Molecular pathology of mismatch repair deficient tumours with emphasis on immune escape mechanisms

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Frequent mutations in the 3' untranslated region of *IFNGR1* lack functional impairment in microsatellite-unstable colorectal tumours

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1. ABSTRACT

Microsatellite repeats are frequently found to be mutated in microsatellite-instable colorectal tumours. This suggests that these mutations are important events during tumour development. We have observed frequent mutations in microsatellite-instable (MSI-H) tumours and cell lines of a conserved A14 repeat within the 30-untranslated region of the *interferon γ receptor 1 gene (IFNGR1)*. The repeat was mutated in 59% (41 of 70) of colon carcinomas and in all four MSI-H colon cancer cell lines tested. In-vitro analysis of these cell lines did not show a decreased responsiveness to standard IFNγ concentrations when compared to microsatellite-stable colon cancer cell lines. A functional consequence of the frequently found microsatellite instability in *IFNGR1* is therefore not evident.

2. INTRODUCTION

DNA mismatch repair deficiency in tumours is characterized by a high frequency of microsatellite instability (MSI-H)[1]. Microsatellite instability is associated with the hereditary non-polyposis colorectal cancer syndrome and also appears in approximately 15% of sporadic colorectal tumours [2]. A subset of genes that encompass coding microsatellites may be specifically targeted in MSI-H tumours. Systemic sequence database searches have identified in the human genome up to 17.000 intra-exon coding repeat sequences with a length of six or more nucleotides [3,4].

Frameshift mutations in MSI-H colorectal tumours have been demonstrated for genes involved in signal transduction (*TGFBRII*, *IGFIIR*, *PTEN*), apoptosis (*BAX*, *CASPASE 5*), DNA repair (*hMSH3*, *hMSH6*, *MBD4*), transcriptional regulation (*TCF-4*) and immune surveillance (*B2M*), with mutation frequencies ranging from 4 to 80% [5]. Non-coding microsatellite repeats, however, may just as well be mutated. Of those with potential functional significance are those that lie within the untranslated regions (UTR) of genes. Elements in such regions can regulate mRNA degradation, translation and localization [6–8]. However, such elements have not been extensively studied for and their identification is complicated by the fact that their activity often depends on specific secondary RNA structures [9]. Suraweera et al [10] characterized frequent mutations in a number of conserved mononucleotide repeats within UTRs and high levels of mutations were found in a novel T25 mononucleotide marker in the 3' UTR of the *CASP2* gene [11].

Interferon γ (IFNγ) is important in regulating cell mediated immune responses. It directly increases the sensitivity of a target cell to CD8+ cytotoxic lymphocyte attack by upregulating the expression of a variety of genes, including the *human leukocyte antigen (HLA) class I genes* and *Fas/CD95* [12]. Mismatch repair-deficient tumours potentially exhibit a large number of tumour-specific 'frameshift' antigens due to instability of coding microsatellite sequences. This has been suggested to render them more susceptible to both native and therapeutically induced antitumour immune responses [13]. Indeed, these tumours are associated with an elevated CD8+ intra-epithelial infiltrate [14,15]. Tumour IFNγ responsiveness is important in regulating anti-tumour immune response in vivo, partly depending on antigen presentation [16,17]. Downregulation of the IFNγ receptor has been shown in hepatocellular carcinoma [18], basal cell carcinoma [19] and virus-associated tumours [20,21], and was correlated with larger

tumour size and a higher frequency of metastases [18]. IFNγ also increases the sensitivity to chemotherapy-induced Fas-mediated apoptosis [22]. Although the use of IFNγ solely has yielded somewhat disappointing results in clinical anticancer treatment trials [23], its use in combination with different chemotherapeutics, such as 5-fluorouracil, indomethacin, phenyl butyrate, mitomycin C, cyclosporine A or clarithromycin has shown synergetic anti-tumour effects [24 – 30]. Thus, loss of IFNγ responsiveness would denote a loss of potential for future immuno- and chemo-based adjuvant therapies.

In this study, we describe frequent mutation of the A14 repeat within the 3' UTR of *IFNGR1*. As will be discussed, it is difficult to draw conclusions regarding its importance on basis of frequencies alone. Therefore, we also analysed IFNγ responsiveness of colon cancer cells bearing these mutations.

3. MATERIALS AND METHODS

3.1. Colon tumour samples and cell culture

Tumour samples were derived from formalin-fixed, paraffin embedded material sent to our department for MSI analysis between 1999 and 2002. Cases were analysed following the medical ethnical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences. The present study falls under approval by the Medical Ethical Committee of the LUMC (protocol P01–019).

Cell lines HCT 15, LoVo, LS180, LS411N, SW480 and SW837 were obtained from the American Type Culture Collection (Atlanta, GA, USA) and cultured and harvested according to standard methods.

3.2. Microsatellite instability and sequence analysis

MSI and sequence analyses were performed as described previously [31]. MSI analysis was performed using the NCI markers BAT25, BAT26, D2S123, D5S346, D17S250 [32], supplemented with BAT40, *MSH3* and *MSH6*. Tumours were classified as MSI-H (instability of at least 30% of the markers) or MSS (no instability). IFNGR1 was analysed using forward primer 50-GAGGATGTGTGGCATTTTCA-30 and reverse primer 50-TGCTATACCAAGGCAGAGAAAAG-30.

3.3. IFNGR1 mRNA expression

Total RNA was isolated using TriZol (Ambion, Austin, TX, USA) following the manufacturer's recommendations. RNA (2 mg) was used for cDNA synthesis using Oligo dT primers and AMV Reverse Transcriptase (Roche Applied Science, Penzberg, Germany). We performed quantitative real-time PCR using SYBR Green (Eurogentec, Seraing, Belgium) on an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Ct values were calibrated to an amount of Human Total Colon RNA (Clontech, BD Biosciences, Palo Alto, CA, USA) and normalized to the level of expression of three stably expressed housekeeping genes *HNRPM*, *CPSF6* and *TBP* [33]. Calibrated Ct values were divided by Ct values of 2 mg Human Total Colon RNA. Primers for amplification of IFNGR1 mRNA used were 50-TCCTCAGTGCCTACACCAACTAA-30 (nucleotides 79–101, exon 1–2), and 50-CTCGTCACAATCATCTTCCTTCTG-30 (nucleotides 568–591, exon 5).

3.4. Flow cytometry of IFNγ-stimulated cells

HLA I and Fas expression was analysed by flow cytometry of viable cells as described previously [34,35] using the primary antibodies W6/32 (supernatant), 2R2 (Alexis Biochemicals,

Carlsbad, CA, USA), and mouse isotype controls (Dako Cytomation, Glostrup, Denmark). Samples containing 1 mM propidium iodide (Calbiochem, San Diego, CA, USA) were collected on an FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Linearized mean fluorescence intensity values were determined by WinList (Verity Software House Inc., Topsham, ME, USA) and subtracted by control values. Means derived from duplicate experiments were used; single experiments encompassed one-session analyses of all cell lines using the same instrument settings. We constructed a dose–response curve using 8, 40, 200 and 1000 U/ml IFNγ (PeproTech Inc., Rocky Hill, NJ, USA) for 48 h. All cell lines were already maximally responsive to a concentration of 8 U/ml. The extent of upregulation was further analysed using 200 U/ml IFNγ.

3.5. Fas-induced apoptosis

To quantify Fas-induced apoptosis using 1 mg/ml 2R2 (Alexis Biochemicals) and 1 mg/ml protein A for 6 h, percentages of apoptotic cells were determined using the Nicoletti assay as described previously [22,36]. For analysis we used a modified ModFit algorithm previously described [37] and kindly provided by Verity Software House Inc. Means derived from duplicate experiments were used.

4. RESULTS AND DISCUSSION

Frequently found MSI suggests a clonally selective advantage, although passenger mutations due to a possible lack of selection pressure of innocent bystander genes, ie genes whose function is irrelevant during tumourigenesis, cannot be excluded [38]. For *TCF-4*, it has been shown that frameshift mutations due to instability of a coding A9 repeat lack functional consequences [39]. Therefore, comprehensive criteria have been proposed at the Bethesda meeting to define valid target genes in MSI-H tumours, including (1) a high frequency of mutation, (2) bi-allelic inactivation, (3) involvement in a tumour suppressor pathway, which is (4) also involved in MSS tumours and (5) functional consequences of the mutation [2]. However, few reports have taken these criteria into account [40,41] and statistical methods have been proposed to identify valid target genes by statistical analysis of mutation frequencies only [42,43].

To examine the (in)stability of the *IFNGR1* repeat we first analysed 17 MSS colon cancer samples and 2 MSS colon cancer cell lines (SW480 and SW837). In all cases the repeat was conserved, ie monomorphic [10]. Subsequently, 70 MSI-H colon tumour specimens were tested from patients complying with the clinical Bethesda criteria [1], 38 of whom a germ line mutation was identified in one of the MMR genes. MSI in the repeat was found in tumours from 12 of 20 (60%) *hMLH1* patients, 6 of 12 (50%) *hMSH2* patients, 2 of 6 (33%) *hMSH6* patients, in 21 of 32 (66%) of the residual MSI-H tumours and in 4 of 4 (100%) MSI-H colon cancer cell lines.

The frequency of mutations in microsatellite tracts is associated with the number and type of repeats [32,44]. Previous studies reported a mutation frequency up to 54% in 29 mononucleotide repeats (8–10 bp) within intronic sequences;38 in conserved repeats instability was found in up to 28% of tumours only [42]. Furthermore, G-repeats are considerably more prone to mutations than A-repeats [38]. In 19 conserved UTR repeats studied [10] a mutation frequency was found up to 95%, but the repeat lengths ranged from 15 up to 32 nucleotides. Led by the Bethesda criteria [2] we decided to study whether 59% MSI in the A14 repeat is truly indicative of a valid target.

First, we studied bi-allelicity of the mutations. The microsatellite analysis used cannot discriminate between mono- or bi-allelic mutations of tumour tissue due to contaminating normal cells. Therefore, we analysed the cell lines described above and performed sequence analysis for confirmation of the observed deletions. A bi-allelic A3 deletion was detected in LoVo and LS411N, in LS180 both an A4 and an A5 deletions were seen and in HCT 15 only a mono-allelic deletion of a single A was observed. No mutations in the MSS cell lines SW480 and SW837 were observed in line with the MSI results.

To detect a possible decline in IFNGR1 mRNA expression upon 3′ UTR mutations we applied quantitative RT-PCR. The receptor is normally expressed on colon epithelium [45] and no decreased IFNGR1 mRNA expression of the MSI-H colon cancer cell lines was observed when compared to the MSS cell lines (Table 1).

To rule out other regulatory effects of the 3′ UTR mutation we investigated the extent of IFNγ-mediated HLA class I and Fas/CD95 upregulation in MSI-H compared to MSS colon cancer cell lines. HLA class I molecules are not present on the cell surface of LoVo and HCT 15 cells, due to mutations of the light chain *β2-microglobulin* [46]. We analysed dose–response curves to make sure the experiments were performed under conditions leading to maximum responsiveness. No decreased responsiveness to IFNγ was observed for MSI-H cell lines compared to the MSS cell lines (Table 1). To study the effect of IFNγ on Fas-induced apoptosis, we measured the fraction of apoptotic cells as quantified by flow cytometry. Again, no impaired IFNγ response was noted in the MSI-H cell lines (Table 1).

Although, the concentrations used are similar to other studies on in-vitro IFNγ responses in colon cancer cell lines [47], we cannot completely rule out a differential response to far lower concentration of IFNγ not yielding maximum response.

In conclusion, we identified a conserved A14 microsatellite repeat within the 3′ UTR of the potential tumour suppressor *IFNGR1* that is mutated in 59% of MSI-H colorectal tumours. Compared to MSI of other conserved, non-coding but transcribed short mononucleotide tracts, the high frequency of instability suggests it to be a valid target during colorectal tumourigenesis. However, *in vitro* analysis did not show an impaired response to IFNγ in any cell line harbouring the mutated gene questioning

		HLA I			*Fas*			*Fas-ind. apoptosis*		
	mRNA	-	+	*f.i.*	-	+	*f.i.*	-	+	*f.i.*
MSS										
SW480	0,1	442,3	1801,8	4,1	28,6	30,3	1,1	17,1	34,1	2,0
SW837	1,1	657,0	1517,4	2,3	16,6	25,6	1,5	11,8	40,5	3,4
MSI-H										
HCT-15	1,0	ne	ne	ne	22,6	32,5	1,4	8,9	85,8	9,6
LoVo	0,9	ne	ne	ne	36,0	22,3	0,9	67,7	81,7	1,2
LS411N	1,3	60,7	375,5	6,2	5,1	22,3	4,4	11,7	61,6	5,3
LS180	1,0	210,6	369,5	1,8	25,0	51,7	2,1	29,2	80,9	2,8

Table 1 IFNγ receptor expression and IFNγ responsiveness of colon cancer cell lines HLA I Fas Fas-induced apoptosis. Abbreviations: f.i., fold increase; NE, not expressed. Displayed are normalized Ct values as derived from IFNGR1 qRT-PCR, fluorescence intensity values of HLA class I and Fas surface expression acquired by flow cytometry and percentages of apopotopic cells after incubation with Fas as determined by propidium iodide staining. + and-: with or without IFNγ stimulation.

the functional consequences of MSI in this repeat. High mutation rates as a consequence of unidentified sequence-dependent factors, chromatin structure or mechanisms associated with transcription may contribute to the high frequency of mutations found in tumours in the absence of selective advantage [38,40,48]. Studying functional consequences seems indispensable to describe the role of these mutations in tumourigenesis rather than the frequency of genomic instability per se [49,50]. Despite a high frequency of MSI of the 3' UTR of *IFNGR1*, it probably does not contribute to colorectal tumour progression.

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