Original article

# Tumour-infiltrating lymphocytes in colorectal cancer with microsatellite instability are activated and cytotoxic

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**Background:** Patients with colorectal cancer that display high-level microsatellite instability (MSI-H) appear to have a better prognosis. This may be explained by the pronounced T cell infiltrate seen in MSI-H tumours that is related to a specific antigen-driven immune response. The nature of tumour-infiltrating lymphocytes in colorectal cancers was investigated using quantitative real-time polymerase chain reaction (PCR) and immunohistochemistry.

**Methods:** Quantitative fluorescent hydrolysis probe-based reverse transcriptase–PCR assays were used to detect levels of mRNA specifying T cell markers in fresh frozen colorectal tissue from MSI-H tumours and those with little or no microsatellite instability (microsatellite stable (MSS) tumours). In addition, immunohistochemistry was performed on paraffin-embedded sections to compare expression of the same T cell markers and the activation markers granzyme B and interleukin 2 receptor α-subunit (IL-2Rα) in MSI-H and MSS tumours.

**Results:** MSI-H tumours contained higher ratios of CD8/CD3 mRNA copy numbers than MSS tumours ($P = 0{\cdot}016$), confirming the cytotoxic nature of lymphocyte infiltrates in this subset of colorectal cancers. Furthermore, immunohistochemistry confirmed that MSI-H tumours contained more infiltrating lymphocytes than MSS tumours, as shown by increased expression of CD3 ($P = 0{\cdot}003$) and CD8 ($P = 0{\cdot}008$). Consistent with other studies, the lymphocytes in MSI-H tumours were activated as indicated by significantly higher granzyme B counts ($P = 0{\cdot}020$) and a significantly higher level of expression of IL-2Rα ($P = 0{\cdot}017$).

**Conclusion:** The results support the hypothesis that MSI-H colorectal cancers may be more immunogenic than MSS tumours.

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## Introduction

Microsatellite instability is a feature of malignancy in the hereditary non-polyposis colorectal cancer (HNPCC) syndrome[1,2]. Germline defects in mismatch repair genes lead to an accumulation of replication errors, deletion and insertion mutations at simple repeated sequences (microsatellites) throughout the genome[3]. Around 15–20 per cent of sporadic colorectal cancers also show microsatellite instability, resulting from defective mismatch repair commonly due to hypermethylation of the *hMLH1* gene[4,5]. HNPCC-associated cancers and sporadic tumours showing high-level microsatellite instability (MSI-H) are associated with an improved 5-year survival rate of up to 10–20 per cent, and this survival advantage is independent of other common prognostic criteria[6,7].

The presence of tumour-infiltrating lymphocytes in colorectal cancer has been associated with an improved prognosis[8,9] and is more marked in MSI-H cancers[10]. Previously, immunohistochemical methods have demonstrated increased expression of cytotoxic CD8-positive T cells and increased apoptosis of tumour cells in MSI-H colorectal cancer[11,12]. Increased levels of perforin and granzyme B, mediators of target cell death[13], were also demonstrated in these studies. Subsequent survival data support the view that the presence of cytotoxic lymphocytes in MSI-H colorectal cancer may enhance patient survival[14].

This study examined the activation of T cell responses in MSI-H colorectal cancer by comparative analysis of interleukin 2 receptor α-subunit (IL-2Rα) expression by immunohistochemistry. The IL-2Rα is upregulated in immune responses and is recognized as a marker of lymphocyte activation[15]. In addition, fluorescent hydrolysis probe-based reverse transcriptase–polymerase chain reaction (RT–PCR) assays were used to detect mRNA levels of T cell markers, allowing a quantitative comparative analysis of lymphocyte infiltration.

## Patients and methods

### Patients

The local research ethics committee gave approval and all patients gave informed consent before operation. Tissue was collected between 1 November 1997 and 1 December 2001 from consecutive patients undergoing operation for colorectal cancer. Eleven patients were excluded because sampling was thought to be potentially detrimental to histopathological assessment (four patients) or because prompt collection of a sample was not possible (seven). Specimens from 164 tumours, arising in 162 patients (two patients had two cancers each), were collected within 30 min of surgical resection. Tumour material was biopsied from the exophytic component, avoiding grossly necrotic areas, and mucosal tissue was sampled at a distance of 10 cm proximal to the cancer (distal in tumours at the caecal pole). The tissue was snap-frozen in liquid nitrogen and stored at − 70°C.

### Microsatellite analysis and fluorescent hydrolysis probe-based polymerase chain reaction

Genomic DNA was prepared from tumour and mucosal specimens using a DNeasy® kit (Qiagen, Hilden, Germany) and quantified on a GeneQuant™ spectrophotometer (Pharmacia Biotech, Cambridge, UK). The two mononucleotide markers, BAT-26 and BAT-40, were chosen from the panel of markers recommended by the National Cancer Institute convention on microsatellite instability analysis[16]. PCR analysis at these polyadenine tracts was performed with 100 ng genomic DNA in a total volume of 40 μl containing 1·5 mmol/l magnesium chloride, 1 × PCR buffer, 200 μmol/l deoxynucleotide triphosphate, 1 unit AmpliTaq Gold™ (Perkin Elmer, Foster City, California, USA) and 1 μmol/l of each primer. PCR conditions were 94°C for 11 min followed by 40 cycles (94°C for 40 s, 50°C for 30 s (45°C BAT-40), 72°C for 30 s) and elongation at 72°C for 7 min. Products were separated on a denaturing polyacrylamide gel (Sequagel XR®; National Diagnostics, Atlanta, Georgia, USA) within a dedicated system (Sequi-Gen® GT sequencing cell/PowerPac 3000; BioRad Laboratories, Hemel Hempstead, UK) and visualized by a modified silver staining procedure[17]. Tumours with MSI-H showed marked differences in tumour DNA product length owing to the quasimonomorphic nature of the BAT-26 marker, and similar bandshifts were seen in BAT-40 analysis. Tumours with no bandshifts in these markers were labelled as microsatellite stable (MSS). In view of the clinicopathological similarities, no distinction was made between HNPCC and sporadic MSI-H colorectal cancers.

For molecular analysis, tissue from 18 MSI-H tumours was available and compared with that from 28 MSS colorectal tumours, chosen to give similar proportions of right- and left-sided tumours (proximal or distal to the splenic flexure) and to minimize the influence of tumour location on the analysis. Total RNA was prepared from samples using an RNeasy® kit (Qiagen) and quantified using spectrophotometry and Ribogreen® quantification kits (Molecular Probes, Leiden, The Netherlands). RNA quality was assessed by measurement of the A260/A280 ratio. Primer and probe sequences were designed for T cell markers (*Table 1*) and quantitative RT–PCR performed on the ABI 7700 Prism sequence detection system using Taqman® EZ RT–PCR reagents (Applied Biosystems,

**Table 1** Primers and probes used for quantitative reverse transcriptase–polymerase chain reaction analysis

| Gene | Exon/exon span | Primers | Probe 5′ FAM 3′ TAMRA | Amplicon length (b.p.) | Genbank accession no. |
|---|---|---|---|---|---|
| *CD3Γ* | 5–7 | F: 5′ GAG TTC GCC AGT CGA GAG CT 3′<br>R: 5′ TCA TCT TCT CGA TCC TTG AGG G 3′ | 5′ CAG ACA AGC AGA CTC TGT TGC CCA ATG A 3′ | 85 | NM_000073 |
| *CD4* | — | F: 5′ CTT CTT CTG TGT CAG GTG CCG 3′<br>R: 5′ AGG AGT CTC TTG ATC TGA GAC ATC C 3′ | 5′ CAC CGA AGG CGC CAA GCA GAG 3′ | 69 | NM_000616 |
| *CD8A* | 2–3 | F: 5′ CTG AGC AAC TCC ATC ATG TAC TTC A 3′<br>R: 5′ GTC GTG GTG GGC TTC GC 3′ | 5′ CCA CTT CGT GCC GGT CTT CCT GC 3′ | 68 | NM_001768 |

F, forward; R, reverse; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

Warrington, UK) and standard cycling parameters. Reactions were processed in duplicate, with reference concentrations of amplicon on the same 96-well plate. Amplicon-specific standard curves were constructed using sense-strand oligonucleotides for each plate, and sample copy numbers of mRNA were calculated for each by reference to this standard curve[18].

## Immunohistochemistry

Tissue from 26 MSI-H tumours was available and 48 MSS tumours were chosen to reflect the proportion of right-sided tumours. Paraffin-embedded stored tissue was retrieved and fresh 3-μm sections were mounted on poly-lysine treated slides (BDH-Merck, Poole, UK). A previously described method was followed for staining of the IL-2Rα subunit in paraffin-embedded tissue[19]. Staining for CD3, CD4, CD8 and granzyme B was performed as follows. Sections were dewaxed by immersion in xylene twice, each for 2 min, and industrial methylated spirits twice, each for 2 min. Endogenous peroxidase was blocked by incubating sections in 1 per cent (v/v) hydrogen peroxide in methanol for 15 min. Sections were washed in tap water for 5 min then placed in 0·05 mol/l Tris-buffered saline (TBS) pH 7.6 for 5 min. Antigen retrieval and visualization was performed according to *Table 2*. TBS washes were included between steps. Washes for CD4 staining included Tween 20 (500 μl/l in TBS). All antibodies were visualized using 3,3′-diaminobenzidine as chromagen (Biogenex, San Ramon, California, USA) and counterstained with Meyer's haemulun (BDH-Merck). Sections were washed in tap water, dehydrated and mounted. For controls, paraffin-embedded appendiceal tissue was used. Negative controls omitted the primary antibody.

## Quantification of results and statistical analysis

Messenger RNA copy numbers for the T cell markers CD4 and CD8 were standardized to CD3 mRNA copy numbers. In immunohistochemical analysis, positively stained intraepithelial cells were counted, as improved prognosis in colorectal cancer has been associated specifically with intraepithelial lymphocytes rather than stromal or peritumoral lymphocytes[20]. Quantification was performed by observation of ten high-power (× 400) fields and taking mean values. Areas were quasirandomly sampled to include representative areas of solid tumour. One author analysed all slides. Some 20 per cent of slides were sampled randomly and reassessed independently by another observer. Both observers were blinded to microsatellite status.

Groups were compared using a non-parametric method, the two-tailed Mann–Whitney *U* test. Correlations between marker expressions were assessed by the non-parametric Spearman's rank test. Clinicopathological data were compared with an unpaired *t* test for mean age and contingency tables and a $\chi^2$ test for associations. Analyses were performed on GraphPad Prism® version 3.0 (GraphPad Software, San Diego, California, USA) and significance was taken at the 5 per cent level.

# Results

## Clinicopathological data

Microsatellite instability was detected in 26 (15·9 per cent) of 164 colorectal cancers using BAT-26 (*Table 3*). Two patients each had two MSI-H cancers. BAT-40 failed to identify any additional cases (data not shown). In this series of 164 colorectal cancers, 59 (36·0 per cent) were located

**Table 2** Antibodies and reagents used for immunostaining

| Antibody (clone) | Supplier | Dilution, time | Antigen retrieval and buffer | Visualization |
|---|---|---|---|---|
| CD3 | Dako | 1 : 400, 40 min | MW, 18 min<br>Citrate pH 6.0 | Vectastain Universal Elite® ABC kit |
| CD4 (IF6) | Novocastra | 1 : 60, 60 min | PC, 3 min<br>EDTA/water, pH 8.0 | Vectastain Universal Elite® ABC kit |
| CD8 | Dako | 1 : 100, 40 min | MW, 18 min<br>Citrate, pH 6.0 | Vectastain Universal Elite® ABC kit |
| IL-2Rα (ACT-1) | Dako | 1 : 1500, 15 min | 99°C, 25 min<br>Dako Target Retrieval Solution, pH 6.1 | Dako CSA system |
| Granzyme B | Dako | 1 : 75, 40 min | MW, 18 min<br>EDTA/water, pH 8.0 | Vectastain Universal Elite® ABC kit |

MW, microwave treatment at 650 W; PC, pressure cooker treatment; citrate buffer, citric acid 0·38 g/l, trisodium citrate 2·38 g/l, adjusted with sodium hydroxide; EDTA/water, EDTA (disodium salt) 0·38 g/l, adjusted with sodium hydroxide IL-2Rα, interleukin 2 receptor α-subunit. Dako, Dako A/S, Glostrup, Denmark; Novocastra, Novocastra Laboratories, Newcastle upon Tyne, UK; Vectastain Universal Elite®, Vector Laboratories, Burlingame, California, USA.

**Table 3** Population statistics for all patients and respective tumour samples

| | MSI-H | MSS |
|---|---|---|
| No. of patients | 24 | 138 |
| Sex ratio (M : F) | 9 : 15 | 79 : 59 |
| Age (years)* | 65·5(13·7) | 69·4(12·5) |
| No. of tumour samples | 26 | 138 |
| Location of tumour† | | |
| Right | 19 (73) | 40 (29·0) |
| Left | 7 (27) | 98 (71·0) |

*Values are mean(s.d); †values in parentheses are percentages. MSI-H, high-level microsatellite instability; MSS, all other phenotypes. Right, tumours from the caecum to the distal transverse colon; left, tumours at or distal to the splenic flexure.

**Table 4** Messenger RNA copy numbers for chosen markers and selected indices

| | Copy no. (per ng tumour RNA) | | |
|---|---|---|---|
| | MSI-H | MSS | *P** |
| CD3 | 3650 (14·8–30 700) | 4410 (1100–19 600) | 0·606 |
| CD4 | 7420 (36·8–133 000) | 2530 (105–31 800) | 0·661 |
| CD8 | 15 700 (660–124 000) | 12 400 (3810–81 200) | 0·105 |
| CD4/CD3 | 0·70 (002–380) | 0·67 (0·03–3·18) | 0·954 |
| CD8/CD3 | 4·20 (2·26–103) | 3·00 (1·05–6·13) | 0·016 |

Values are median (5th–95th percentiles). MSI-H, high-level microsatellite instability; MSS, all other phenotypes. *Two-tailed Mann–Whitney *U* test.

**Table 5** Immunohistochemistry counts for intraepithelial lymphocytes

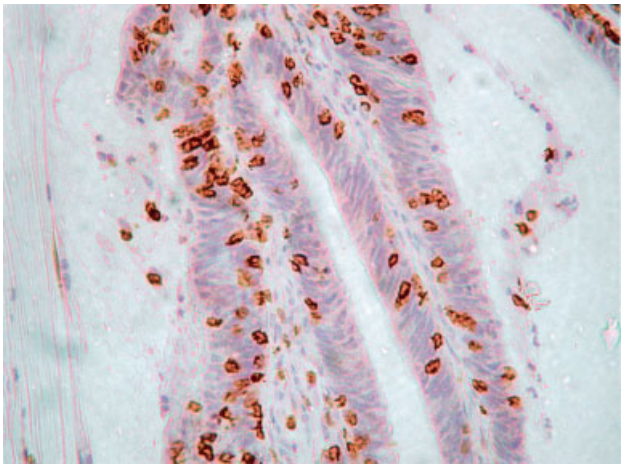
| | Counts per high-power field | | |
|---|---|---|---|
| | MSI-H | MSS | *P** |
| CD3 | 14·6 (1·0–58·5) | 3·9 (0·1–21·8) | 0·003 |
| CD4 | 3·9 (1·0–41·3) | 3·1 (0·2–20·4) | 0·212 |
| CD8 | 18·8 (0·7–139) | 3·3 (0·6–19·0) | 0·008 |
| IL-2Rα | 1·5 (0·3–9·5) | 1·1 (0·1–2·7) | 0·017 |
| Granzyme B | 23·5 (5·2–78·0) | 6·3 (1·0–24·2) | 0·020 |

Values are median (5th–95th percentiles). MSI-H, high-level microsatellite instability; MSS, all other phenotypes. IL-2Rα, interleukin 2 receptor α-subunit. *Two-tailed Mann–Whitney *U* test.

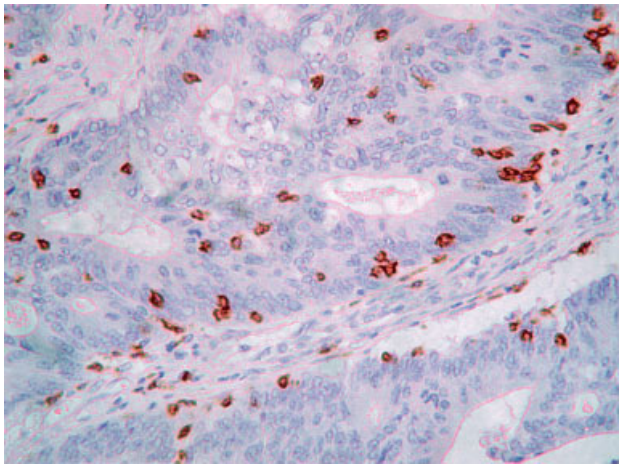
proximal to the splenic flexure and MSI-H was found in 19 (32 per cent) of these tumours ($P < 0·001$). The MSI-H population was younger, probably owing to the inclusion of patients with HNPCC, and two-thirds were women, but these trends were not significant.

## Reverse transcriptase–polymerase chain reaction

Comparison of absolute values for mRNA copy numbers between samples is restricted by lack of ability to control for cellular constituents. Although analysis of frozen sections has confirmed a tumour epithelial proportion of 80–90 per cent, RNA extraction is affected by sample heterogeneity between cases. This variability was allowed for by using ratios of mRNA copy numbers. The number of mRNA copies of T cell subset markers CD4 and CD8 was standardized to that of CD3. There were significant differences in CD8/CD3 ratios between MSI-H and MSS tumours (*Table 4*).

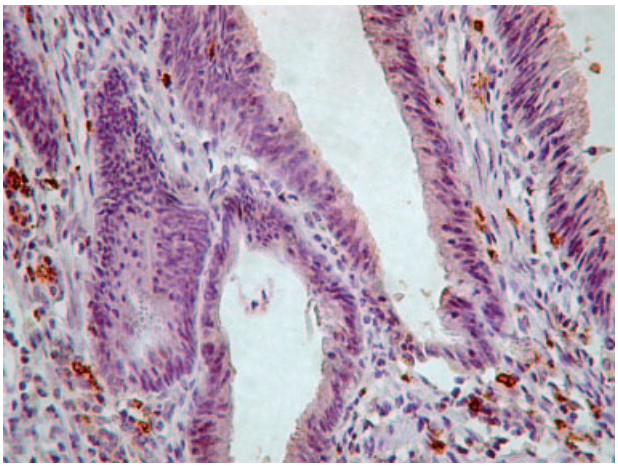


**a** MSI-H

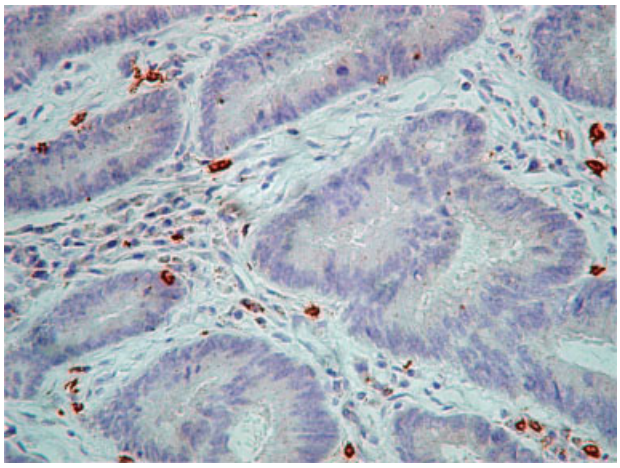


**b** MSS

**Fig. 1** Immunohistochemical staining for CD8 antibodies on intraepithelial lymphocytes in colorectal cancers **a** with high-level microsatellite instability (MSI-H) and **b** all other phenotypes (MSS) (Meyer's haemulun counterstain, original magnification × 400)



**a** MSI-H



**b** MSS

**Fig. 2** Immunohistochemical staining for interleukin 2 receptor α antibodies on intraepithelial lymphocytes in colorectal cancers **a** with high-level microsatellite instability (MSI-H) and **b** all other phenotypes (MSS) (Haematoxylin counterstain, original magnification × 400)

## Immunohistochemistry

Clear differences in immunohistochemical staining were noted between MSI-H and MSS tumours (*Table 5*). MSI-H tumours showed significantly higher counts for CD3-positive and CD8-positive cells (*Fig. 1*), but no differences were found in CD4 counts. IL-2Rα (*Fig. 2*) and granzyme B levels were also significantly higher in MSI-H tumours. Overall CD3 expression correlated most strongly with CD8 expression ($r_s^2 = 0{\cdot}64$) and, similarly, IL-2Rα expression correlated most strongly with CD8 expression ($r_s^2 = 0{\cdot}70$). The correlation between IL-2Rα and CD4 was relatively poor ($r_s^2 = 0{\cdot}24$).

To assess variability, interobserver scores were analysed by method comparison analysis and agreed within 5 per cent[21].

## Discussion

This analysis revealed microsatellite instability in 15·9 per cent of 164 consecutive colorectal cancers. There was a significant association with the right side of the colon, consistent with accepted figures and indicating that the choice of microsatellite marker is valid. Since the International Collaborative Group tentatively endorsed the division of microsatellite status into three groups (MSI-H, low-level microsatellite instability (MSI-L) and MSS) in 1997, several studies have highlighted the biological distinction between MSI-H and the two other groups[22,23]. However, clinicopathological differences between MSI-L and MSS cancers are not well defined, so MSI-L was not defined separately in this study but simply included in the MSS group. The BAT-26 marker has been shown to be highly specific for the MSI-H phenotype and consistent with larger panels, including dinucleotide markers[24–26]. Several studies have highlighted the value of BAT-26 as a marker for MSI-H, and inclusion of BAT-40 revealed no additional benefit in terms of sensitivity.

In this study MSI-H colorectal cancers displayed significantly higher immunostaining of CD3 and CD8 T lymphocyte markers. The significant correlation between CD8 and CD3 markers confirmed that the lymphocyte infiltrate was predominantly cytotoxic (CD8 positive), as shown in previous work[11]. Notably the mRNA analysis confirmed this finding by demonstrating significantly increased CD8/CD3 mRNA levels.

Immunohistochemical staining of paraffin-embedded colorectal tissue for IL-2Rα demonstrated that this marker of activation was also raised in MSI-H tumours. IL-2Rα is expressed by a variety of immune cells other than cytotoxic lymphocytes. Indeed, a subset of CD4-positive cells that expresses high levels of IL-2Rα appears to have immune suppressive actions[27]. However, expression of IL-2Rα correlated more strongly with CD8-positive lymphocytes, whereas correlation with CD4-positive lymphocytes was relatively poor. This suggests that the population of T cells was dominated by activated cytotoxic CD8-positive IL-2Rα-positive lymphocytes rather than regulatory CD4-positive IL-2Rα-positive lymphocytes.

There were no significant correlations between T cell marker mRNA copy numbers and immunohistochemical counts in these specimens (data not shown). This may be explained by the fact that there are obvious and significant differences between the two techniques.

Immunohistochemistry on paraffin sections allows global assessment of a tumour sample and is, at best, semiquantitative. The mRNA analysis is quantitative but is performed on only a tiny fraction of the tumour sample. The use of ratios of marker copy numbers corrects for tumour heterogeneity but there remain clear sampling differences. These differences may contribute to the discrepancy seen between levels of protein and mRNA detection. In addition, mRNA analysis makes no differentiation between all lymphocytes in the sample whereas only intraepithelial lymphocytes were counted in the immunohistochemical analyses. Despite these limitations, both techniques provided evidence for greater activation of a predominantly cytotoxic T cell infiltrate in MSI-H colorectal cancers.

The mutator phenotype characteristic of MSI-H produces mutated peptides that may act as tumour-specific antigens[28,29]. For an antigen-specific response such antigens must be presented in an HLA-restricted manner, but the higher frequency of mutations in β2-microglobulin, necessary for major histocompatibility complex class I presentation, in MSI-H tumours may preclude direct antigen presentation in these cancers[30,31]. However, cross-priming of dendritic cells by released intracellular antigens may provide an alternative pathway of immune stimulation[32]. Increased expression of HLA-DR has been demonstrated in these tumours[33] and HLA-DM, involved in endosomal processing during class II presentation, is also overexpressed in MSI-H[34]. Thus, activation of the helper arm of the immune system may have a pivotal role, and interaction between the helper and killer arms of the immune system is well recognized. Increased IL-2Rα expression might be a manifestation of this interaction.

There is now considerable evidence that MSI-H colorectal cancer represents a potential *in vivo* model of a host immune response to malignant cells. It is attractive to propose that such a reaction is responsible for the improved prognosis in patients with these tumours. Whether this immune activity is indeed driven by tumour-specific antigens remains to be determined. Further studies of MSI-H colorectal cancer might yield vital knowledge regarding this model, leading to significant improvements in understanding of host–tumour interactions and improvement of current immunotherapeutic strategies.

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