*

Differences in tissue transcript levels were analysed using Student's independent samples *t*-test. The significance of differences in CD74 and MIF protein expression related to clinico-pathological parameters was tested using the Mann–Whitney *U*-test and Kruskal–Wallis one-way analysis of variance. Logistic regression with stepwise forward selection was performed to identify independent factors that predicted CD74 and MIF expression within adenomas. The relationship between IHC scores for CD74 and MIF was explored using Spearman's correlation coefficient. Statistical significance was assumed if the *P* value was less than 0.05. All analyses were performed using SPSS v14.0.

3. Results

3.1. *CD74, mRNA and protein levels are reduced in $Apc^{Min/+}$ mouse intestinal adenomas*

As we had previously demonstrated the up-regulation of *Mif* expression in $Apc^{Min/+}$ mouse SI and colorectal adenomas,[6] we firstly investigated CD74 mRNA and protein expression in this well-established model of the early stages of human colorectal carcinogenesis.[17] For real-time RT-PCR analysis of CD74 transcript levels, we used LCM in order to measure epithelial and stromal cell mRNA levels separately. RT-PCR for the intermediate filament gene *vimentin* was used as a mesenchymal cell marker in order to exclude inadvertent contamination of epithelial cell samples with stromal cell mRNA (Supplementary Fig. 2).

There are two alternatively spliced mouse CD74 mRNAs that encode 31 kDa and 41 kDa CD74 protein isoforms termed p31 and p41.[18] Therefore, we designed PCR primers (see Supplementary Table) that would distinguish between p31 and p41 mRNAs, as well as those that would measure total CD74 transcript levels (termed p31 + p41). Transcripts encoding p31 protein were more abundant in mouse SI and colon than p41 mRNA consistent with the previous data (Fig. 1).[19] p31 and p41 mRNA levels were consistently higher in the stromal cell compartment of histologically normal (HN) mucosa than in HN epithelium (Fig. 1), particularly in the colon ($P < 0.01$). Similar levels of CD74 mRNA were demonstrated in epithelium and the stromal compartment of SI and colonic mucosa from wild-type C57Bl/6 littermates (Supplementary Fig. 3). Levels of p31 and p41 transcripts, as well as the total CD74 mRNA level measured using a separate primer pair, were significantly lower in both epithelial and stromal cell compartments of adenomas (Ad) compared with neighbouring HN mucosa in $Apc^{Min/+}$ mouse distal SI (Fig. 1) and in the stromal cell compartment of $Apc^{Min/+}$ mouse colonic adenomas versus HN colonic mucosa (Fig. 1).

Next, we examined CD74 protein expression in $Apc^{Min/+}$ mouse intestinal adenomas by IHC using a polyclonal goat anti-CD74 antibody (C-16). Staining specificity was confirmed using peptide pre-absorption and CD74-null tissue (Supplementary Fig. 4). Consistent with the RT-PCR findings, there was a reduced cytoplasmic CD74 immunoreactivity in dysplastic epithelial cells in $Apc^{Min/+}$ mouse SI adenomas compared with epithelial cells in neighbouring HN mucosa (Fig. 2A). Strongly CD74-positive stromal cells were present within SI adenomas, but they were fewer in number than in HN mucosa (Fig. 2A). Reduced CD74 immunostaining was also evident in epithelial cells in $Apc^{Min/+}$ mouse colonic adenomas (Fig. 2B) compared with HN colonic mucosa (Fig. 2C). Similar findings were obtained using a direct immunofluorescence technique with a different anti-CD74 antibody (In-1; Fig. 2D–E).

3.2. *Down-regulation of MHC Class II and Class II trans-activator accompany the reduction in CD74 expression in $Apc^{Min/+}$ mouse adenomas*

The reduction in CD74 protein levels in epithelial cells in SI and colonic $Apc^{Min/+}$ mouse adenomas was mirrored by lower MHC Class II immunoreactivity associated with epithelial cells in SI adenomas compared with HN epithelium (Fig. 2F). Epithelial cells in HN $Apc^{Min/+}$ mouse colonic mucosa were only weakly positive for MHC Class II (data not shown).

Mouse CD74 and MHC Class II gene expression are known to be regulated coordinately by a family of Class II *trans*-activator (CIITA) transcription factors termed I, III and IV each encoded by the *Mhc2ta* gene, which contains three independent promoters each linked to a distinct first exon.[20,21] CIITA transcript levels are assumed to correlate with CIITA protein isoform abundance, which is considered to be too low for

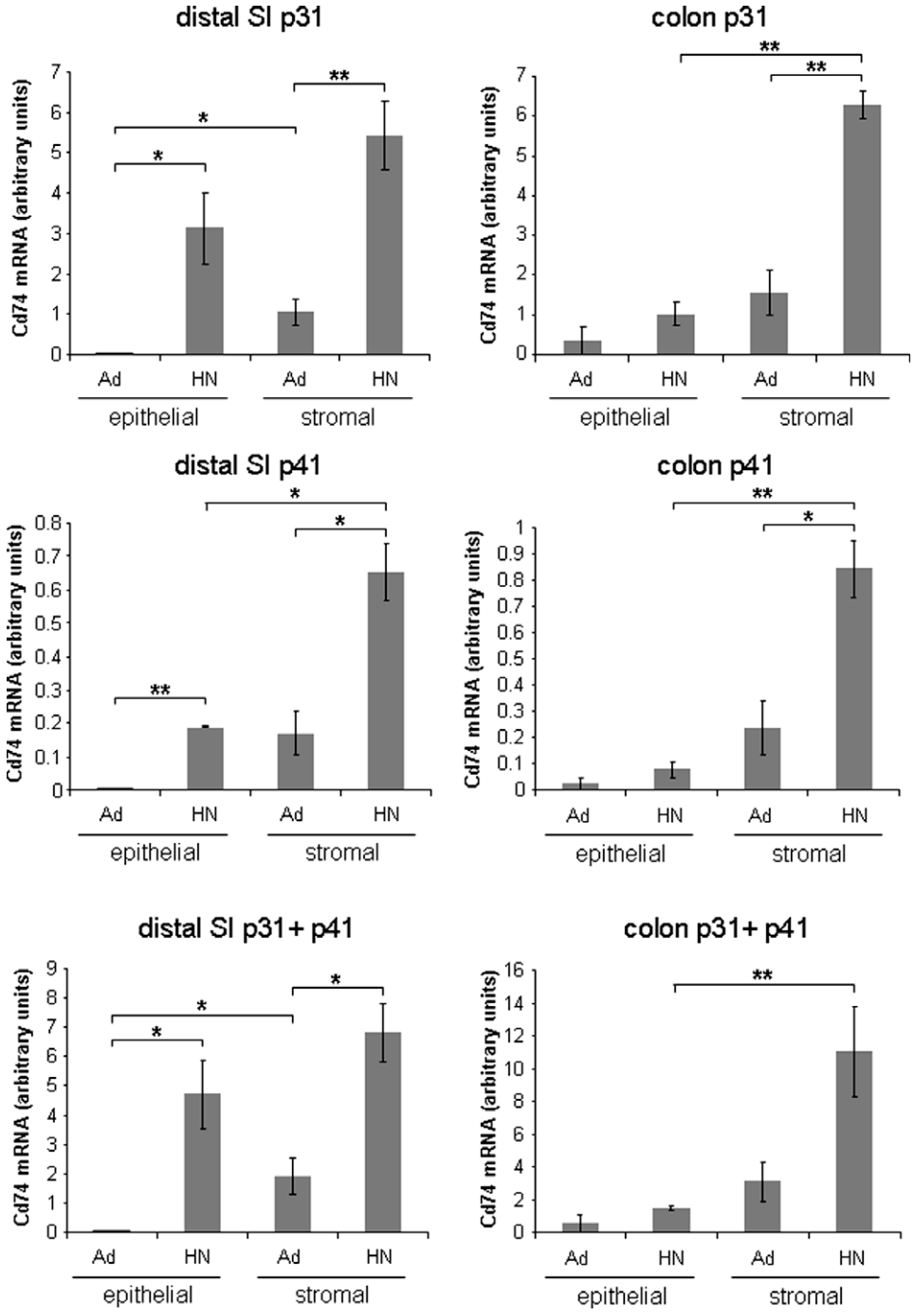

Fig. 1 – Real-time RT-PCR analysis of CD74 mRNA levels in epithelial cell and stromal cell elements of histologically normal (HN) mucosa and adenoma (Ad) tissue from 120 d-old *Apc*$^{Min/+}$ mice. Separate primer pairs were used to distinguish between p31 CD74 mRNA, p41 CD74 mRNA and total (p31 + p41) CD74 mRNA levels. Data represent the mean and standard error of the mean normalised transcript level from LCM tissue from three different animals. *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).

detection by available techniques.[22] Therefore, we measured CIITA I, II and IV mRNA levels in *Apc*$^{Min/+}$ mouse intestine by real-time RT-PCR. For these experiments, we micro-dissected frozen adenoma tissue from HN mucosa, including both epithelial and stromal cell elements, in order to maximise template cDNA for PCR of these low abundance mRNAs. There was approximately 10-fold less mRNA for CIITA III than for CIITA I and IV in *Apc*$^{Min/+}$ mouse SI and colon (Fig. 3). Consistent with the down-regulation of CD74 and MHC Class II expression in *Apc*$^{Min/+}$ mouse adenomas, levels of all the three CIITA transcripts were lower in adenomas than in HN mucosa in both SI and colon (Fig. 3). This difference reached statistical significance ($P = 0.02$) for the reduction in CIITA IV mRNA, which was the most abundant CIITA transcript in SI (Fig. 3).

In summary, we have demonstrated a parallel decrease in expression of CD74, MHC Class II and their shared master

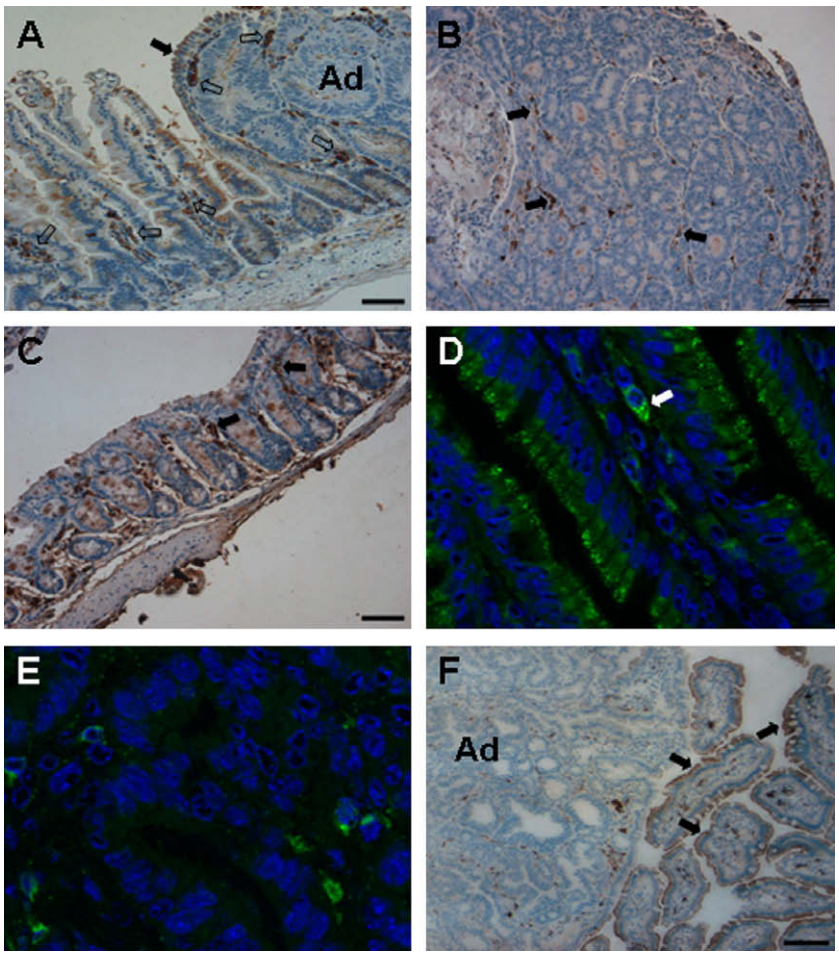

Fig. 2 – Immunohistochemistry for CD74 protein in $Apc^{Min/+}$ mouse intestine. (A) SI mucosa including an adenoma (Ad) surrounded by a layer of CD74-positive non-neoplastic epithelium (closed arrow). CD74-positive stromal cells in the adenoma and HN mucosa are denoted by open arrows. Size bar = 50 μm. (B) Colonic adenoma containing CD74-negative dysplastic epithelial cells interspersed with CD74-positive stromal cells (closed arrows). Size bar = 50 μm. (C) HN colonic mucosa demonstrating weak staining of epithelial cells (compare with CD74-negative adenoma cells in part (B)) with stronger CD74 immunoreactivity in neighbouring stromal cells (closed arrows). Size bar = 50 μm. Controls for CD74 IHC are demonstrated in *Supplementary* Fig. 3. (D) Direct immunofluorescence for CD74 in HN SI. CD74 immunoreactivity (green) was noted in villus epithelial cells, and was particularly marked in the apical portion of cells. CD74 immunofluorescence was stronger in stromal cells (arrow) than in neighbouring epithelial cells, in keeping with the colorimetric IHC performed with a different primary antibody (A). Nuclei visualised with DAPI (blue). (E) Loss of CD74 immunoreactivity in dysplastic epithelial cells within an adenoma. CD74-positive stromal cells act as an internal control. (F) IHC for Class II on SI tissue. Class II-negative dysplastic epithelial cells within an adenoma (Ad) adjacent to HN mucosa lined by Class II-positive epithelium (arrows). Size bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transcriptional regulator CIITA in $Apc^{Min/+}$ mouse adenomas compared with HN SI and colonic mucosa.

3.3. *Increased CD74 protein expression in human colorectal adenomas*

The $Apc^{Min/+}$ mouse CD74 data prompted us to examine whether similar changes occurred in 'sporadic' human colorectal adenomas. Surprisingly, IHC using the same C-16 antibody used for the mouse IHC experiments, as well as a second, independent anti-CD74 antibody LN2, demonstrated that CD74 immunoreactivity was actually increased in dysplastic epithelial cells compared with HN colonic mucosa (Fig. 4A and B), which invariably contained prominent CD74-positive stromal cells but CD74-negative epithelium (Fig. 4C–E), in keeping with previous reports.[8,9] CD74 staining with C-16 and LN2 antibodies was remarkably similar in both adenomatous and HN colorectal mucosa (compare Fig. 4A and B, and C and D). There was uniform, intense staining of human CRC cells (Fig. 4F) and splenocytes by C-16 as previously reported.[8]

CD74 immunoreactivity was variable with respect to distribution and intensity in individual colorectal adenomas (Fig. 4B and H–L). In some adenomas, CD74 staining was uniform in all epithelial cells within the tumour (Fig. 4H and K), although in other adenomas there was patchy immunoreactivity (Fig. 4B,

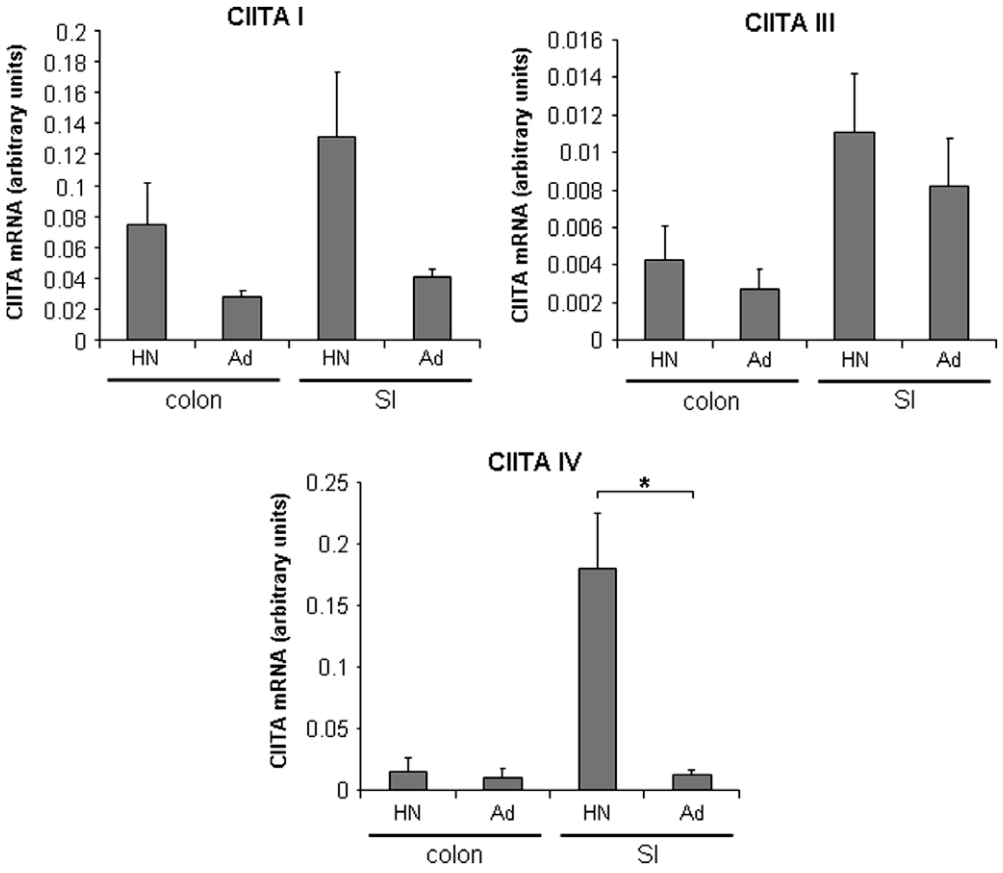

Fig. 3 – Real-time RT-PCR analysis of CIITA mRNA levels in micro-dissected adenoma (Ad) tissue and histologically normal (HN) mucosa from the colon or SI of 120 d-old $Apc^{Min/+}$ mice. Data represent the mean and standard error of the mean normalised transcript level from LCM tissue from three different animals. *$P < 0.05$ (Student's t-test).

I–J and L). In general, there were higher CD74 protein levels in surface epithelial cells compared with deeper crypt epithelium (Fig. 4I). The predominant CD74-expressing stromal cells in colorectal adenomas and HN mucosa were morphologically B lymphocytes and, to a lesser extent, macrophages.

3.4. *CD74 immunoreactivity is associated with features of 'advanced' human colorectal adenomas*

IHC using LN2 antibody was performed in a single run on sections of 55 human sporadic colorectal adenomas, adjacent to those sections that were previously stained for MIF protein.[6]

CD74 staining was present in dysplastic epithelial cells in 47 of 55 (85%) adenomas, and in stromal cells in all the cases, thus acting as an internal positive control for epithelial CD74-negative adenomas. Individual CD74 epithelial (EP) scores are displayed in Fig. 5. Representative sections from adenomas with EP scores 1 and 2 are displayed in Fig. 4H–L. There was a significant difference in EP CD74 scores between adenomas from patients aged above or below 65 years (Fig. 5A; $P = 0.006$). There was no difference between male and female patients (Fig. 5B). Higher EP scores were more frequent in larger ($\geqslant$10 mm) adenomas (Fig. 5C; $P = 0.07$). There was no difference in epithelial CD74 scores between adenomas proximal and distal to the splenic flexure (Fig. 5D). However, there was a significant difference in CD74 scores between adenomas with different histology (Fig. 5E; $P = 0.019$) and grade of dysplasia (Fig. 5F; $P = 0.013$). Stepwise logistic regression, including factors identified as significant by univariate analysis, identified the grade of dysplasia as the only significant independent predictor of epithelial CD74 protein expression ($P = 0.01$).

All adenomas contained CD74-positive stromal cells. There were no statistically significant associations between CD74 stromal (STR) scores and any clinico-pathological factor (data not shown). There was no correlation between CD74 EP and STR scores for individual adenomas (Spearman's correlation coefficient = 0.201).

3.5. *The relationship between CD74 and MIF protein expression in human colorectal adenomas*

The function of cell surface CD74 as a receptor for MIF (3–4) provided the rationale for dual analysis of CD74 and MIF immunoreactivity in human colorectal adenomas. There was a significant correlation between MIF and CD74 EP scores in individual adenomas (Spearman's correlation coefficient 0.326; $P = 0.015$). If CD74 expression is necessary for the protumorigenic activity of MIF during colorectal carcinogenesis *in vivo*, a combined MIF and CD74 EP score might have a higher predictive value than either parameter alone. In order to test the hypothesis that combined high MIF and CD74 EP

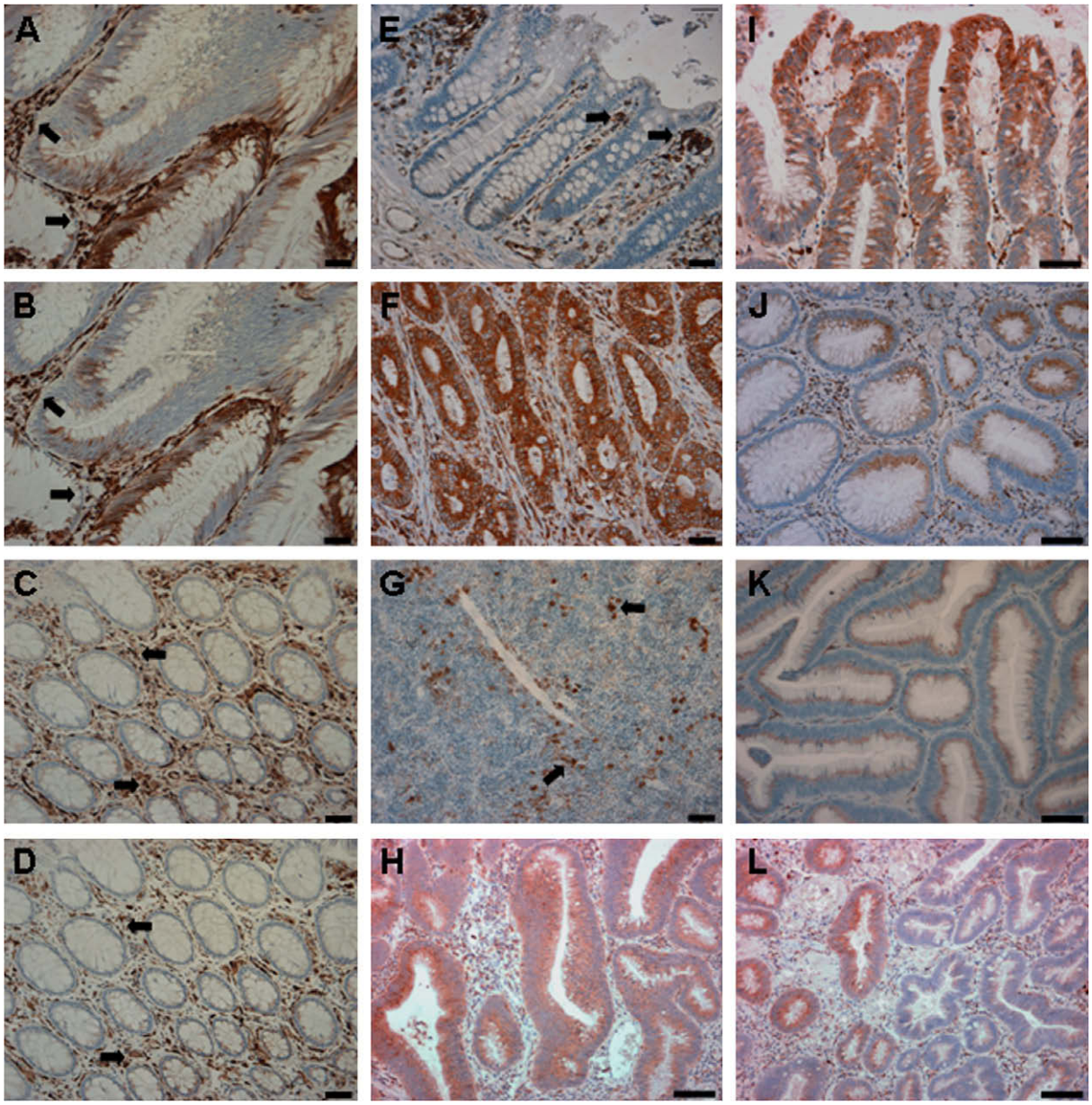

Fig. 4 – Immunohistochemistry for CD74 on sporadic human colorectal adenomas. Immunohistochemistry for CD74 protein on adenoma tissue (A and B) and HN mucosa (C and D) using C-16 (A and C) or LN2 (B and D) antibodies on adjacent sections. The same pattern and intensity of CD74 immunoreactivity are evident with the two antibodies. A and B demonstrate variable epithelial cell staining in adjacent crypt structures surrounded by CD74-positive stromal cells (arrows). Size bars = 50 μm. C and D show CD74-negative epithelium within normal lamina propria containing large numbers of CD74-positive stromal cells (arrows). Size bars = 50 μm. (E) Cross-section of HN colorectal mucosa stained with C-16 antibody. Arrows highlight CD74-positive stromal cells adjacent to CD74-negative epithelium. Size bar = 50 μm. (F) Human CRC tissue demonstrating intense staining of cancer cells. Size bar = 50 μm. (G) Human spleen containing scattered CD74-positive cells. Size bar = 50 μm. (H–L) Representative human colorectal adenomas with variable epithelial cell immunoreactivity. (H) EP score 2. Size bar = 100 μm. (I) EP score 2. Size bar = 50 μm. (J) EP score 1. Size bar = 50 μm. (K) EP score 1. Size bar = 100 μm. (L) EP score 2. Size bar = 100 μm.

scores were associated with factors known to be related to malignant progression of adenomas, tumours were split into CD74–MIF^{low} ($n = 15$) and CD74–MIF^{high} ($n = 12$) groups (Table 1). Univariate statistical analysis by Pearson's χ^2 test confirmed that the same clinico-pathological factors that were associated with differences in adenoma CD74 EP score were also significantly associated with combined adenoma MIF–CD74 status stratified as CD74–MIF^{low} or CD74–MIF^{high} (size, $P = 0.031$; histology, $P = 0.037$; grade of dysplasia, $P = 0.016$). Stepwise logistic regression demonstrated that the CD74–MIF status of an adenoma was a stronger predictor of high-grade dysplasia ($P = 0.003$) than the CD74 IHC score alone.

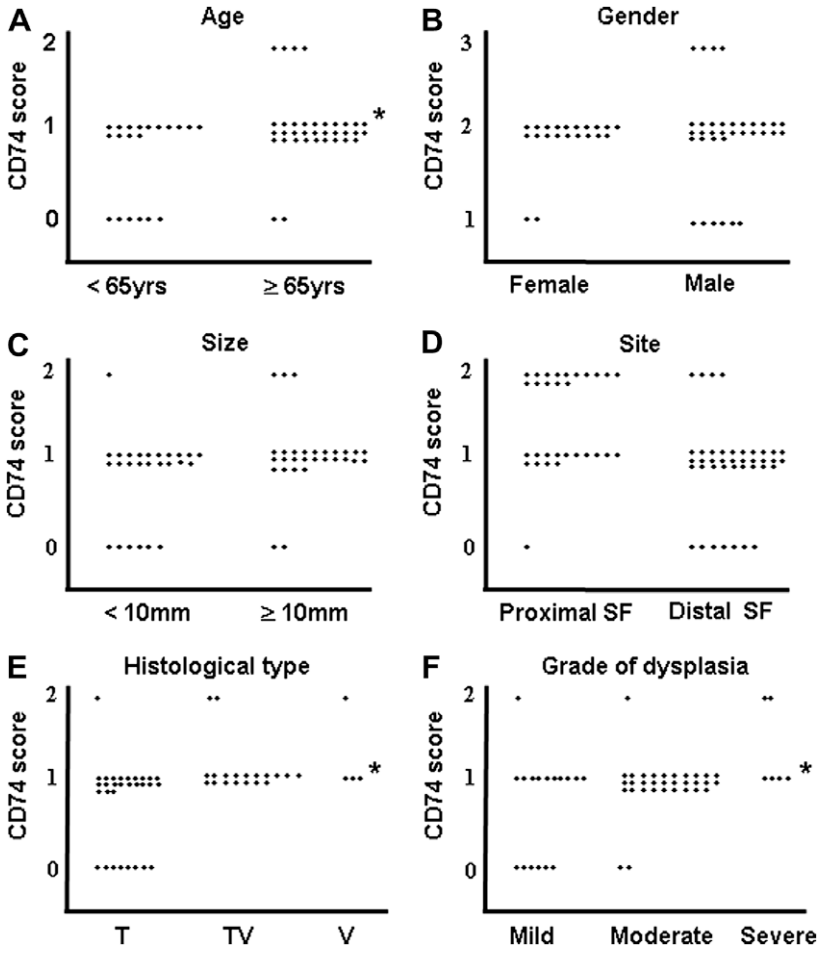

Fig. 5 – Individual CD74 epithelial (EP) scores of human colorectal adenomas. SF, splenic flexure; T, tubular; TV, tubulo-villous; V, villous. *P < 0.05. All adenomas contained CD74-positive stromal cells (STR score 1 61.8% and STR score 2 38.2%).

Table 1 – Distribution of CD74 and MIF immunohistochemistry EP scores in 55 human colorectal adenomas.

MIF EP score* \ CD74 EP score	0	1	2
1	3†‡	7	0
2	5	28	2
3	0	8	2

*See Ref. [6].
†The number of adenomas in each score category.
‡Light-shaded boxes are CD74–MIF^{low}, and dark-shaded boxes are CD74–MIF^{high}.

4. Discussion

This is the largest study to date describing CD74 protein expression in human colorectal adenomas and is the first since the discovery that cell surface CD74 acts as a receptor for MIF.[3,4] Herein, we demonstrate that CD74 protein is up-regulated in 85% of human 'sporadic' colorectal adenomas, and that increased levels of epithelial cell CD74 (particularly in combination with elevated epithelial cell MIF immunoreactivity) are associated with increasing epithelial cell dysplasia. It is difficult to compare our results directly with previous smaller studies[7–9] due to the use of different primary antibodies and scoring systems. However, O'Keane et al. used the LN2 antibody clone and also observed an association between LN2 staining and high-grade dysplasia in human colorectal adenomas, although only 45% of adenomas were positive for LN2 immunoreactivity.[9] A similar co-localisation of CD74 and MIF has recently been reported in malignant cells in human non-small cell lung tumours.[23]

Up-regulation of CD74 expression during human colorectal carcinogenesis has important potential implications for use of anti-cancer therapy directed at CD74, particularly as we and others have demonstrated that neighbouring HN epithelium

is CD74-negative.[8,9] Rapid internalisation of cell surface CD74 has already been used to target anti-cancer antibody conjugates to malignant B-cells in pre-clinical models.[11] Alternatively, CD74 acts as a survival receptor in cells and may be amenable to direct targeting in colorectal neoplasms.[24]

Importantly, we have uncovered profound differences in CD74 expression between $Apc^{Min/+}$ mouse adenomas (both SI and colon) and counterpart human adenomas. The $Apc^{Min/+}$ mouse is a precise genetic model of the rare, inherited CRC predisposition syndrome familial adenomatous polyposis.[17] For unknown reasons, $Apc^{Min/+}$ mice develop the majority of intestinal adenomas in the SI, with far fewer colorectal adenomas.[17] Despite this, the $Apc^{Min/+}$ mouse model has become established as a major tool for study of the biology and for chemoprevention of the early stages of human colorectal carcinogenesis based on the shared features of the molecular pathogenesis of tumour initiation and growth in both species.[25] Down-regulation of CD74 expression in $Apc^{Min/+}$ mouse intestinal adenomas (coordinately with MHC Class II and the shared transcriptional regulator CIITA) represents a major difference from that which occurs in benign human colorectal tumours. We observed CD74 down-regulation in $Apc^{Min/+}$ mouse intestinal adenomas at both the mRNA and the protein level (using the same antibody employed for human IHC studies). In addition, CD74 transcript levels in $Apc^{Min/+}$ mouse colonic adenomas have been shown to be significantly lower than in paired HN mucosa by cDNA microarray analysis (A.R. Clarke, University of Cardiff, UK). These observations have important implications for the future studies utilising the $Apc^{Min/+}$ mouse model for investigation of intestinal tumour immunology, including continued analysis of the role of the CD74 ligand MIF. It is interesting to note that in our previous study of the role of MIF during intestinal tumorigenesis,[6] we demonstrated that exogenous MIF induced resistance to apoptosis of CD74-positive VACO-235 human colorectal adenoma cells but that, unexpectedly, there was no increase in epithelial cell apoptosis frequency in *Mif*-null $Apc^{Min/+}$ mouse intestinal adenomas compared with $Apc^{Min/+}$ mouse littermates with wild-type *Mif* alleles. One explanation is that the lack of epithelial cell CD74 expression by epithelial cells in $Apc^{Min/+}$ mouse adenomas could explain the absence of a direct pro-apoptotic effect of *Mif* gene deletion (although *Mif*-null tumours had reduced microvessel density[6]).

In the absence of CD74-dependent MIF signalling, several other potential mechanisms of action of MIF have been described.[26] These include intracellular binding and negative regulation of *c-JUN*-activating binding protein 1 (JAB1) and tautomerase activity.[26] The relative contributions of these different signalling mechanisms to the pro-tumorigenic activity of MIF remain to be elucidated.

The mechanistic basis of CD74 down-regulation in $Apc^{Min/+}$ mouse intestinal adenomas is unknown. One valid hypothesis is that reduced CD74 (and MHC Class II) expression is secondary to methylation silencing of *Mhc2ta*.[27] Epigenetic CpG island methylation silencing of the *Mhc2ta* promoter is recognised in human CRC and gastric cancer cells.[28] However, it is not known whether CIITA gene silencing in human CRC cells is associated with reduced expression of CD74.

In summary, down-regulation of *CD74* expression in $Apc^{Min/+}$ mouse SI and colonic adenomas does not mirror CD74 expression patterns in human colorectal adenomas during the early stages of 'sporadic' colorectal carcinogenesis. Future pre-clinical studies investigating the function of CD74, and its known ligand MIF, during intestinal tumorigenesis should be designed with this in mind. Up-regulation of CD74 in the majority (85%) of human colorectal adenomas was associated with increasing grade of epithelial cell dysplasia and provided an opportunity to target the cell-surface pool of CD74 as a novel CRC chemoprevention and/or treatment strategy.

5. Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.02.005.

REFERENCES

1. Stumptner-Cuvelette P, Benaroch P. Multiple roles of the invariant chain in MHC Class II function. *Biochim Biophys Acta* 2002;**1542**:1–13.
2. Ong GL, Goldenberg DM, Hansen HJ, Mattes MJ. Cell surface expression and metabolism of major histocompatibility complex class II invariant chain (CD74) by diverse cell lines. *Immunology* 1999;**98**:296–302.
3. Leng L, Metz CN, Fang Y, et al. MIF signal transduction initiated by binding to CD74. *J Exp Med* 2003;**197**:1467–76.
4. Shi X, Leng L, Wang T, et al. CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity* 2006;**25**:595–606.
5. Bucala R, Donnelly SC. Macrophage migration inhibitory factor: a probable link between inflammation and cancer. *Immunity* 2007;**26**:281–5.
6. Wilson JM, Coletta PL, Cuthbert RJ, et al. Macrophage migration inhibitory factor promotes intestinal tumorigenesis. *Gastroenterology* 2005;**129**:1485–503.
7. Degener T, Momburg F, Moller P. Differential expression of HLA-DP, HLA-DQ and associated invariant chain (Ii) in normal colorectal mucosa, adenoma and carcinoma. *Virchows Arch A Pathol Anat Histopathol* 1988;**412**:315–22.
8. Jiang Z, Xu M, Savas L, LeClair P, Banner BF. Invariant chain expression in colon neoplasms. *Virchows Arch* 1999;**435**:32–6.
9. O'Keane JC, Stahl D, Sheahan K, Burke B, Gottlieb LS, O'Brien MJ. Analysis of epithelial and lymphoid phenotypic markers in relation to growth pattern of colorectal adenomas. *Hum Pathol* 1992;**23**:1038–43.
10. Morand EF, Leech M, Bernhagen J. MIF: a new cytokine link between rheumatoid arthritis and atherosclerosis. *Nature Rev Drug Discov* 2006;**5**:399–411.

11. Stein R, Mattes MJ, Cardillo TM, et al. CD74: a new candidate target for the immunotherapy of B-cell neoplasms. *Clin Cancer Res* 2007;**13**(18 Suppl):5556s–63s.
12. Scott DJ, Hull MA, Cartwright EJ, et al. Lack of inducible nitric oxide synthase promotes intestinal tumorigenesis in the $Apc^{Min/+}$ mouse. *Gastroenterology* 2001;**121**:889–99.
13. Hull MA, Faluwi OO, Ko CWS, et al. Regulation of stromal cell *cyclooxygenase-2* expression in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis. *Carcinogenesis* 2006;**27**:382–91.
14. Elliott EA, Drake JR, Amigorena S, et al. The invariant chain is required for intracellular transport and function of major histocompatibility complex Class II molecules. *J Exp Med* 1994;**179**:681–94.
15. Ko CWS, Cuthbert RJ, Orsi NM, et al. Lack of interleukin-4 receptor α chain-dependent signalling promotes azoxymethane-induced colonic aberrant crypt focus formation in *Balb/c* mice. *J Pathol* 2008;**214**:603–9.
16. Vandesompele J, de Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**. research0034.
17. Boivin GP, Washington K, Yang K, et al. Pathology of mouse models of intestinal cancer: consensus report and recommendations. *Gastroenterology* 2003;**124**:762–77.
18. Koch N, Lauer W, Habicht J, Dobberstein B. Primary structure of the gene for the murine Ia antigen-associated invariant chains (Ii). An alternatively spliced exon encodes a cysteine-rich domain highly homologous to a repetitive sequence of thyroglobulin. *EMBO J* 1987;**6**:1677–83.
19. Yamamoto K, Koch N, Steinmetz M, Hammerling GJ. One gene encodes two distinct Ia-associated invariant chains. *J Immunol* 1985;**134**:3461–7.
20. Wright KL, Ting JP-Y. Epigenetic regulation of MHC-II and CIITA genes. *Trends Immunol* 2006;**27**:405–12.
21. Sanderson IR, Bustin SA, Dziennis S, Paraszczuk J, Stamm DS. Age and diet act through distinct isoforms of the Class II transactivator gene in mouse intestinal epithelium. *Gastroenterology* 2004;**127**:203–12.
22. Steimle V, Siegrist CA, Mottet A, Lisowska-Grospierre B, Mach B. Regulation of MHC Class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science* 1994;**265**:106–9.
23. McClelland M, Zhao L, Carskadon S, Arenberg D. Expression of CD74, the receptor for macrophage migration inhibitory factor, in non-small cell lung cancer. *Am J Pathol* 2009;**174**:638–46.
24. Starlets D, Gore Y, Binsky I, et al. Cell-surface CD74 initiates a signalling cascade leading to cell proliferation and survival. *Blood* 2006;**107**:4807–16.
25. Corpet DE, Pierre F. How good are rodent models of carcinogenesis in predicting efficacy in humans? Systematic review and meta-analysis of colon tumour chemoprevention in rats, mice and men. *Eur J Cancer* 2005;**41**:1911–22.
26. Mitchell RA. Mechanisms and effectors of MIF-dependent promotion of tumourigenesis. *Cell Signal* 2004;**16**:13–9.
27. Holling TM, van Eggermond MCJA, Jager MJ, van den Elsen PJ. Epigenetic silencing of *MHC2TA* transcription in cancer. *Biochem Pharmacol* 2006;**72**:1570–6.
28. Satoh A, Toyota M, Ikeda H, et al. Epigenetic inactivation of class II transactivator (CIITA) is associated with the absence of interferon-γ-induced HLA-DR expression in colorectal and gastric cancer cells. *Oncogene* 2004;**23**:8876–86.