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Expression of the hMSH6 mismatch-repair protein in colon cancer and HeLa cells

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Summary

Principles: 10 to 15% of human colon cancers are associated with an inherited or somatic defect of the DNA Mismatch Repair (MMR) system, which has evolved to correct biosynthetic errors such as nucleotide mis-incorporations or misalignments arising during DNA replication in the S phase of the cell-cycle. Although expression of the MMR genes was expected to be cell-cycle dependent, we and others observed that the MMR proteins hMSH2 and hMLH1 are expressed constitutively in proliferating cells.

Methods: In this study we extend our observations to another essential MMR protein, hMSH6. We used immunohistochemistry to evaluate the expression pattern of this protein in human colorectal mucosa and tumours, as well as in synchronised HeLa-S3 cells, in which we analysed its steady-state levels during the cell-cycle.

Results: We show that the immunohistochemical pattern of expression of hMSH6 in normal colorectal crypts and in colon cancers differs significantly from that of the other MMR proteins, with a much lower percentage of replicating cells being hMSH6-positive. This implies that hMSH6 could be cell-cycle regulated. In order to test this hypothesis in a model system, we synchronised HeLa-S3 cells with mitotic shake-off and found that the hMSH6 protein was detectable throughout the cell-cycle, but that its steady-state level increased when cells progressed from G1 to S-phase.

Discussion: The increase of hMSH6 steady-state level when cells enter S-phase was expected, since MMR acts during DNA replication. However, the overall low level of oscillations of hMSH6 during the cell-cycle in this cellular model apparently does not fit the immunohistochemical phenotype. We believe that this discrepancy is due to the fact that human cell lines proliferate at a much higher rate than normal and neoplastic colorectal cells *in vivo*.

Key words: mismatch repair, *hMSH6*, *cell proliferation*, *colon cancer*

Introduction

A phenotype characterised by widespread instability of microsatellite sequences in DNA has been reported to occur in 10–15% of human colon cancers [1, 2]. Microsatellites are stretches of repeated DNA sequences consisting of mono-, di- or tri-nucleotide repeats. Mono- and di-nucleotide microsatellites are either shorter or longer by a few repeat units in this subset of colon cancers, when compared to those in the DNA of normal tissues. Microsatellite instability (MSI) is caused by a defect of the Mismatch Repair (MMR) system, which has evolved to correct errors of DNA polymerases [3, 4]. These enzymes are error-prone and, during DNA replication, they can cause strand misalignments with the formation of loops of extrahelical bases in the newly synthesised or in the template strand. These intermediates (i.e., insertion/deletion loops), if not repaired, result in insertions or deletions of repeat units in the microsatellites of the progeny DNA. In addition, DNA polymerases incorporate non-complementary nucleotides into the newly synthesised DNA strand (i.e., base mismatches) with a frequency of approximately one error per 100 000 nucleotides synthesised. Also base misincorporations are substrates for the MMR system, which thus efficiently reduces the mutation rate from one error every 10^7 to one error every 10^{10} nucleotides. Base mismatches and insertion/deletion loops are recognised by a complex consisting of the hMSH2 and hMSH6 proteins [5] (figure 1). Another complex (hMSH2/hMSH3) (not shown in figure 1), which is less represented

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in human cells, plays a back-up role only in the recognition of loops, since it does not recognise base mismatches [6, 7]. After recognition, a second heterodimeric complex, hMLH1/hPMS2, is recruited. Although the function of this latter complex is still unknown, it has been suggested that it acts as a molecular matchmaker between the mismatch recognition complex and a series of proteins (helicases, exonucleases, etc.) necessary to excise the tract of the newly synthesised strand carrying the aberrantly paired bases (figure 1, see legend for details) [4].

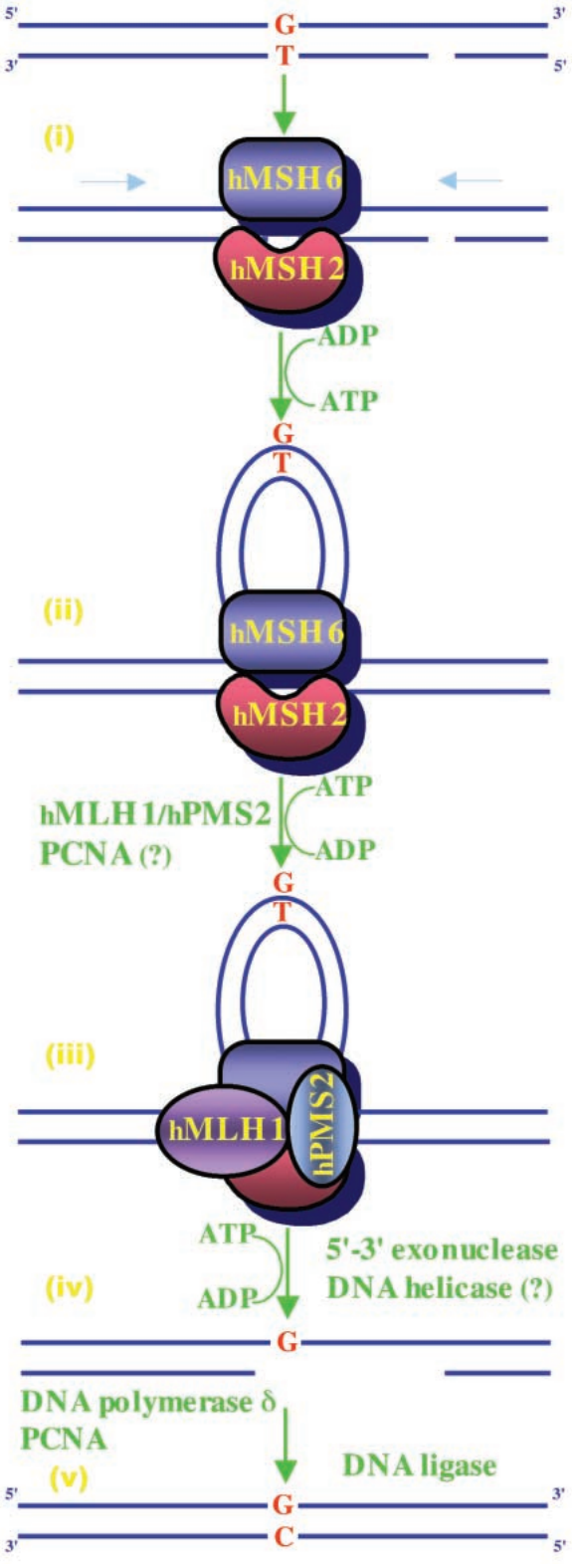


Figure 1

Putative model of human MMR. (1) The mismatched "T", introduced into the newly synthesised strand by the replication complex, is recognised by the hMSH2/hMSH6 heterodimer. (2) ATP drives the bi-directional threading of DNA, which makes a loop and (3) recruits other essential members of the MMR complex, such as the hMLH1/hPMS2 heterodimer and PCNA. (4) Exonucleolytic degradation of the T-containing strand is initiated by an as yet unidentified helicase(s) and exonuclease(s). (5) DNA synthesis is re-initiated by the replication complex and a "C" is normally paired with "G".
The recognition complex hMSH2/hMSH3, not included in this figure, does not recognise base mismatches, but only insertion/deletion loops (see Introduction). However, the repair process downstream of the DNA binding is essentially the same as that described in this figure for base mismatches.

Of particular interest is a biochemical property of the three above-mentioned heterodimers: one partner is stable also in monomeric form (hMSH2 in the hMSH2/hMSH6 and hMSH2/hMSH3 heterodimers, and hMLH1 in the hMLH1/hPMS2 heterodimer), whereas hMSH6, hMSH3 and hPMS2 are unstable as monomers [7, 8]. Most human MMR-deficient colon cancers have genetic or epigenetic alterations of the *hMSH2* and *hMLH1* genes (http://www.nfdh.nl/database/mdbchoice.htm) [2]. In particular, 2–5% of colon cancers are associated with a germ-line mutation (inherited in all cells of the body) in one of these two genes in members of Hereditary Nonpolyposis Colon Cancer (HNPCC) families, and 8–12% of colon cancers are associated with promoter hypermethylation of both *hMLH1* alleles at the somatic level (in the colonocytes) [2]. As a consequence, hMSH6 is degraded in *hMSH2*-negative tumours, and hPMS2 is degraded in *hMLH1*-negative tumours. The functional significance of this phenomenon is still unknown. One possibility could be that the stable partner is involved in other processes other than MMR, and the degradation of the unstable partner is necessary for the former to function in these pathways, alone or in combination with other proteins. In fact, yeast homologs of hMSH2, hMSH3, hMLH1 and hPMS2 have also been found to be involved in DNA recombination [9, 10].

In contrast, the only role found to date for hMSH6 is in MMR. MMR occurs during DNA replication in S phase, thus we hypothesised that an assembled hMSH2/hMSH6 complex is necessary in this phase of the cell-cycle. We and others [11, 12] previously found that the steady-state levels of hMSH2 are very low in cell lysates of resting cells, but that a considerable increase of its levels is observed when cells enter the cell-cycle. However, only minor oscillations of the steady-state levels of hMSH2 are observed during the cell-cycle, as is usually observed with products of housekeeping genes. We set out to extend our observations to the expression and degradation patterns of hMSH6 during the cell-cycle.

Due to the intrinsic instability of hMSH6 in monomeric form, we supposed that higher oscillations of its steady-state levels could be found. hMSH6 is essential in MMR because it is the actual mismatch-recognition protein of the hMSH2/hMSH6 complex [5, 13, 14]. Its specificity for the recognition of base mismatches and its redundancy with hMSH3 in the repair of loops explains why human cell lines with mutations in *hMSH6* show only a very low degree of MSI, mainly in mononucleotide repeats [1]. Thus, in addition to colon cancers with a high degree of MSI, MMR-deficient tumours with a high mutation rate but without or with only low-degree MSI could be found. Recently, familial aggregations of colon cancer with germ-line mutations in *hMSH6* have been described [15–18], and hypermethylation of the *hMSH6* promoter has also been demonstrated in a

cellular model [19]. Thus, it is crucial to characterise the expression and degradation patterns of hMSH6, because abnormalities of these patterns could result from missense mutations or epigenetic modifications of this gene. We report here the results obtained by using both an immunohistochemical approach in human colorectal samples and a cellular model suitable for cell-cycle analysis.

Methods

Immunohistochemistry

Normal and tumour colorectal samples were obtained from the department of Pathology of Triemli hospital in Zürich involved in a national screening program aimed at the identification of MMR-deficient colon cancers (www.imr.unizh.ch/research/jiricny/HNPCC/hnpcc.html). 4 µm serial sections from paraffin blocks were mounted on silanised slides, deparaffinised and rehydrated. Antigen retrieval was accomplished by heating the sections in a pressure cooker at 120 °C for 2 min in 10 mM citrate-buffered solution (pH 6.0). DAKO peroxidase blocking reagent and goat serum were sequentially used to suppress non-specific staining due to endogenous peroxidase activity and non-specific binding of antibodies, respectively. Incubation with primary monoclonal antibodies was performed as follows: anti-hMSH2: 24 hours at 4 °C with Ab NA26 (Oncogene Research), 1 µg/ml; anti-hMSH6: 2 hours at RT with Ab G70220 (Transduction Laboratories), 4 µg/ml; anti-hMLH1: 1 hour at RT with Ab 13271A (PharMingen), 1.2 µg/ml; anti-hPMS2: 24 hours at 4 °C with Ab 65861A (PharMingen), 3 µg/ml. After washing, anti-mouse secondary antibodies conjugated to peroxidase labelled polymer (DAKO EnVision+ kit) were applied for 30 min at RT, and the peroxidase activity was developed by incubation with 3,3'diaminobenzidine (DAB) chromogen solution (DAKO). The sections were then counterstained slightly with haematoxylin.

Cell synchronisation of HeLa-S3 cells by mitotic detachment

Synchronous cultures of HeLa-S3 (cultured in Iscove's modified Dulbecco's medium supplemented with 10% FBS and 2 mM L-glutamine, Life Technologies) were obtained using a manual system for partitioning of detached cells [20]. Cells were plated into 150 mm dishes at a density of $1.5\times10^4/cm^2$. When they were exponentially growing, detached rounded cells were removed and discarded, and fresh pre-warmed (37 °C) serum-supplemented medium was added. Newly-detached cells were collected from the cell culture dishes every 1.5 h, replated, and incubated for various lengths of time from 0 to 15 h (5 time points). An aliquot of cells at each time point was fixed for cell-cycle analysis, whereas another aliquot was harvested for Western blot analysis.

Cell-cycle analysis

1×10^6 cells per time point were washed with PBS and fixed in ice-cold 70% ethanol. The cells were treated with 200 U/ml RNase A and stained with 20 µg/ml propidium iodide. Cell-cycle analysis was performed using a Becton Dickinson FACscan flow cytometer and Cell Quest software.

Preparation of total protein extracts

8×10^6 cells were washed twice with PBS, collected and dissolved in 180 µl of ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0, 350 mM NaCl, 1% NP40, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 2 µg/ml aprotinin, 0.7 µg/ml pepstatin, and 1× EDTA-free Complete – Boehringer). After 10–20 minutes incubation on ice, the completion of the cell lysis was verified under the microscope with trypan blue staining. The lysate was centrifuged in an Eppendorf microfuge for 5 minutes at 12 000 *xg* and the supernatant was collected, snap frozen in small aliquots in liquid nitrogen and stored at –80 °C.

Gel electrophoresis and Western blot analysis

50 µg of total proteins from each sample were loaded on a 7.5% SDS-PAGE and electroblotted onto poly (vinylidene difluoride) membranes. The blots were first blocked in TBST (0.1% Tween 20 in Tris-buffered saline) containing 5% non-fat dry milk for 1 h at 37 °C, and then incubated with the respective primary monoclonal antibodies for 1 h at room temperature. The primary antibody concentrations were: anti-hMSH6 (no. G70220–050, Transduction Laboratories), 0.125 µg/ml; anti-hMSH2 (no. NA26, Oncogene Science), 0.65 µg/ml; anti-TFIIH p89 (no. sc-293, Santa Cruz), 0.04 µg/ml; anti-cyclin E (no. MS-870–P, NeoMarkers), 0.2 µg/ml, and cyclin B1 (no. 05–373, Upstate biotechnology), 1 µg/ml. After washing with TBST, the blots were incubated with horseradish peroxidase conjugated sheep anti-mouse Ig (no. NXA 931, Amersham Pharmacia, diluted 1:5000) for 1 h at room temperature. The protein-antibody complexes were detected with the enhanced chemiluminescence system (Amersham Pharmacia). The pre-stained molecular weight marker (no. 161–0318, BIO-RAD) was loaded alongside the samples on each gel. The intensity of the bands was quantified relative to TFIIH p89, using the ImageQuant software of the Computing Densitometer (Molecular Dynamics).

Statistical analysis

Nuclei stained and unstained with anti hMSH2 or anti hMSH6 antibodies were counted in 22 colorectal crypts and 18 tumours. Multiple logistic regression was used to evaluate the odds ratio associated with being stained when treated with anti hMSH2 versus anti hMSH6 antibodies after adjusting for the confounding effects of the differences between patients and the different number of nuclei in each sample. To evaluate whether the expression of hMSH6 significantly increased from time points 3 h to 9 h when compared with the changes of the housekeeping gene TFIIH p89 at the same time points, the natural logarithms of the expression were compared using the t test (data obtained from three independent experiments). The use of logarithms enabled normalisation of the distribution and comparison by ratios rather than absolute differences.

Results

Immunohistochemistry for MMR proteins has been performed in our laboratory for the past 3 years as a tool to screen colorectal cancers for MMR deficiency. Four proteins were routinely tested in these tumours: hMSH2, hMSH6, hMLH1 and hPMS2. As expected, MMR proteins were expressed in proliferating cells, i.e., in the lower two thirds of the normal colorectal crypts and in most of the tumour cells (figure 2, panels A and B; hMLH1 and hPMS2, not shown, have a staining pattern similar to that of hMSH2). However, we found a consistent difference in the expression pattern between hMSH6 and the other three proteins. In the normal colorectal crypt, most of the nuclei of proliferating cells were positive for hMSH2, hMLH1 and hPMS2, whereas hMSH6 expression was limited to only a few of the cells of the proliferative compartment (figure 2, panel A). Also in neoplastic colorectal samples (figure 2, panel B), the expression of hMSH6 was more "selective" among proliferating cells, displaying an interspersed pattern. In contrast, the other three MMR proteins were expressed abundantly in most of the tumour cells. Multiple logistic regression analysis showed that the difference in number of nuclei stained with anti hMSH6 or anti hMSH2 antibodies was statistical significant ($p < 0.00001$), both in colorectal crypts and in tumours.

This staining pattern prompted us to investi-

Figure 2

Imunohistochemical expression patterns of hMSH2 and hMSH6 in colorectal mucosa and tumours.
A) normal mucosa: MMR proteins are expressed in the proliferative compartment of the colorectal crypts (lower $^2/_3$) where most of the nuclei are positive for hMSH2, whereas hMSH6 expression is confined to only some of the cells of this compartment.
B) adenomatous region of a colon cancer: also in these lesions, the pattern of expression of hMSH6 is "interspersed", whereas hMSH2 is expressed in most of the tumour cells.
C) MMR-deficient colon carcinoma: this tumour was excised from a member of a HNPCC family carrying a germ-line mutation in *hMSH2*.
Usually, a second alteration occurs in the wild-type allele of *hMSH2* in the tumour and hMSH2 is not expressed at all. Consequently, hMSH6 is degraded (see Introduction) and the same tumour is also negative for this latter protein. Some non-neoplastic cells in the lamina propria are positive for hMSH2 and hMSH6 as expected, and the other MMR proteins are normally expressed in this tumour as shown for hMLH1 in the superimposed image in C.

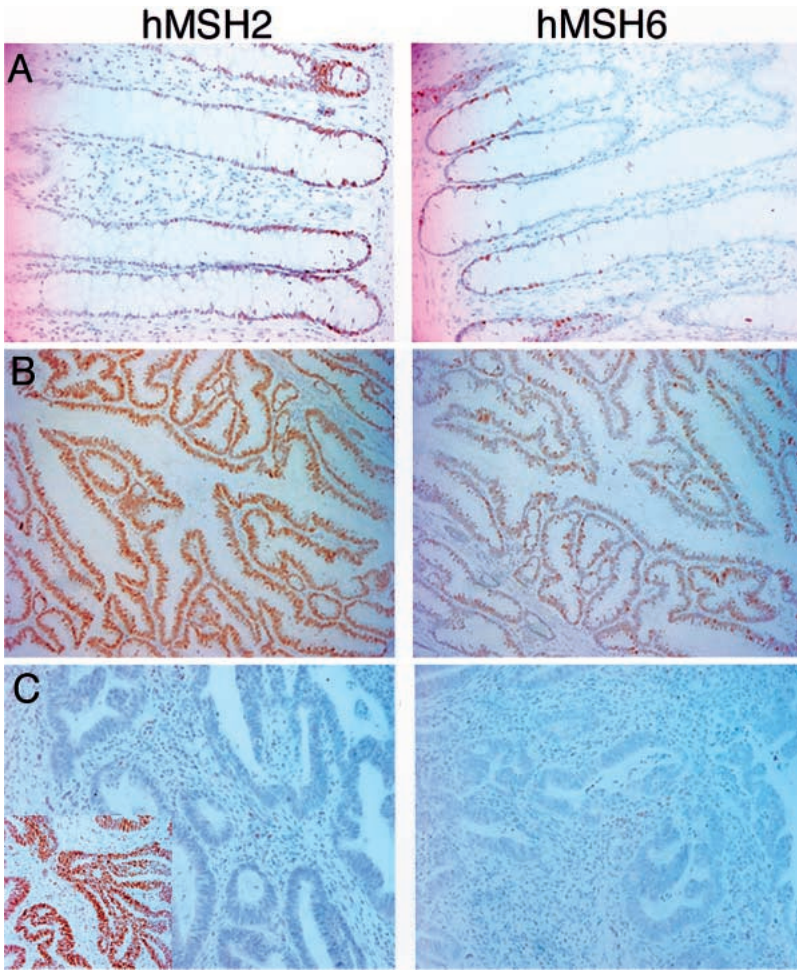
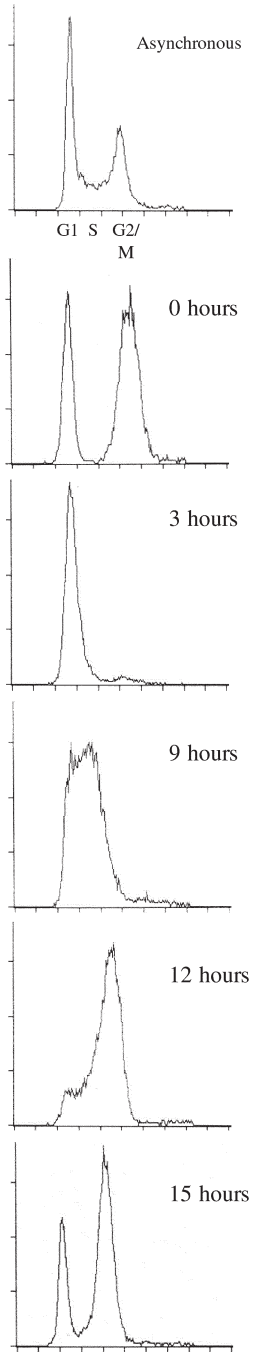


Figure 3

Flow cytometry analysis of HeLa-S3 cells synchronised by mitotic shake-off (see methods). Compare the DNA content of asynchronous cells with that of cells at different time points after mitosis. *0 h* (suspension cells harvested and fixed): a portion had already passed to G1, presumably during the harvesting period;
3 h after re-plating of suspension cells: most of the cells were in G1;
9 h: cells were traversing S phase; 12 h: they reached G2; 15 h: a second G1 phase was reached.



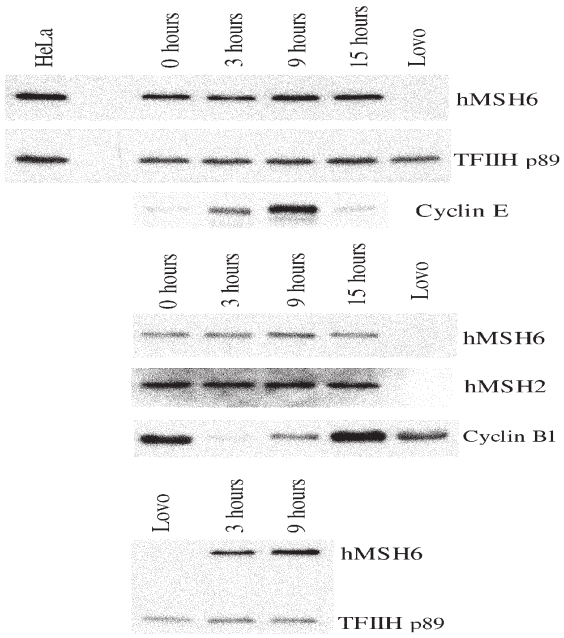


Figure 4

Western blot analysis of proteins extracted from HeLa-S3 cells at different time points after mitosis. The steady-state levels of hMSH6 and hMSH2 are high in every phase of the cell-cycle, although a consistent increase (25%, p = 0.01) of hMSH6 was always observed when cells entered S phase (compare 3 h vs 9 h; three of the several blots performed are shown here). Data from three independent experiments were compared with the steady-state level of the housekeeping protein TFIIH p89. Cyclin E and B1 expression was used as a control of cell synchronisation. Extracts from HeLa and LoVo cell lines were used as positive and negative controls of hMSH2/hMSH6 expression, respectively.

gate if hMSH6 steady state levels could be regulated during the cell-cycle in an *in vitro* cellular model. We chose HeLa-S3 cells because these cells have a particular behaviour in tissue culture: they detach in mitosis. This phenotype allowed us to synchronise them with a method called mitotic shake-off [20] and to avoid perturbations in cell metabolism introduced by synchronising agents. Mitotic cells in suspension were harvested at different time points and re-plated in new flasks for the time lag needed to reach different cell-cycle phases after cell division. Re-plated cells were harvested after 3, 9, 12 and 15 h and their progression throughout the cell-cycle was monitored by flow cytometry (figure 3). Suspension cells were also harvested and immediately fixed for flow cytometry: as shown in figure 3, most were in the G2/M peak, even though a portion had already passed to G1 (compare with the flow cytometric pattern of asynchronous cells), presumably during the harvesting period. At 3 h, most of the cells were in G1, as expected. They passed through S phase at 9 h and reached G2 phase at 12 h. At 15 h a second G1 phase was reached.

Proteins were extracted at these time points and Western blots for MMR proteins were performed. The steady state levels of cyclin E and B1 were also analysed to verify the progression through the cell-cycle. The expression of the housekeeping protein TFIIH p89 was used as an internal control. Dramatic increases of cyclin E in S phase and cyclin B1 in G2/M were observed (figure 4) confirming the good degree of cell-cycle synchronisation. The steady-state levels of hMSH2, hMSH6, hMSH3, hMLH1 and hPMS2 did not show dramatic variations during the cell-cycle in proliferating cells (data shown for hMSH6 and hMSH2 in figure 4), confirming the data already published by us and other groups (see Introduction). However, a 25% increase of hMSH6 steady-state levels was observed when HeLa-S3 cells entered S phase (figure 4, compare 3 h vs 9 h). This increase, although of low degree, was consistent in different experiments (three of which are shown in figure 4) and was statistical significant (p = 0.01).

Discussion

As already mentioned in the Introduction, hMSH6 is an essential protein in DNA mismatch repair because it is the actual molecule that recognises the mismatched bases, even though this function is active only when hMSH6 is heterodimerised with hMSH2 [5, 21]. In addition, hMSH6 is >10 times more abundant than hMSH3 in human cells [6] suggesting that also the repair of insertion/deletion loops is primarily performed by hMSH2/hMSH6. However, the hMSH2/hMSH3 complex is efficient in repairing loops in *hMSH6*$^{-/-}$ cells [7] which, for this reason, show a high mutation rate but do not display a high degree of MSI. In our opinion, this biochemical finding could explain why most of the MMR-deficient tumours in the colon (and in other organs where MMR alterations were investigated, such as in the endometrium) have been mostly associated with mutations in *hMSH2* and *hMLH1*. The fact that MSI has been extensively used to screen for MMR deficiency in tumours could have underestimated MMR-deficient tumours without MSI. However, familial aggregations of colorectal and endometrial cancers associated with germ-line mutations in *hMSH6* have been frequently described in the recent years [15–18], and the possibility thus exists that a subset of sporadic (i.e., non-familial) cancers arises due to a high mutation rate caused by genetic or epigenetic alterations of *hMSH6*. We are at present analysing, in a prospective study, consecutive colon cancers from several hospitals in Switzerland for alterations in MMR genes using different techniques in order to evaluate the real incidence and the type of *hMSH6* alterations.

In this study, we have used immunohistochemistry to confirm that the expression of MMR proteins is strictly associated with cell proliferation. Four of the MMR proteins we have studied were indeed expressed in the nuclei of cells lining the proliferating zone of the normal colorectal

crypt, ie, its lower two thirds. Their expression was lost in the upper third of the crypt, where cells are non-proliferating and differentiated. The expression level was high in most of the cells in the proliferative compartment, suggesting that MMR proteins are expressed in all phases of the cell-cycle. However, hMSH6 showed a different pattern: only a portion of the cells in the proliferative compartment were strongly positive, whereas other cells were either only weakly positive or negative. The interspersed staining pattern of hMSH6 was also apparent in colorectal adenomas and carcinomas, and we found similar expression patterns in the skin and in melanomas (S. D'Atri et al., Journal of Investigative Dermatology, in press). This pattern suggests that hMSH6 expression and/or degradation are more tightly regulated *in vivo* than those of the other MMR proteins. We plan to investigate this phenotype in more detail with fresh colorectal samples and immunofluorescence in order to identify the cell-cycle phase(s) in which hMSH6 is expressed. Biochemical studies are also in progress to study the kinetics of MMR protein degradation in human cells.

However, we suppose that the cells with higher expression of hMSH6 *in vivo* might be in the S phase of the cell-cycle, as is expected for proteins that function during DNA replication. This hypothesis is also supported by the experiments reported in this study using HeLa-S3 cultures, which allowed us to isolate cells in different cell-cycle phases in the absence of synchronising agents. In this system, we consistently found an increase of the steady-state level of hMSH6 when the cells entered S phase. In these cells however, hMSH6 was also expressed in every phase of the cell-cycle, as demonstrated for the other MMR proteins [11, 12]. The proliferative rate of HeLa-S3 cells *in vitro* is very high, with a doubling time of 15 h. On the contrary, the rate of cell proliferation in the normal colorectal mucosa is lower, since the cell-cycle duration of colorectal epithelial cells has been estimated to be between 30 and 40 h [22]. During the transit throughout the cell-cycle phases, colonocytes also migrate from the bottom of the crypts to the lumen with a cellular turnover time of 4–8 days [22]. The rate of cell proliferation in colorectal cancers is highly variable, depending mainly on the stage and vascularisation of the tumour; however, colon cancer cells often have a longer cell generation time than normal colonocytes [22]. Thus, the oscillation of the steady-state level of hMSH6 suggested by the immunohistochemical pattern could be less pronounced in cells, such as HeLa-S3, which proliferate quickly *in vitro*, especially in the eventuality that the degradation of hMSH6 is slow.

One explanation of the tighter regulation of hMSH6 levels compared to those of hMSH2 is that *in vitro* and *in vivo* (in yeast) studies [9, 10] have shown the involvement of the latter protein in other pathways of DNA metabolism, in particular in DNA recombination and in the repair of large single-strand DNA loops. Yeast homologues of hMLH1 and hPMS2 were also found to be involved in recombination [9, 10]. We found that the steady-state levels of these two latter proteins were also similar throughout the cell-cycle ([11, 12], and data not shown) and the immunohistochemical pattern of hMLH1 and hPMS2, similar to that of hMSH2, suggests that they are probably needed in all phases of the cell-cycle. The competition between hMSH6 and hMSH3 for hMSH2 suggests that hMSH6 may be degraded in order to make hMSH2 available for hMSH3 to function in other pathways. We did not find significant variations of hMSH3 steady-state levels during the cell-cycle (data not shown) and the expression pattern of this protein in colon samples was not performed due to the unavailability of reliable anti-hMSH3 antibodies for immunohistochemistry.

At present, different morphological and biochemical approaches are exploited in our laboratories to better understand the regulation of hMSH6 expression. Our effort to evaluate the expression/degradation pattern of the hMSH6 protein is crucial to understand the mechanism of dysfunction of the MMR system and, with regard to its interaction with hMSH2, of other pathways of DNA metabolism. We believe that these studies will elucidate the mechanisms of abnormal DNA metabolism in a subset of human tumours.

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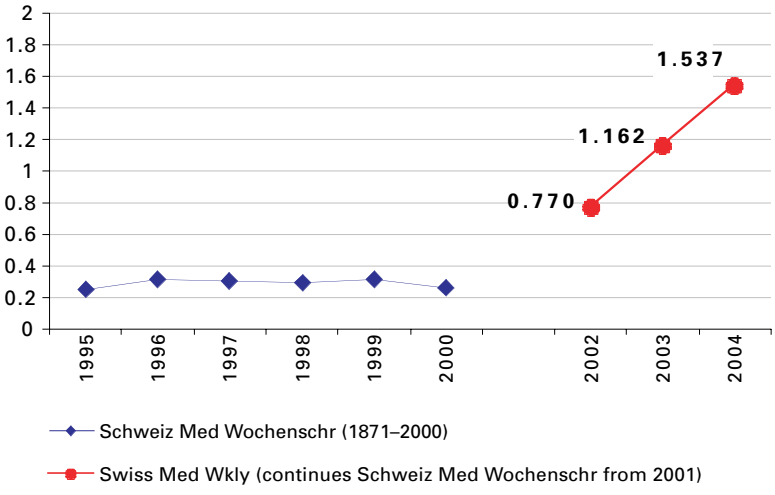

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