

GASTROINTESTINAL CANCER

Transient mismatch repair gene transfection for functional analysis of genetic hMLH1 and hMSH2 variants

A Brieger, J Trojan, J Raedle, G Plotz, S Zeuzem

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See end of article for authors' affiliations

Correspondence to:
Dr S Zeuzem, Medizinische Klinik II, Zentrum der Inneren Medizin, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt a M, Germany;
Zeuzem@em.uni-frankfurt.de

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Background: Germline mutations in the mismatch repair (MMR) genes *hMLH1* and *hMSH2* can cause hereditary non-polyposis colorectal cancer (HNPCC). However, the functional in vitro analysis of *hMLH1* and *hMSH2* mutations remains difficult.

Aims: To establish an in vitro method for the functional characterisation of *hMLH1* and *hMSH2* mutations.

Methods: *hMLH1* and *hMSH2* wild type (wt) genes and several mutated subclones were transiently transfected in mismatch repair deficient cell lines (HCT-116 and LOVO). Apoptosis, proliferation, and regulation of mRNA expression and protein expression of interacting proteins were analysed by Hoechst staining, AlamarBlue staining, real time polymerase chain reaction, and western blotting, respectively.

Results: The protein expression of *hMLH1* and *hMSH2* mutants was significantly decreased after transfection compared with wild type transfections. The *hMLH1* and *hMSH2* interacting proteins hPMS2 and hMSH6 became detectable only after transfection of the respective wild type genes. In parallel, *hMSH6* mRNA levels were increased in *hMSH2* wt transfected cells. However, *hPMS2* mRNA levels were independent of the mutation status of its interacting partner *hMLH1*, indicating a post-transcriptional regulating pathway. In the *hMLH1* deficient HCT-116 cell line apoptosis was not affected by transfection of any mismatch repair gene, whereas complementation of *hMSH2* deficiency in LOVO cells increased apoptosis. Conversely, proliferative activity of HCT-116 was decreased by complementation with *hMLH1*wt and unaffected in *hMSH2* deficient LOVO cells.

Conclusion: These data show that the cellular role of the MMR genes and its mutations are assessable in a simple transient transfection system and show the influence of MMR gene regulation on major cell growth regulating mechanisms. This method is applicable for the functional definition of mutations in *hMLH1* and *hMSH2* genes observed in patients with suspected HNPCC.

Mismatch repair (MMR) is an important cellular pathway that facilitates genome stability by excising mismatched nucleotides of the DNA. Germline mutations in two human MMR genes, *hMSH2* and *hMLH1*, account for approximately 98% of hereditary non-polyposis colorectal cancer (HNPCC) cases.¹ *hMSH2*, a homologue of *Escherichia coli* MutS protein, is required for mismatch recognition.² Heterodimers formed between *hMSH2* and substrate specificity modifying MutS homologues (*hMSH3* and *hMSH6*) are essential for the following repair of mismatches. *hMutS α* , the heterodimer of *hMSH2* and *hMSH6* is known to recognise base-base mispairs, or single insertion/deletion loops, while *hMutS β* , the heterodimer of *hMSH2* and *hMSH3* is primarily involved in the correction of larger DNA insertion/deletion loops.^{3,4} For the DNA repair process, activated *hMutS α* or *hMutS β* interact with *hMLH1*, the human homologue of the *E coli* MMR gene *MutL*.^{5,6} In addition to *hMLH1*, other human *MutL* homologues have been identified (*hMLH3*, *hPMS1*, and *hPMS2*). While interactions between *hMLH1*, *hMLH3*, and *hPMS1* have been reported,⁷⁻⁹ only the *hMLH1/hPMS2* heterodimer has been shown to participate in mismatch repair.¹⁰

Recently, specific mutational inactivation of *hMLH1* and *hMSH2* leading to post-translational downregulation of the heterodimerising partners was proposed.¹¹ Furthermore, MMR proteins were suggested to be involved in the regulation of apoptosis and proliferation.¹²⁻¹⁴ In these studies, however, different experimental models such as transfection of whole chromosomes and co-microinjection of expression plasmids and GFP vectors were used. The interpretation of these data remain difficult because effects caused by multiple gene

expression and accumulation of multiple defects in different tumour cells cannot be excluded. No data have yet been reported determining the different cellular roles of *hMLH1* and *hMSH2* in one comprehensive system. Moreover, the clinical role of specific MMR mutations in the progression of disease and for the efficacy of chemotherapy is widely unclear. Thus, the functional analysis of MMR mutations may help to define the clinical significance of mutations.

In this study we expressed *hMLH1* wild type (*hMLH1* wt), *hMSH2* wild type (*hMSH2* wt), and several mutants in a well defined transient transfection system to investigate the interaction of *hMSH2/hMSH6* and *hMLH1/hPMS2* as well as the functional role of *hMLH1* and *hMSH2* in apoptosis and proliferation.

METHODS

Cell lines and cultures

The cell lines used in this study were purchased from the American Type Culture Collection (ATCC). HCT-116 and LOVO cells were cultured in a humidified atmosphere containing 5% carbon dioxide in McCoy's 5A (Gibco, Karlsruhe, Germany) and RPMI 1640 medium (Gibco), respectively. All media contained 10% fetal calf serum (Gibco).

Abbreviations: HNPCC; hereditary non-polyposis colorectal cancer; MMR, mismatch repair; PCR, polymerase chain reaction; PBS, phosphate buffered saline

Table 1 Primer for real time PCR. Probes for detection were labelled with FAM (6-carboxy-fluorescein), TAMRA (6-carboxy-N,N,N,N-tetramethylrhodamin) or VIC (fluorescein derivat)

Primer	Sequence (5' → 3')
hMSH6-probe	FAM -CAGGAGCTTTTATCAATGGCTA- TAMRA
hMSH6-Taq-S	TCTAGGTGGTTGTCTCTTACCTCA
hMSH6-Taq-AS	TAGTGCTGACTGTGTCAGAATCCA
hPMS2-probe	FAM -ACTGCTCTAACACAAGCGAGATGAAGAA- TAMRA
hPMS2-Taq-S	CGGAAGTCGGTGATGATTGG
hPMS2-Taq-AS	TTGGCGATGTGTCATGGTT
GAPDH-probe	VIC -CAAGCTTCCCCTTCTCAGCC- TAMRA
GAPDH-S	GAAGGTGAAGGTCGGAGTC
GAPDH-AS	GAAGATGGTGATGGGATTTC

S, sense; AS, antisense.

Construction of hMLH1 and hMSH2 mutants

The complete wild type cDNA for *hMSH2* was subcloned from laboratory isolates into the pcDNA3.1+ vector (Invitrogen, Groningen, Netherlands), placing the cDNA under the control of the CMV promoter. Inserts for *hMSH2* wt cDNA were amplified using primers bearing BamHI (sense) or XhoI (antisense) restriction sites and ligated in the appropriate sites of the vector. The pcDNA3.1+ vector containing the cDNA of *hMLH1* was kindly provided by Dr Hong Zhang (University of Utah, Salt Lake City, Utah). The expression constructs for the mutant proteins were designed using the corresponding pcDNA3.1+ wild type vectors (*hMLH1* or *hMSH2*) and site directed mutagenesis (Promega, Madison, UK). The following cDNA-mutant constructs were generated: *hMLH1* Thr117Met, *hMLH1* Lys618Thr, *hMSH2* DEL782FS, and *hMSH2* Cys697Arg, representing four mutations of patients with HNPCC identified in our clinic. Expression vectors containing the genes of interest were confirmed by sequencing and transcription/

translation (Promega). Proteins were detected by appropriate monoclonal antibodies (anti-hMLH1, clone G168-728, PharMingen, Stuttgart, Germany; anti-hMSH2, Ab2, Calbiochem, La Jolla, CA).

Transcription/translation of plasmids

Protein production of the used plasmids was verified by the in vitro TNT Quick Coupled Transcription/Translation system (Promega).

Preparation of cells for transfection

HCT-116 and LOVO cells were grown to 50%–80% confluency and subsequently transfected with 2 µg Genomed purified plasmid DNA (Genomed, Bad Oeynhausen, Germany) mixed with 6 µl FuGENE 6 (Boehringer Mannheim, Mannheim, Germany) and with 2 µg plasmid DNA mixed with 7.9 µl Tfx-20 reagent (Promega), respectively. pcDNA3.1+ vector without insert was transfected in both cell lines as mock control.

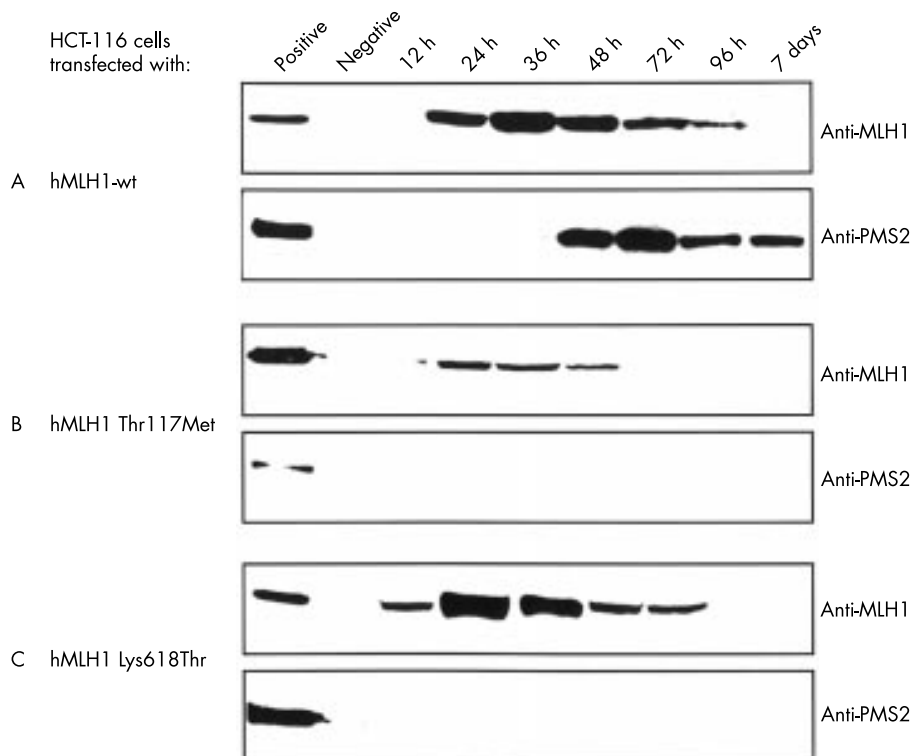


Figure 1 Expression of hMLH1 in human colorectal cancer cell line HCT-116. HCT-116 cells were transiently transfected either with pcDNA3.1+ vectors expressing hMLH1 wt (A), hMLH1 Thr117Met (B), hMLH1 Lys618Thr (C), or pcDNA3.1+ vector without insert (negative control). Total extracts of transiently transfected HCT-116 cells (20 µg/lane) were separated on a 7.5% SDS-PAGE and blotted onto PVDF membranes. The transferred proteins were visualised with either anti-hMLH1 antibody (upper lane) or anti-hPMS2 antibody (lower lane). *hMLH1* proficient LOVO cells were used as positive control. Data shown are representative of four independent experiments.

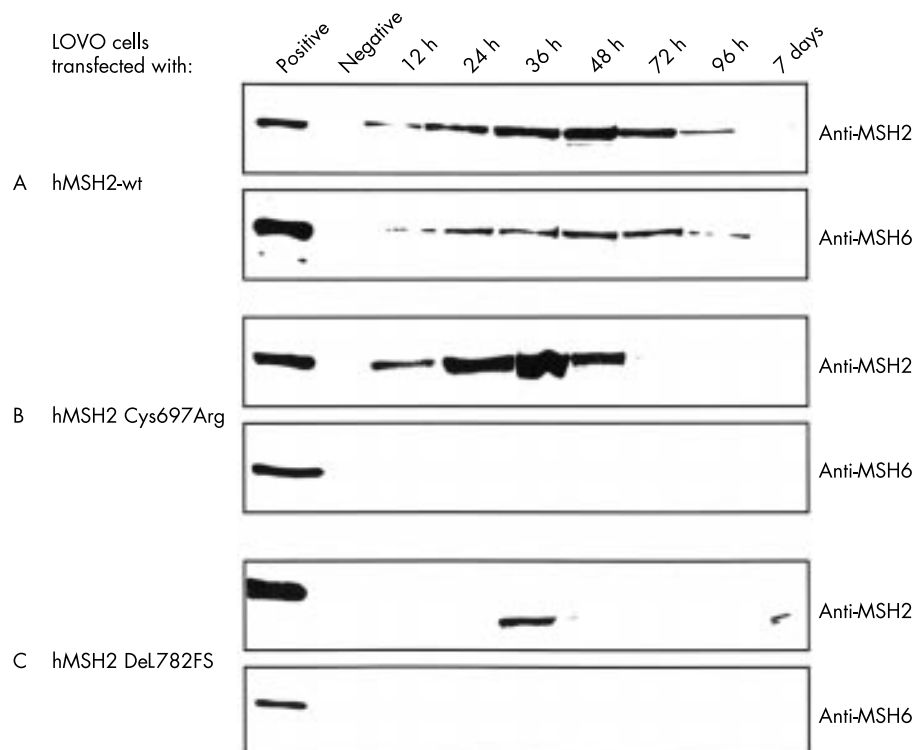


Figure 2 Expression of hMSH2 in human colorectal cancer cell line LOVO. LOVO cells were transiently transfected either with pcDNA3+ vectors expressing hMSH2 wt (A), hMSH2 Cys697Arg (B), hMSH2 DEL782FS (C), or pcDNA3+ vector without insert (negative control). Total extracts of transiently transfected LOVO cells (50 µg/lane) were separated on a 7.5% SDS-PAGE and blotted onto PVDF membranes. The transferred proteins were visualised with either anti-hMSH2 antibody (upper lane) or anti-hMSH6 antibody (lower lane). hMSH2 proficient HCT-116 cell were used as positive control. Data shown are representative of four independent experiments.

Western blotting and immunoprecipitation

Proteins were separated on denaturing 7.5% SDS-polyacrylamide gels, blotted onto PVDF membranes, and detected with the ECL-Kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The purified mouse antihuman antibodies used for western blotting were the monoclonal anti-hMLH1 (Clone: G168-728, Pharmingen), the monoclonal anti-hPMS2 (Clone: A16-4, Pharmingen), the monoclonal anti-hMSH2 (Clone 27, Transduction Laboratories), and the monoclonal anti-GTBP (hMSH6, Clone: 44, Transduction Laboratories). Immunoprecipitation was carried out using a cellular labelling and immunoprecipitation kit (Boehringer Mannheim). The resulting precipitate was subsequently analysed by SDS-PAGE (7.5%).

Immunofluorescence

Cells were washed with phosphate buffered saline (PBS) 12, 24, 36, 48, 72, 96 hours, and seven days after transfection and fixed in 100% prechilled acetone overnight at -20°C . Fluorescence microscopy (Axiovert 135, Zeiss, Jena, Germany) was performed after incubation of the cells for five minutes with blocking buffer (PBS, 1% BSA, 0.2% Tween 20, 5 mM TRIS/HCl, pH 7.6), with the indicated primary antibody in blocking buffer for one hour at 37°C , and the corresponding Cy³ conjugated secondary antibody (antimouse IgH/L, Jackson Laboratories, UK) at room temperature for 30 minutes in the dark.

Quantitative PCR analysis of hPMS2 and hMSH6 mRNA

Total RNA was extracted from transfected HCT-116 and LOVO cells using Tristar (AGS GmbH, Heidelberg, Germany). First strand cDNA was prepared from total cellular RNA using random hexadeoxynucleotide primers and reverse transcriptase (SuperScriptII, Gibco). PCR was performed in a volume of 50 µl containing 50 mM KCl, 10 mM TRIS-HCl, 0.01 mM EDTA and 60 nM ROX (6-carboxy-x-rhodamine), 6 mM MgCl₂,

400 µM of dATP, dCTP, dGTP and dTTP each, 100 nM of primers each (table 1), 100 nM of probe and 1.25 U of AmpliTaq Gold DNA polymerase. Thermal cycling involved preincubation at 95°C for 12 minutes, followed by 50 cycles at 95°C for 20 seconds and 55°C for 20 seconds. Fluorescence was measured real time during PCR (Taq-Man-PCR, Applied Biosystems, Weiterstadt, Germany). Emission ranges of both fluorescent dyes were detected independently by wavelength in the Perkin-Elmer 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). The relative hPMS2 and hMSH6 RNA quantification values were obtained from the threshold cycle number from which the increase in signal associated with an exponential growth of PCR product became detectable (Sequence detection system software version 1.6, Applied Biosystems). The resulting $\Delta\Delta\text{CT}$ -factor enables relative quantification of the RNA of interest (Sequence Detector User Bulletin, Applied Biosystems). For quantification normalised to an endogenous control, standard curves were prepared for both the target (hPMS2 and hMSH6) and the endogenous reference (GAPDH). For calibration hPMS2 RNA and hMSH6 RNA were prepared from mismatch repair proficient colorectal tumour cells (SW 480). Quantitative PCR was performed in duplicate for each primer set and the mean of the two experiments was used as relative quantification value. Contamination precautions according to Kwok *et al* were strictly complied.¹⁵

Apoptosis

Air dried cells were fixed 12, 36, 48, and 72 hours after transfection with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 minutes at room temperature, washed with PBS and incubated with permeabilisation solution (1% Triton X-100, 0.1% sodium citrate in PBS) for two minutes at 4°C . Fluorescence microscopy (Axiovert 135, Zeiss) was performed after washing with PBS and staining with the

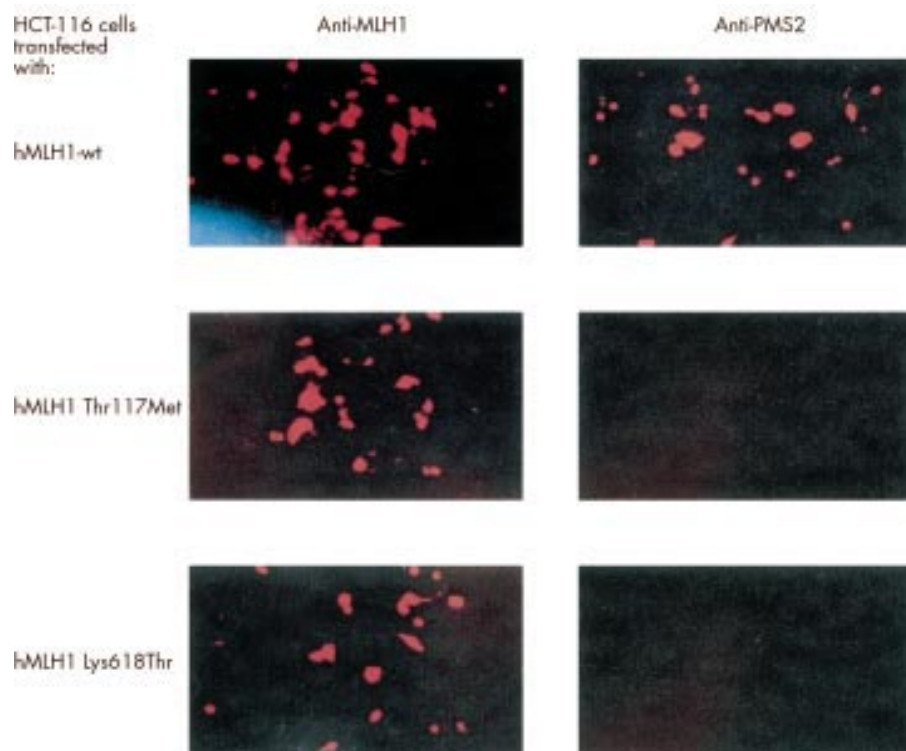


Figure 3 Immunofluorescence of hMLH1 deficient tumour cell line HCT-116 after transfection with hMLH1 wt, hMLH1 Thr117Met, or hMLH1 Lys618Thr. Protein expression after 48 hours was detected using anti-hMLH1 (left) or anti-hPMS2 antibody (right). Data shown are representative of four independent experiments.

DNA specific dye Hoechst 33342 (Sigma, Deisenhofen, Germany) in PBS (10 µg/ml).¹⁶ The intercalation of Hoechst 33342 in the DNA allows to distinguish compressed apoptotic from intact DNA.

Proliferation

Proliferation of transiently transfected HCT-116 and LOVO cells was quantified using the AlamarBlue Assay (Serotec, Oxford, Kidlington, UK). The assay works analogous to the

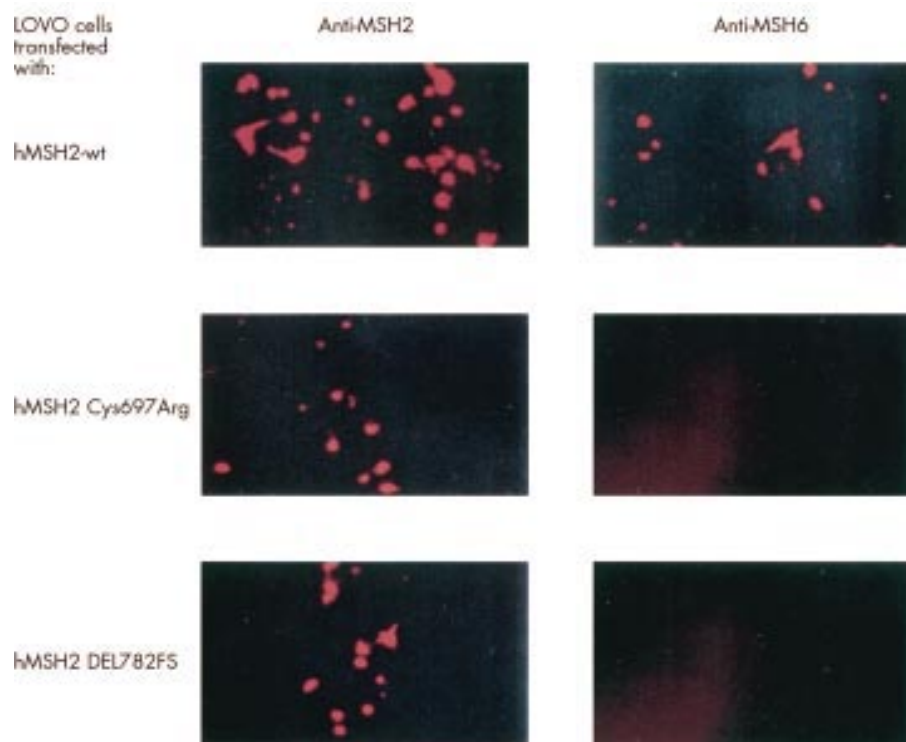


Figure 4 Immunofluorescence of hMSH2 deficient tumour cell line LOVO after transfection with hMSH2 wt, hMSH2 Cys697Arg, or hMSH2 DEL782FS. Protein expression after 48 hours was detected using anti-hMSH2 (left) or anti-hMSH6 antibody (right). Data shown are representative of four independent experiments.

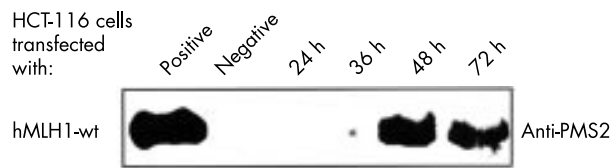


Figure 5 Immunoprecipitation of hMLH1. HCT-116 cells were transiently transfected with hMLH1 wt protein and harvested 24, 36, 48, and 72 hours after transfection. Immunoprecipitation was carried out with 5 μ g monoclonal anti-hMLH1 antibody. Protein-A-agarose was resuspended in 40 μ l SDS loading buffer and protein was resolved by 7.5% SDS-PAGE. hPMS2 was detected by monoclonal antibody. Data shown are mean (SD) of four individual experiments.

MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (De Fries *et al*¹⁷; Goegan *et al*¹⁸). The native, oxidised form of the AlamarBlue reagent is readily taken up by the cells and reduced intracellularly by oxidoreductases and the mitochondrial electron transport chain, with a corresponding shift in its absorbance.^{17,18} HCT-116 or LOVO cells were plated in 96 wells and transiently transfected with wild type proteins, mutant proteins or pcDNA3.1+ vector without insert as control. At 24, 48, and 96 hours after transfection cells were incubated with the alamarBlue dye (10% alamarBlue in growth medium) for three hours at 37°C. Absorption was monitored at 570/600 nm using an ELISA reader (SLT Rainbow, TECAN, Germany). Baseline readings were assessed from medium and alamarBlue without cells.

Data presentation and statistical analysis

Data are shown either as representative experiments or as mean (SD). Differences in apoptosis and proliferation data were analysed by Fisher's exact test (BIAS Software version 7.04, Epsilon Verlag, Darmstadt, Germany). All p values are two tailed, and $p < 0.05$ was considered significant.

RESULTS

Expression of mismatch repair proteins in deficient cell lines

hMLH1 expression was performed in the hMLH1 mismatch repair deficient human colon carcinoma HCT-116 cell line containing a homozygous non-sense mutation at codon 252 (stop codon) in exon 9. hMSH2 proteins were expressed in the hMSH2 deficient human colon carcinoma LOVO cell line that has a homozygous deletion in the hMSH2 gene from exon 3 to exon 8.¹⁹ The investigated mutants comprise mutations found in four patients with HNPCC: a well characterised *hMLH1* mutation (*hMLH1* Thr117Met) affecting the essential ATP binding domain in the aminoterminal portions,²⁰ a *hMLH1* mutation affecting the hPMS2 interaction domain (*hMLH1* Lys618Thr)²¹ and two *hMSH2* mutations, a 1-bp deletion-mutation leading to a truncated hMSH2 protein (hMSH2 DEL782FS), and a *hMSH2* missense mutation (hMSH2 Cys697Arg). All expression plasmids were tested to produce protein using a coupled transcription/translation assay.

The time course of transient expression of wild type and mutant hMLH1 and hMSH2 was investigated by immunoblot (fig 1 and 2). Expression started 12 hours after transfection of mutant *hMLH1* (fig 1B, C), *hMSH2* wt, and mutant *hMSH2* (fig 2A, B, C) and 24 hours after transfection of *hMLH1* wt (fig 1A). Maximum expression was achieved after 24 hours (hMLH1 Thr117Met and hMLH1 Lys618Thr), 36 hours (hMLH1 wt, hMSH2 Cys697Arg and hMSH2 DEL782FS), and 48 hours (hMSH2 wt). Four to seven days after transfection, wild type and mutant MMR proteins declined to pre-transfection levels. In general, expression of mutant proteins decreased earlier than the expression of wild type proteins (fig 1 and 2). The expression of hMLH1 Thr117Met and hMSH2 DEL782FS was

approximately 20% and 80% lower compared with the expression of hMLH1 wt and hMSH2 wt, respectively (fig 1A, B; fig 2A, C). However, similar expression levels were observed for hMLH1 wt and hMLH1 Lys618Thr as well as hMSH2 wt and hMSH2 Cys697Arg (fig 1A, C; fig 2A, B).

Stabilisation of interacting mismatch repair proteins

Expression of interacting proteins was analysed to determine functional consequences of transfected proteins. In HCT-116 cells hPMS2 became detectable by western blot analysis approximately 48 hours after transfection of hMLH1 wt gene (fig 1A), while hPMS2 remained undetectable in cells expressing mutant hMLH1 proteins (fig 1B and 1C). The presence of hPMS2 protein was examined in LOVO cells transiently transfected with *hMSH2* wt or *hMSH2* mutants to obtain further evidence for the specific hMLH1 dependent expression of hPMS2 protein. The transfected plasmids, however, did not affect hPMS2 expression levels (data not shown).

Similarly, hMSH6 was only detectable in LOVO cells expressing hMSH2-wt protein (fig 2). The time course of hMSH6 expression was parallel to the expression of hMSH2 protein (fig 2A). In contrast, hMSH6 protein was not detectable in LOVO cells expressing mutant hMSH2 proteins (hMSH2 Cys697Arg and hMSH2 DEL782FS) (fig 2B and 2C). hMSH6 expression was not affected in HCT-116 cells transiently transfected with *hMLH1* wt or *hMLH1* mutant constructs, indicating specific hMSH2 dependent expression of hMSH6.

Both, hMLH1 wt and mutant proteins as well as hMSH2 wt and mutant proteins were detected by immunofluorescence in transfected HCT-116 and LOVO cells, respectively, with a similar time course as shown by western blot analysis. hPMS2 protein was detectable by immunofluorescence during expression of hMLH1 wt but not mutant protein in HCT-116 cells (fig 3). Similarly, hMSH6 protein was present in LOVO cells complemented with hMSH2 wt, but not in cells transfected with mutant hMSH2 constructs (fig 4). None of the respective proteins were detectable in HCT-116 and LOVO cells transfected with an empty pcDNA3.1+ vector (data not shown).

Coimmunoprecipitation of hMLH1-hPMS2 and hMSH2-hMSH6

hMLH1 was immunoprecipitated from HCT-116 cells transfected with *hMLH1* wt, *hMLH1* Thr117Met, and *hMLH1*

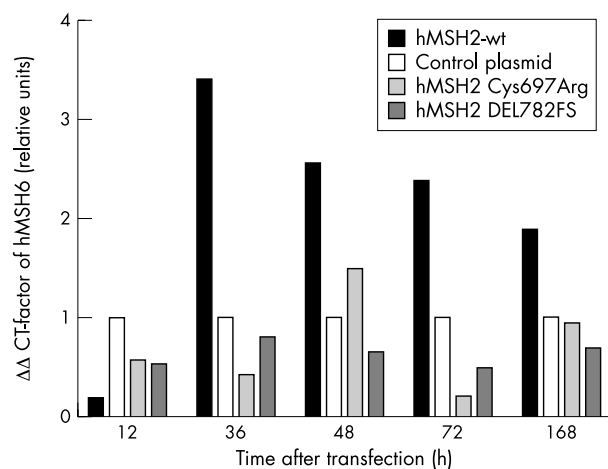


Figure 6 Quantitative PCR-analysis. Total RNA of transiently transfected LOVO cells expressing hMSH2 wt protein, hMSH2 Cys697Arg, hMSH2 DEL782FS, or control plasmid were extracted 12, 36, 48, 72 hours and seven days after transfection. *hMSH6* mRNA levels were quantified using real time PCR. Results show the $\Delta\Delta$ CT-factor of hMSH6, representing the relative mRNA quantity of sample to control. $\Delta\Delta$ CT-factor was calculated for four independent experiments.

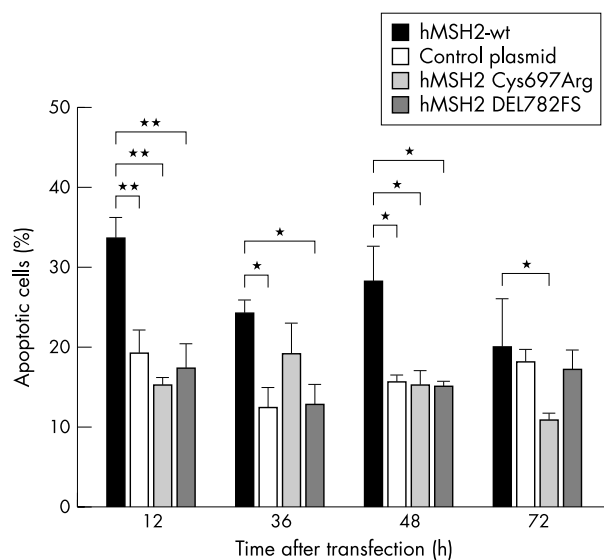


Figure 7 Measurement of apoptosis. LOVO cells were transiently transfected either with *hMSH2* wt or mutant *hMSH2*. Cells were fixed 12, 36, 48, and 72 hours after transfection. Apoptosis was examined by fluorescence microscopy after membrane permeabilisation and staining of the nuclei with Hoechst 33342. Data shown are mean (SD) of four independent experiments. * $p < 0.05$; ** $p < 0.01$.

Lys618Thr using monoclonal anti-hMLH1 antibody. Analysis of the precipitates by western blotting showed the presence of hPMS2 only in HCT-116 cells expressing hMLH1 wt (fig 5), while hPMS2 protein was not detectable in precipitates from HCT-116 cells expressing hMLH1 Thr117Met or hMLH1 Lys618Thr and in HCT-116 cells transfected with empty pcDNA3.1+ plasmid (data not shown). These results indicate that hMLH1 wt protein interacts *in vivo* with hPMS2 to form the heterodimer hMutL α and that mutant hMLH1 lose function to stabilise hPMS2 protein. Experiments using LOVO cell extracts resulted in an increased background signal as a result of polyclonal anti-hMSH2 antibody binding. Using several antibodies, coimmunoprecipitation was not sufficiently sensitive to detect hMSH2 expression and interaction with hMSH6.

Quantitative PCR analysis

mRNA levels of *hPMS2* and *hMSH6* were quantified in transiently transfected HCT-116 and LOVO cells to differentiate between reduced transcription and protein stabilisation, respectively. Quantification of *hPMS2* mRNA showed no difference at any time point between HCT-116 cells expressing hMLH1 wt or mutant proteins (data not shown). In contrast, two, three, and seven days after transfection, *hMSH6* mRNA levels were increased in LOVO cells transfected with hMSH2 wt protein compared with LOVO cells transfected with *hMSH2* Cys697Arg or *hMSH2* DEL782FS (fig 6).

Expression of hMSH2 wt induces apoptosis

Apoptosis of HCT-116 and LOVO cells expressing hMLH1 wt, hMSH2 wt or mutant proteins was investigated to determine loss of function arising by mutant MMR proteins. Apoptosis of hMLH1 wt or hMLH1 mutant transfected HCT-116 cells was similar (data not shown). However, apoptosis of LOVO cells complemented with hMSH2 wt protein was increased compared with *hMSH2* Cys697Arg or *hMSH2* DEL782FS transfected LOVO cells (fig 7). Parallel to hMSH2 wt protein expression, apoptosis was observed 12 hours to 72 hours after transfection (fig 1A).

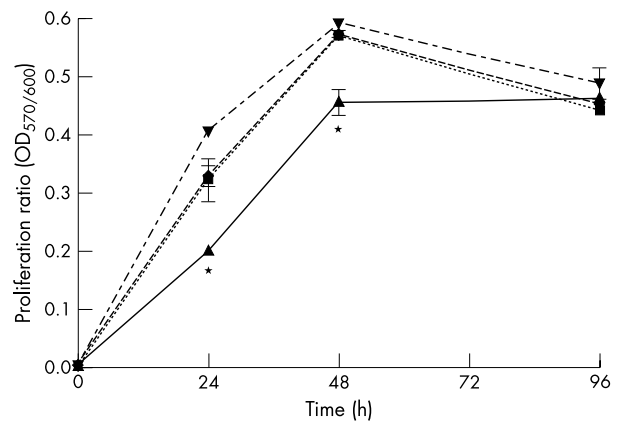


Figure 8 Proliferative activity of transiently hMLH1 transfected HCT-116 cells. The *hMLH1* deficient cell line HCT-116 was transfected with *hMLH1* wt (triangles), *hMLH1* Thr117Met (inverted triangles), *hMLH1* Lys618Thr (diamonds), or control plasmid (squares). The mitochondrial activity as a parameter of proliferation was determined 24, 48, and 96 hours after transfection. Data shown are mean (SD) of four individual experiments. * $p < 0.05$.

Proliferation in transiently transfected HCT-116 and LOVO cells

Conversion of AlamarBlue dye, an indicator of mitochondrial enzyme function, was measured to analyse the influence of hMLH1 and hMSH2 on cell proliferation. The assay has been previously validated as a substitute for the 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay.^{16,17} Proliferation of HCT-116 cells expressing hMLH1 wt was lower compared with HCT-116 cells transfected with an empty pcDNA3.1+ vector or cells expressing hMLH1 mutants (hMLH1 Thr117Met, hMLH1 Lys618Thr) (fig 8). Changes in proliferation of HCT-116 cells were similar to the time course of hMLH1 protein expression (fig 1A)—that is, HCT-116 proliferation normalised approximately four days after transfection as hMLH1 wt protein expression declined. Proliferation was not different in LOVO cells transiently transfected with *hMSH2* wt, *hMSH2* Cys697Arg or *hMSH2* DEL782FS mutants (fig 9).

DISCUSSION

The role of mismatch repair proteins was previously analysed *in vitro* by methods such as whole chromosome transfer,

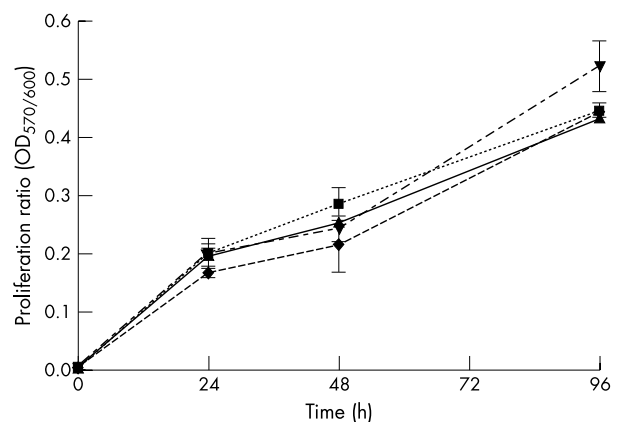


Figure 9 Proliferative activity of transiently hMSH2 transfected LOVO cells. The *hMSH2* deficient LOVO cells were transfected with *hMSH2* wt (triangles), *hMSH2* Cys697Arg (inverted triangles), *hMSH2* DEL782FS (diamonds) or control plasmid (squares). The mitochondrial activity as a parameter of proliferation was determined 24, 48, and 96 hours after transfection. Data shown are mean (SD) of four individual experiments. Differences observed did not achieve statistical significance.

microinjections, and MMR assays, which are less suitable for large scale analyses of HNPCC mutations. Moreover, Umar *et al*²² indicated difficulties in chromosome complemented MMR deficient cell lines corresponding to unpredictable side effects of different amounts of transferred chromosomes. In contrast, transient transfection of mismatch repair deficient colorectal cancer cell lines with different *hMLH1* and *hMSH2* cDNA constructs permits direct functional study of mutations found in patients with HNPCC. Thus, in the present study wild type, *hMLH1* (*hMLH1* Thr117Met and *hMLH1* Lys618Thr) and *hMSH2* mutants (*hMSH2* Cys697Arg and *hMSH2* DEL782FS) were cloned and expressed in HCT-116 and LOVO cells, respectively.

MMR protein expression in transiently transfected cells was analysed by western blotting and immunofluorescence. Impaired expression of the *hMLH1* Thr117Met mutant and shorter expression of *hMLH1* Thr117Met and *hMLH1* Lys618Thr mutants compared with *hMLH1* wt protein was observed in HCT-116 cells. In LOVO cells, expression of *hMSH2* DEL782FS mutant was impaired and expression of *hMSH2* Cys697Arg and *hMSH2* DEL782FS mutants was shorter compared with *hMSH2* wt protein. These results indicate that mutant *hMLH1* and *hMSH2* proteins are either less stable than the *hMLH1* and *hMSH2* wt proteins, respectively, or less efficiently synthesised, for example, because of lower mutant MMR mRNA levels. These observations are in accordance with Curia *et al* who failed to extract and sequence mutant *hMLH1* and *hMSH2* mRNA but successfully extracted and sequenced *hMLH1* wt and *hMSH2* wt mRNA from patients with HNPCC.²³ The data of this study may explain the inability to detect mutant MMR proteins in human tumour tissue of patients with HNPCC by immunohistochemistry.^{24, 25}

In addition, the expression of heterodimeric proteins of *hMLH1* and *hMSH2* were analysed. hPMS2 protein was restored in HCT-116 cells transfected with *hMLH1* wt but not in cells transfected with *hMLH1* mutants. *hPMS2* mRNA levels as quantified by real time PCR showed no difference in *hMLH1* transfected cells indicating that *hMLH1* wt protein stabilises hPMS2 protein post-translationally. Moreover, the data shown by western blotting could be verified by coimmunoprecipitation. hPMS2 coimmunoprecipitated with *hMLH1* wt but not with mutant *hMLH1* proteins that failed to restore the expression of hPMS2. These results are in accordance with data discussed by Chang *et al*, Curia *et al*, and Guerrette *et al*.^{11, 23, 25}

The interaction of hMSH6 with *hMSH2* wt or mutant proteins was investigated in LOVO cells. hMSH6 protein was detectable after expression of *hMSH2* wt, but not of *hMSH2* mutants. Similar data were previously reported by whole chromosome transfection experiments.^{27, 28} Quantification of *hMSH6* mRNA levels by real time PCR showed reduced *hMSH6* mRNA levels in *hMSH2* wt transfected LOVO cells indicating transcription associated regulation of *hMSH2* and *hMSH6*. This may further account for the inability to detect hMSH6 in *hMSH2* wt deficient cells, although it was previously attributed to post-translational stabilisation of hMSH6 by hMSH2 wt.¹¹ However, hMSH2/hMSH6 interaction could not be demonstrated by coimmunoprecipitation in LOVO cells transfected with *hMSH2*. Because coimmunoprecipitation of hMSH6 with *hMSH2* in MMR proficient cell lines was consistently successful, the efficiency of expression *hMSH2* mutants in transfected LOVO cells may be insufficient for coimmunoprecipitation of hMSH6 and *hMSH2* protein.

In this study the inability of *hMLH1* mutants and *hMSH2* mutants to complement deficiency of hMutL α or hMutS α in HCT-116 and LOVO cells, respectively, was demonstrated. It was previously shown in a yeast based *hMLH1* functional assay that the investigated *hMLH1* mutations alter mismatch repair.²⁹ Moreover, all colorectal tumours of the patients with HNPCC from whom the *hMLH1* and *hMSH2* mutants were derived showed microsatellite instability.³⁰ This provides

evidence that the investigated mutations are associated with a defective DNA mismatch repair system. In this study we further focused on the possible functional consequences of wild type and mutant MMR proteins in the regulation of apoptosis and proliferation.

Apoptosis of LOVO cells was increased by *hMSH2* wt but not by *hMSH2* mutant expression, while no changes were observed after transfection of HCT-116 cells with *hMLH1* wt or mutants. In contrast, Zhang *et al* showed that apoptosis was induced after microinjection of *hMLH1* wt in HCT-116 cells.¹³ Because the GFP-plasmid was coinjected with the *hMLH1* construct in that study, synergistic effects of overexpressed *hMLH1* and GFP protein cannot be excluded. Indeed, we have obtained evidence that the GFP-plasmid transfection in colon carcinoma cell lines can induce apoptosis (unpublished data). The results suggesting that *hMSH2* wt but not *hMLH1* wt protein is required for induction of apoptosis, may have implications for the use of several apoptosis inducing chemotherapeutic agents.³¹⁻³³

Furthermore, the proliferative activity of MMR transfected cells was investigated. HCT-116 cells complemented by *hMLH1* wt protein showed reduced proliferative activity (24 hours and 48 hours after transfection) compared with cells transfected with the control plasmid or *hMLH1* mutants. In accordance with Shin and coworkers,¹⁴ these data indicate that *hMLH1* mutants fail to regulate proliferation. In contrast, no effect on proliferation was observed by complementation with *hMLH2* wt or mutants, indicating that *hMSH2* is not involved in the regulation of proliferation.

The analysis of microsatellite instability in colorectal tumours has substantially improved the management of patients with HNPCC.^{34, 35} Nevertheless, the clinical role of specific MMR mutations in the progression of disease and for the efficacy of chemotherapy is widely unclear. Our experimental set up for the functional analysis of MMR mutations may be useful to evaluate their clinical significance. Chemotherapeutic agents act by induction of apoptosis or inhibition of proliferation.³⁶ The data of this study show that mutations in the *hMLH1* and *hMSH2* genes lead to decreased apoptosis and increased proliferation, respectively. Chemotherapeutic agents may directly interact with the mismatch repair system,³⁷⁻³⁹ for example, *hMLH1* but not *hMSH2* seems to be involved in processing topoisomerase I poison induced damage.⁴⁰ Our system may be suitable for further in vitro analysis of interaction of chemotherapeutic agents with the MMR system and may help to optimise and individualise treatment.

In conclusion, the data of this study demonstrate that *hMLH1* wt protein, but not *hMLH1* mutants increase hPMS2 protein levels and decrease proliferation. In contrast, *hMSH2* wt protein but not *hMSH2* mutants increase *hMSH6* mRNA and protein levels as well as apoptosis. Thus, using these well defined transient transfection system the functional role of genetic variants (mutations/polymorphisms) of MMR genes found in HNPCC families can be easily studied.

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Authors' affiliations

A Brieger, J Trojan, J Raedle, G Plotz, S Zeuzem, Medizinische Klinik II, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt, Germany

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