SPANDIDOS PUBLICATIONS

Conditional nuclear localization of hMLH3 suggests a minor activity in mismatch repair and supports its role as a low-risk gene in HNPCC

MARI K. KORHONEN[1], TIINA E. RAEVAARA[1], HANNES LOHI[2] and MINNA NYSTRÖM[1]

[1]Department of Biological and Environmental Sciences, Genetics, University of Helsinki; [2]Department of Genetics, Folkhälsan Institute and Program in Molecular Medicine, Faculty of Medicine, University of Helsinki, FI-00014 Helsinki, Finland

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Abstract. DNA mismatch repair (MMR) mechanism contributes to the maintenance of genomic stability. Loss of MMR function predisposes to a mutator cell phenotype, microsatellite instability (MSI) and cancer, especially hereditary non-polyposis colorectal cancer (HNPCC). To date, five MMR genes, *hMSH2*, *hMSH6*, *hMLH1*, *hPMS2*, and *hMLH3* are associated with HNPCC. Although, hMLH3 is suggested to be causative in HNPCC, its relevance to MMR needs to be confirmed to reliably assess significance of the inherited sequence variations in it. Recently, a human heterodimer hMLH1/hMLH3 (hMutLγ) was shown to be able to assist hMLH1/hPMS2 (hMutLα) in the repair of mismatches *in vitro*. To repair mismatches *in vivo*, hMLH3 ought to localize in the nucleus. Our immunofluorescence analyses indicated that when all the three MutL homologues are natively expressed in human cells, endogenous hMLH1 and hPMS2 localize in the nucleus, whereas hMLH3 stays in the cytoplasm. Absence of hPMS2 and co-expression of hMLH3 with hMLH1 results in its partial nuclear localization. Our results are clinically relevant since they show that in the nuclear localization hMLH3 is dependent on hMLH1 and competitive with hPMS2. The continuous nuclear localization of hMLH1 and hPMS2 suggests that *in vivo*, hPMS2 (hMutLα) has a major activity in MMR. In absence of hPMS2, hMLH3 (hMutLγ) is located in the nucleus, suggesting a conditional activity in MMR and supporting its role as a low-risk gene in HNPCC.

Introduction

DNA mismatch repair (MMR) mechanism eliminates DNA polymerase errors such as base/base mismatches and insertion/deletion loops from the newly synthesised strand during replication and recombination. MMR is highly conserved pathway and numerous human genes have homology to MMR genes in *E. coli*. To date, five MMR genes, MutS homologues *hMSH2*, *hMSH6*, *hMSH3* and MutL homologues *hMLH1* and *hPMS2*, are known to be involved in human MMR function (1,2). Consistent with the role of MMR proteins in the maintenance of genomic stability, loss of MMR function predisposes to cancer. About 25% of colon tumours, as well as a number of tumours of endometrium, ovary and some other organs or tissues, are deficient in MMR (3). Moreover, germline mutations in the MMR genes are associated with hereditary non-polyposis colorectal cancer (HNPCC), although with varying frequencies [(3), http://www.insight-group.org/]. The *hMLH1* and *hMSH2* are the most common susceptibility genes for HNPCC, *hMSH6* mutations account for 5-10% of the families, while *hPMS2* is seldom, and *hMSH3* not at all reported to be involved in HNPCC. Remarkably, the third MutL homologue in human, *hMLH3*, without confirmed role in MMR is suggested to be a susceptibility gene for HNPCC (4,5).

In accord with cancer predisposition, hMSH2, hMSH6, hMLH1 and hPMS2 are responsible for important steps in MMR. A heterodimer of hMSH2 and hMSH6, hMutSα, is responsible for mismatch recognition and a heterodimer of hMLH1 and hPMS2, hMutLα, functions as a 'molecular matchmaker' assembling the repairosome to degrade the error-containing newly synthesized strand and to repair synthesis (1,2,6). The challenge is *hMLH3* whose contribution to MMR is unclear and thus, the interpretation of the found sequence variations difficult. Altogether 19 different putative pathogenic variations have been reported in *hMLH3* gene, some are found in the low-risk patients lacking the typical molecular characteristics of the HNPCC syndrome, such as high microsatellite instability (MSI) [(5), http://www.insight-group.org/], whereas some mutations are associated with high MSI (MSI-H) in the tumours (4). Overall, germline *hMLH3* mutations are only rarely found in familial colorectal cancer (CRC) (7,8), suggesting low or no role for hMLH3 in CRC predisposition.

The fact that *hMLH1* and *hMSH2* are the most common susceptibility genes for HNPCC is explained by their unreplaceable roles in MMR function, whereas mutations in

Correspondence to: Dr Minna Nyström, Department of Biological and Environmental Sciences, Genetics, University of Helsinki, Viikinkaari 5, FI-00014 Helsinki, Finland
E-mail: minna.nystrom@helsinki.fi

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hMSH6 and *hMSH3*, which are shown to be functionally redundant (2,9), are associated with no or low cancer susceptibility (10-12). Correspondingly, the functional overlap of hMLH3 and hPMS2 could explain why their mutations are only rarely found in HNPCC families. This assumption originally suggested by results from the yeast studies (13) was recently supported by the study, in which a heterodimer of Mlh1 and Mlh3 was shown to contribute to mechanisms of tumour suppression in the mouse (14). Furthermore, a human homologue, hMutLγ (hMLH1/hMLH3) was shown with low efficiency to be able to repair mismatches *in vitro* (15). Since MMR occurs in the nucleus, to be functional *in vivo*, hMLH3 ought to localize in the nucleus. Here, by using immuno-fluorescence we analyzed the subcellular localization of hMLH3 and thus its relevance to MMR function.

Materials and methods

Human cell lines. For localization studies, we used cervical carcinoma cell line HeLa and colorectal carcinoma cell line HCT116, both purchased from American Type Culture Collection. HeLa cells express hMLH3, hPMS2 and hMLH1, while HCT116 lacks hMLH1 and hPMS2 (15). HeLa cells were cultured in MEM + Earle's medium (Invitrogen/Gibco) and HCT116 cells in McCoy's 5a media (Invitrogen/Gibco) with 10% fetal bovine serum (Invitrogen/Gibco) at 37˚C in a 5% CO_2-humidified atmosphere.

Expression vector constructs. For protein expression in human cells, the entire human MLH3, PMS2 and MLH1 cDNAs were cloned into pEGFP-N1 plasmid (BD Biosciences) between BamHI and NotI restriction sites so that the EGFP gene was replaced. Cloning was done as previously described (16). The expression constructs were verified by sequencing and named as phMLH3-N1, phPMS2-N1 and phMLH1-N1.

Transfections. For transfection, 30000 cells were seeded on a 6-well plate for 4 h. The transfection was performed by using a total of 1 μg of DNA with 3 μl of FuGENE 6 reagent (Roche Applied Science) per well, according to the manufacturer's instructions. In HeLa cells, we expressed phMLH3-N1 separately and together with phMLH1-N1. In HCT116 cells, each three expression vectors were transfected separately and phMLH3-N1 also together with phMLH1-N1 and with phPMS2-N1.

Immunofluorescence staining and microscopy. Twenty-four hours after transfection cells were washed with PBS, fixed with ice cold methanol for 15 min at -20˚C, and again washed with PBS. The cells were blocked with 1% bovine serum albumin (Sigma Aldrich) in PBS at 37˚C for at least 1 h. Incubations with primary and secondary antibodies were performed at 37˚C for 1 h each. Antibodies were diluted in PBS including 1% bovine serum albumin as follows: anti-MLH1 G168-728 (BD Biosciences Pharmingen), 1 μg/ml; anti-PMS2 clone A16-4 (BD Biosciences Pharmingen), 2 μg/ml; anti-MLH3 sc-25313 (Santa Cruz Biotechnology), 10 μg/ml; and the secondary antibody fluorescein-conjugated AffinityPure donkey anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Labs Inc.), 4 μg/ml. The nuclei were stained by incubating cells in PBS with 300 nM 4',6'-diamindino-2-phenylindole (DAPI) (Sigma Aldrich) for 3 min. Slides were mountained with Fluorescence Mounting Medium (Dako).

Subcellular localization of recombinant proteins was analyzed by direct fluorescence using Axioplan 2 microscope (Zeiss) with 63x and 100x objectives. Each experiment was done at least three times, and at least 100 cells from each individual experiment were analyzed. Representative images were taken with Isis 5 software (Metasystems) and processed with Adobe Photoshop 7.0 (Adobe).

Results

Subcellular localization of endogenous hMLH3. Subcellular localization of endogenous MutL homologues, hMLH3, hPMS2 and hMLH1 was studied in HeLa and HCT116 cells, which express all the three proteins or only hMLH3, respectively. When all the three homologues were natively expressed, endogenous hMLH1 and hPMS2 localize in the nucleus, while hMLH3 stays in the cytoplasm (Fig. 1A). Also in HCT116 cells, which lack hMLH1 and hPMS2, hMLH3 stays in the cytoplasm (Fig. 1B). Most probably because of its low amount *in vivo* (15) hMLH3 protein was detectable only in about 70% of the analyzed cells.

Subcellular localization of transfected hMLH3. For human cell transfections, we constructed expression vectors phMLH3-N1, phPMS2-N1 and phMLH1-N1, which include the full-length hMLH3, hPMS2 and hMLH1, respectively. The expression level of hMLH3 varied between cells but in about 50% of the stained cells the amount was high enough to be analyzed. After co-transfection of hMLH3 and hMLH1 into HeLa cells, in which endogenous hMLH3 was shown to stay in the cytoplasm (see above), all the three proteins were located in the nucleus, though hMLH3 only partially (Fig. 2A). In HCT116 cells, which lack hMLH1 and hPMS2, hMLH3 stayed in the cytoplasm also after transfection (Fig. 2B). After co-transfection with hMLH1, it was partially nuclear, as in HeLa cells (Fig. 2C). When hMLH3 and hPMS2 were co-transfected into HCT116 cells both proteins stayed in the cytoplasm (Figure not shown).

Discussion

Germline mutations in the *hMLH3* gene are reported to predispose to HNPCC syndrome. Nineteen different hMLH3 variations are found from putative HNPCC patients, 17 amino acid substitutions and 2 amino acid deletions [(4,5), http://www.insight-group.org/]. Ten different mutations were found in families fulfilling the Amsterdam clinical criteria for HNPCC (17) and most of the studied tumours showed high microsatellite instability (MSI). However, a segregation analysis was not done in families and 3 out of 12 patients carried also germline mutations in *hMSH6* (4), which complicates the interpretation of their pathogenicity. In another study, *hMLH3* mutations were found in low-risk patients, whose families displayed diverse and less typical clinical features, and none of the tumours showed MSI (5). The MSI phenotype

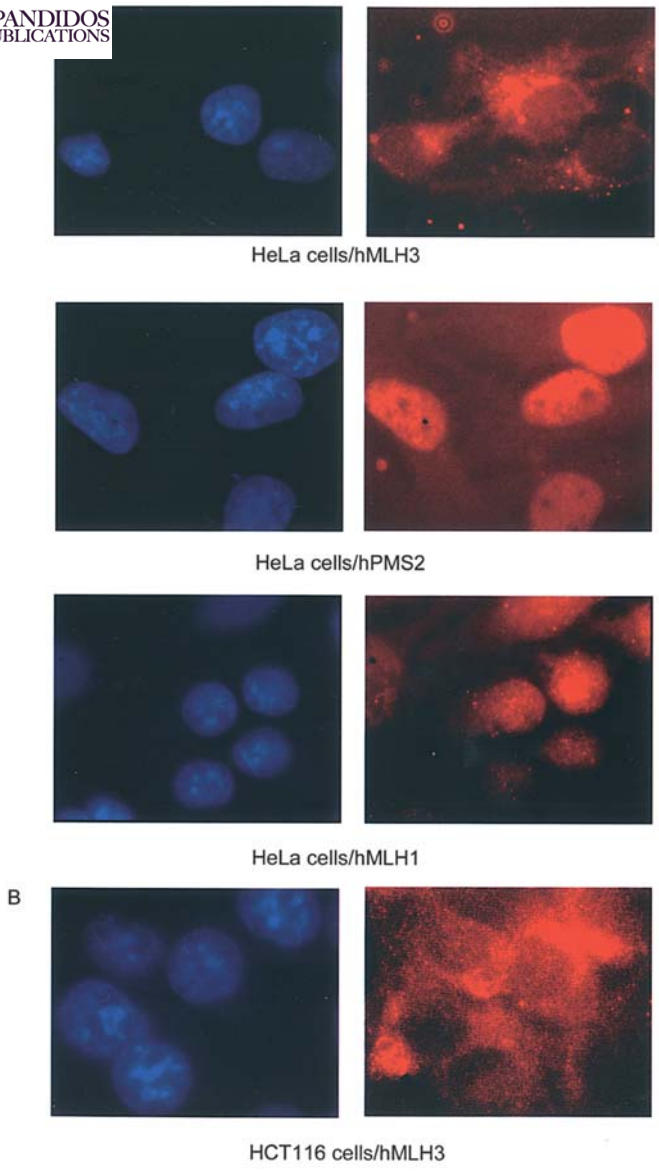

Figure 1. (A), Immunofluorescence analysis to detect the subcellular localization of endogenous hMLH3, hPMS2 and hMLH1. Left panel shows the nuclei stained with DAPI and right panel shows the same cells detected with immunofluorescence. In HeLa cells, the hMLH3 protein is located in the cytoplasm, whereas hPMS2 and hMLH1 are located in the nucleus. (B), In HCT116 cells, which lack hMLH1 and hPMS2, hMLH3 is located in the cytoplasm.

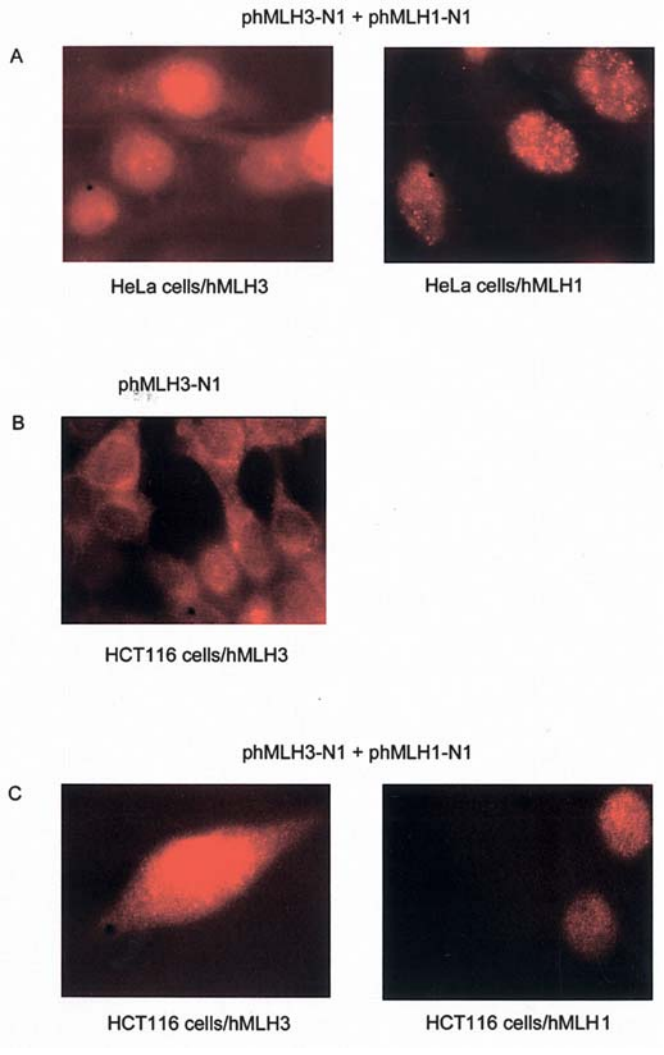

Figure 2. (A), Immunofluorescence analysis of HeLa cells to detect the subcellular localization of hMLH3 and hMLH1 after co-transfection with expression vectors phMLH3-N1 and phMLH1-N1. The hMLH3 protein is located partially and hMLH1 totally in the nucleus. (B), In HCT116 cells, after cell transfection with expression vector, phMLH3-N1, hMLH3 stays in the cytoplasm. (C), In HCT116 cells, after co-transfection with expression vectors phMLH3-N1 and phMLH1-N1, the hMLH3 protein is located partially and hMLH1 totally in the nucleus.

typical to HNPCC tumours is a consequence of MMR defect in the cell. Overall, the clinical data including many tumours without MSI suggests that *hMLH3* may have a role in MMR but only as a low-risk gene in familial colorectal cancer. To assess the pathogenicity of the hMLH3 variations, more information is needed of its functional role *in vivo*.

Since MMR occurs in the nucleus, nuclear import is suggested to be a mechanism to regulate MMR (18). A previous study performed with mouse proteins showed that Pms2 expressed alone does not reside in the nucleus because of impaired nuclear import and that dimerization of Mlh1 and Pms2 is essential and limits nuclear localization of MutLα (19). In human, hMLH1 interacts with 36 homologous amino acid residues within hPMS2, hMLH3 and hPMS1 (20) supporting the idea of their redundant function in MMR. So far, only hMutLα (hMLH1/hPMS2) was shown to be involved in human MMR (21). The hMutLß (hMLH1/hPMS1) complex was demonstrated to be totally deficient in an *in vitro* MMR assay (22), whereas hMutLγ (hMLH1/hMLH3), when expressed in adequate amounts, seems to have an ability to repair base-base mismatches and single extrahelical nucleotides *in vitro* (15). In this study, we examined the nuclear localization of hMLH3 with and without hMLH1 or hPMS2 to evaluate the contribution of hMLH3 to MMR. To be functional *in vivo* MMR mechanism, hMLH3 ought to localize in the nucleus. Consistent with previously published data that the endogenous hMLH3 is ~60 times less abundant than hPMS2 (15), the hMLH3 protein was not always detected by immunofluorescence. Although, its fluctuating expression levels in different cells (15) hampered quantitative analysis, our analyses clearly show that when all the three MutL homologues are natively expressed in human cells (HeLa), endogenous hMLH1 and hPMS2 localize in the nucleus, while hMLH3 stays in the

cytoplasm. Moreover, if hMLH3 is expressed alone (HCT116), it stays in the cytoplasm, whereas co-expression with hMLH1 results in its partial nuclear localization. The results indicate that the nuclear import of hMLH3 is dependent on interaction with hMLH1, resembling the nuclear import of Pms2 and Mlh1 in the mouse (19). Remarkably, in HeLa cells, which express all the three MutL homologues, the partial nuclear localization of hMLH3 was detected only after co-transfection of hMLH3 and hMLH1, suggesting that hPMS2 and hMLH3 are competing for hMLH1 and that the amount of endogenous hMLH1 is not enough for hMLH3.

In two recent reports, a reconstitution of human MMR system was described to need seven purified human activities including hMutSα (hMSH2/hMSH6) or hMutSß (hMSH2/hMSH3) and hMutLα (hMLH1/hPMS2) (1,2). The hMutSß complex was shown to play a limited role in repair of base-base mismatches, but it processed insertion/deletion mispairs much more efficiently than hMutSα, which efficiently corrected both types of mismatches. This explains, why mutations in hMSH2 and hMLH1 account for most HNPCC families, whereas mutations in hMSH6 and hMSH3 are associated with no or low cancer susceptibility. Correspondingly, the functional overlap of hMLH3 and hPMS2 could explain why hPMS2 and hMLH3 mutations are only rarely found in HNPCC families. This assumption originally supported by results from the yeast studies (13) was recently strengthen by studies, in which the heterodimer of Mlh1 and Mlh3 was shown to contribute to mechanisms of tumour suppression in the mouse (14), and a human homologue, hMutLγ, with low efficiency to be able to repair mismatches *in vitro* (15).

To address the question of whether hMutLα and hMutLγ could have similar partially redundant roles in MMR as hMutSα and hMutSß, we studied subcellular localization of the three MutL homologues, hMLH3, hPMS2 and hMLH1, in human cells. Although, the expression level of hMLH3 was previously detected to be unaffected by hMLH1 or hPMS2 levels (15), our study demonstrates that nuclear localization of hMLH3 is dependent on hMLH1 expression and affected by abundance of hPMS2 in the cell. The continuous nuclear localization of hMLH1 and hPMS2 suggests that *in vivo*, hPMS2 (hMutLα) has a major activity in MMR. In absence of hPMS2, hMLH3 (hMutLγ) is located in the nucleus, suggesting a conditional activity in MMR and supporting its role as a low-risk gene in HNPCC.

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References

1. Constantin N, Dzantiev L, Kadyrov FA and Modrich P: Human mismatch repair reconstitution of a nick-directed bidirectional reaction. J Biol Chem 280: 39752-39761, 2005.
2. Zhang Y, Yuan F, Presnell SR, Tian K, Gao Y, Tomkinson AE, Gu L and Li G-M: Reconstitution of 5'-directed human mismatch repair in a purified system. Cell 122: 693-705, 2005.
3. Peltomäki P: Role of DNA mismatch repair defects in the pathogenesis of human cancer. J Clin Oncol 21: 174-179, 2003.
4. Wu Y, Berends MJW, Sijmons RH, Mensink RGJ, Verlind E, Kooi KA, van der Sluis T, Kempinga C, van der Zee AGJ, Hollema H, Buys CHCM, Kleibeuker JH and Hofstra RMW: A role for MLH3 in hereditary non-polyposis colorectal cancer. Nature Genet 29: 137-138, 2001.
5. Liu H-X, Zhou X-L, Liu T, Werelius B, Lindmark G, Dahl N and Lindblom A: The role of hMLH3 in familial colorectal cancer. Cancer Res 63: 1894-1899, 2003.
6. Jiricny J and Nyström-Lahti M: Mismatch repair defects in cancer. Curr Opin Genet Dev 10: 157-161, 2000.
7. Lipkin SM, Wang V, Stoler DL, Anderson GR, Kirch I, Hadley D, Lynch HT and Collins FS: Germline and somatic mutation analysis in the DNA mismatch repair gene MLH3: evidence for somatic mutation in colorectal cancer. Hum Mutat 17: 389-396, 2001.
8. Hienonen T, Laiho P, Salovaara R, Mecklin J-P, Järvinen H, Sistonen P, Peltomäki P, Lehtonen R, Nupponen NN, Launonen V, Karhu A and Aaltonen LA: Little evidence for involvement of MLH3 in colorectal cancer predisposition. Int J Cancer 106: 292-296, 2003.
9. Marsischky GT, Filosi N, Kane MF and Kolodner R: Redundancy of Saccharomyces cerevisiae MSH3 and MSH6 in MSH2-dependent mismatch repair. Genes Dev 10: 407-420, 1996.
10. Huang J, Kuismanen SA, Liu T, Chadwick RB, Johnson CK, Stevens MW, Richards SK, Meek JE, Gao X, Wright FA, Mecklin JP, Järvinen HJ, Gronberg H, Bisgaard ML, Lindblom A and Peltomäki P: MSH6 and MSH3 are rarely involved in genetic predisposition to non-polypotic colon cancer. Cancer Res 61: 1619-1623, 2001.
11. Kariola R, Raevaara TE, Lönnqvist KE and Nyström-Lahti M: Functional analysis of MSH6 mutations linked to kindreds with putative hereditary non-polyposis colorectal cancer syndrome. Hum Mol Genet 11: 1303-1310, 2002.
12. Kariola R, Hampel H, Frankel WL, Raevaara TE, de la Chapelle A and Nyström-Lahti M: MSH6 missense mutations are often associated with no or low cancer susceptibility. Br J Cancer 91: 1287-1292, 2004.
13. Kolodner RD and Marsischky GT: Eukaryotic DNA mismatch repair. Curr Opin Genet Dev 9: 89-96, 1999.
14. Chen PC, Dudley S, Hagen W, Dizon D, Paxton L, Reichow D, Yoon SR, Yang K, Arnheim N, Liskay RM and Lipkin SM: Contributions by MutL homologues Mlh3 and Pms2 to DNA mismatch repair and tumor suppression in the mouse. Cancer Res 65: 8662-8670, 2005.
15. Cannavo E, Marra G, Sabates-Bellver J, Menigatti M, Lipkin SM, Fischer F, Cejka P and Jiricny J: Expression of the MutL homologue hMLH3 in human cells and its role in DNA mismatch repair. Cancer Res 65: 10759-10766, 2005.
16. Raevaara TE, Vaccaro C, Abdel-Rahman WM, Mocetti E, Bala S, Lönnqvist KE, Kariola R, Lynch HT, Peltomäki P and Nyström-Lahti M: Pathogenicity of the hereditary colorectal cancer mutation hMLH1 del616 linked to shortage of the functional protein. Gastroenterology 125: 501-509, 2003.
17. Vasen HF, Watson P, Mecklin JP and Lynch HT: New clinical criteria for hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology 116: 1453-1456, 1999.
18. Brieger A, Plotz G, Raedle J, Weber N, Baum W, Caspary WF, Zeuzem S and Trojan J: Characterization of the nuclear import of human MutLalpha. Mol Carcinog 43: 51-58, 2005.
19. Wu X, Platt JL and Cascalho M: Dimerization of MLH1 and PMS2 limits nuclear localization of MutLα. Mol Cell Biol 23: 3320-3328, 2003.
20. Kondo E, Horii A and Fukushige S: The interacting domains of three MutL heterodimers in man: hMLH1 interacts with 36 homologous amino acid residues within hMLH3, hPMS1 and hPMS2. Nucleic Acids Res 29: 1695-1702, 2001.
21. Li GM and Modrich P: Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human MutL homologs. Proc Natl Acad Sci USA 92: 1950-1954, 1995.
22. Räschle M, Marra G, Nyström-Lahti M, Schär P and Jiricny J: Identification of hMutLß, a heterodimer of hMLH1 and hPMS1. J Biol Chem 274: 32368-32375, 1999.