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***PMS2* expression decrease causes severe problems in mismatch repair**

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Abstract

PMS2 is one of the four susceptibility genes in Lynch syndrome (LS), the most common cancer syndrome in the world. Inherited mutations in DNA mismatch repair (MMR) genes, *MLH1*, *MSH2*, and *MSH6*, account for approximately 90% of LS, while a relatively small number of LS families segregate a *PMS2* mutation. This and the low cancer penetrance in *PMS2* families suggest that *PMS2* is only a moderate or

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low risk susceptibility gene. We have previously shown that even a partial expression decrease in *MLH1*, *MSH2* or *MSH6* suggests that heterozygous LS mutation carriers have MMR malfunction in constitutive tissues. Whether and how *PMS2* expression decrease affects the repair capability is not known. Here, we show that *PMS2* knockdown cells retaining 19%, 33% or 53% of *PMS2* expression all have significantly reduced MMR efficiency. Surprisingly, the cells retaining expression levels comparable to *PMS2* mutation carriers indicate the lowest repair efficiency.

Key Words:

Lynch syndrome; *PMS2*; mismatch repair; colorectal cancer; mRNA expression

As a component of the MutL α (MLH1+PMS2) heterodimer, PMS2 (postmeiotic segregation increased 2) is central in the post-replicative human DNA mismatch repair (MMR) mechanism (Kunkel & Erie, 2005). Heterozygous germline mutations in MMR genes cause Lynch syndrome (LS; MIM# 120435), the most common cancer predisposition syndrome in human (de la Chapelle, 2004; Lynch, Snyder, Shaw, Heinen, & Hitchins, 2015). Mutations in *MLH1* (MIM# 120436) and *MSH2* (MIM# 609309) are the most prevalent in known LS families (36.2% and 35.3%, respectively), while the number of *MSH6* (MIM# 600678) and *PMS2* (MIM# 600259) mutations is lower (19.4% and 9.1%, respectively) (www.insight-database.org, data accessed 22.01.2019). The absence of functional MMR protein results in DNA repair malfunction, which is the cause of early onset colorectal and endometrial cancers and less frequently cancer of the ovaries, stomach, pancreas, prostate and breast in LS families (Lynch et al., 2015). However, in *PMS2* families the prevalence of stomach, prostate and breast cancers is unusually high and compared to *MLH1* and *MSH2* families, also the mean age of cancer onset is higher and the

penetrance of colorectal and endometrial cancers lower, resembling the situation in *MSH6* families (Hendriks et al., 2006; Roberts et al., 2018; Senter et al., 2008; ten Broeke et al., 2015; Truninger et al., 2005). One explanation for the lower cancer penetrance in *PMS2* families might be the protein homologue MLH3, which is also able to bind and partially function with MLH1 (Cannavo et al., 2005; Korhonen, Raevaara, Lohi, & Nystrom, 2007; Senter et al., 2008; ten Broeke et al., 2015).

The diagnostic workflow to identify Lynch syndrome typically involves tumor studies and DNA analyses. The first clinical step in diagnosing LS includes tumor immunohistochemistry (IHC) and microsatellite instability (MSI) analyses followed by mutation screening dictated by the IHC and MSI results (Kansikas, Kariola, & Nystrom, 2011; Yurgelun & Hampel, 2018). While IHC is a valuable method for distinguishing *PMS2* carriers, who generally lose only *PMS2*, from *MLH1* carriers, who frequently lose both MLH1 and *PMS2* protein expressions in a tumor (Niessen et al., 2009), it took a decade to differentiate sequence between *PMS2* and its 15 pseudogenes for reliable sequencing and mutation detection (De Vos, Hayward, Picton, Sheridan, & Bonthron, 2004; van der Klift et al., 2010; Vaughn et al., 2010). As an outcome, the prevalence of *PMS2* mutations in suspected LS families has increased according to the InSiGHT database, from approximately 2% to 9% during the last two decades (www.insight-database.org). Furthermore, the population-based *PMS2* variant prevalence was recently reported to be relatively high (1:714) compared to the variant prevalence in *MLH1* (1:1946) and *MSH2* (1:2841) (Win et al., 2017) and a strikingly high prevalence (96%) was reported from an Icelandic population using whole genome sequencing data of a colorectal cancer cohort (Haraldsdottir et al., 2017).

Nevertheless, atypical clinical phenotypes and a low cancer penetrance in *PMS2* families still question its role in Lynch syndrome. Hence, this study aimed to determine the significance of *PMS2* as an LS susceptibility gene by analyzing whether a partial *PMS2* expression decrease, comparable to the expression decrease in a Lynch syndrome mutation carrier, causes mismatch repair malfunction, as was previously shown to be the case with the susceptibility genes *MLH1*, *MSH2* and *MSH6*.

Accordingly, stable shRNA mediated *PMS2* knockdown (KD) clones were established and characterized by real-time PCR (RT-Q-PCR), and the clones with reduced *PMS2* expression were included in functional MMR analyses (Supporting Materials, Materials & Methods). Each KD clone had its own specific control across all studies to avoid experimental artefacts. A prerequisite for the functional analysis was that the *PMS2* mRNA expression level should be at most ~50% of the normal level, a level comparable to that of an LS mutation carrier. From a total of 16 clones, the three retaining 19%, 33% and 53% of *PMS2* mRNA expression met the criteria for further MMR analysis.

In order to be able to assess the repair efficiency of each clone, the nuclear proteins of the cells in chosen clones and their controls were extracted in parallel as described previously (Holmes, Clark, & Modrich, 1990; Kantelinen et al., 2010). The nuclear protein expression was analyzed by Western blot (WB) (Supporting Materials, Materials & Methods) (**Figure 1**). Despite the semi-quantitative nature of the method, each KD clone clearly showed decreased *PMS2* protein expression compared to the level in its respective control (NC), indicating that the amount of protein reflects the reduced mRNA expression in the cell extract. Importantly, the *MLH1* protein expression was shown to be normal in the KD extracts.

For a long time, *PMS2* mutation prevalence was underestimated, suggesting that *PMS2* is not an important LS susceptibility gene. Its significance in LS has been questioned by the atypically low cancer penetrance in *PMS2* families (Senter et al., 2008; ten Broeke et al., 2015), an unclear risk for extra-colonic cancers (Roberts et al., 2018; Ten Broeke et al., 2018), as well as inconsistencies across studies due to variable cohort sizes and carrier ascertainment (compliant with Amsterdam II criteria and/or revised Bethesda Guidelines) (Senter et al., 2008; ten Broeke et al., 2015; Ten Broeke, Suerink, & Nielsen, 2018; Ten Broeke et al., 2018). For these reasons, *PMS2*-specific surveillance methods remain under debate (Espenschied et al., 2017; Ten Broeke et al., 2018).

Nevertheless, over 20% of *PMS2* carriers are still estimated to be missed with the conventional criteria used for LS identification (Senter et al., 2008; ten Broeke et al., 2015). The IHC- and MSI- studies help the pathogenicity assessment since up to 99% of *PMS2* carriers have been shown to lack *PMS2* in tumor tissue, and 98% of tumors have been characterized as MSI-high (Goodenberger et al., 2016). Since both these tumor phenotypes signal MMR deficiency, it was anticipated that *PMS2* expression decrease should also interfere with MMR capability of the cells, as was previously shown to be the case with the other LS susceptibility genes (Kansikas, Kasela, Kantelinen, & Nystrom, 2014). Indeed, the functional *in vitro* MMR assay (Nystrom-Lahti et al., 2002; Raevaara et al., 2003) (Supporting Materials, Materials & Methods) demonstrated lowered MMR efficiency in cells with reduced *PMS2* mRNA expression, while all controls maintained higher repair proficiencies throughout three independent assays (**Figure 2a**). The reduction in MMR capability in cells retaining 19%, 33% or 53% of *PMS2* mRNA expression was statistically significant (according to Student's T-Test) in all, showing 21%, 15% and 12% of average repair efficiency,

while their controls showed 38%, 55% and 48% of repair capability, respectively ($p = 0.002, 0.001, \text{ and } 0.0002$) (**Figure 2a**). Interestingly, the most severe repair defect was in cells retaining 53% of *PMS2* expression. The relative repair efficiencies (RR%) of cells retaining 19%, 33% and 53% of *PMS2* mRNA expression were 55%, 27% and 25%, respectively (**Figure 2b**), confirming the finding that the KD cells with 53% *PMS2* expression had the lowest repair capability.

Previously, a significant reduction in MMR efficiency was detected in cells retaining 75% of *MSH6*, 50% of *MSH2*, and 25% of *MLH1* mRNA expression (Kansikas et al., 2014). In this study, we show that *PMS2* expression decrease also affects MMR capability but unexpectedly in a different way than what was observed with the other MMR genes. Surprisingly, the lower *PMS2* expression levels (19% and 33%) had higher repair efficiencies than the carrier-like level (53%), while in *MLH1* and *MSH2*, 25% expression level was not enough to maintain any repair capability (25% level in *MSH6* was not studied) (Kansikas et al., 2014). The severity of *MLH1* and *MSH2* expression decrease may be due to their obligatory roles in the heterodimeric protein complexes (Acharya et al., 1996; Kondo, Horii, & Fukushige, 2001), while such a low level of *PMS2* may be compensated by some homologous protein. Compensation of MutL α with MutL γ (MLH1+MLH3) has been suggested to explain the low penetrance of *PMS2* mutations (Cannavo et al., 2005; Korhonen et al., 2007). Although *PMS2* is the main partner of MLH1 among MutL homologues, MutL γ has shown at least low MMR capability (Cannavo et al., 2005; Raschle, Marra, Nystrom-Lahti, Schar, & Jiricny, 1999). The nuclear localization of MLH3 seems to be dependent on the absence of *PMS2* in the cell (Korhonen et al., 2007), and while excess of MLH3 has been shown to reduce *PMS2* binding to MLH1, most probably MLH3 is not a significant counterpart to MLH1 in a normal cell due to the 60-fold

higher expression of PMS2 (Cannavo et al., 2005; Kondo et al., 2001). To determine whether MLH3 could compensate MMR efficiency in 19% and 33% *PMS2* KD clones and improve their repair capability compared to the 53% clone, we further studied *MLH3* mRNA expression and protein expression in the clones. Although, the *MLH3* mRNA expression levels were decreased, they did not differ between the KD cells (Supporting Materials, **Supp. Figure S1**), even though slightly lowered MLH3 protein expression in 53% KD clone compared to 19% clone was detected by WB (Supporting Materials, **Supp. Figure S2**). While MLH3 expression differences do not explain the lowered MMR efficiency in KD clones, it is still possible that the cells expressing only 19% or 33% of *PMS2* are able to activate a “survival mechanism” that sustains MMR capability.

By using a functional approach to assess the significance of *PMS2* in Lynch syndrome, we show that *PMS2* expression decrease causes severe problems in mismatch repair, which is the hallmark of LS susceptibility genes.

Disclosure statement: Nyström and Kansikas are inventors on patent number PCT/EP2012/062708. Nyström is a shareholder and a board member and Kansikas and Kasela are employees of LS CancerDiag Ltd.

References

- Acharya, S., Wilson, T., Gradia, S., Kane, M. F., Guerrette, S., Marsischky, G. T., . . . Fishel, R. (1996). hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. *Proceedings of the National Academy of Sciences of the United States of America*, 93(24), 13629-13634.

- Cannavo, E., Marra, G., Sabates-Bellver, J., Menigatti, M., Lipkin, S. M., Fischer, F., . . . Jiricny, J. (2005). Expression of the MutL homologue hMLH3 in human cells and its role in DNA mismatch repair. *Cancer Research*, *65*(23), 10759-10766. doi:65/23/10759 [pii]
- de la Chapelle, A. (2004). Genetic predisposition to colorectal cancer. *Nature Reviews.Cancer*, *4*(10), 769-780. doi:nrc1453 [pii]
- De Vos, M., Hayward, B. E., Picton, S., Sheridan, E., & Bonthron, D. T. (2004). Novel PMS2 pseudogenes can conceal recessive mutations causing a distinctive childhood cancer syndrome. *American Journal of Human Genetics*, *74*(5), 954-964. doi:10.1086/420796 [doi]
- Espenschied, C. R., LaDuca, H., Li, S., McFarland, R., Gau, C. L., & Hampel, H. (2017). Multigene panel testing provides a new perspective on lynch syndrome. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *35*(22), 2568-2575. doi:10.1200/JCO.2016.71.9260 [doi]
- Goodenberger, M. L., Thomas, B. C., Riegert-Johnson, D., Boland, C. R., Plon, S. E., Clendenning, M., . . . Lindor, N. M. (2016). PMS2 monoallelic mutation carriers: The known unknown. *Genetics in Medicine : Official Journal of the American College of Medical Genetics*, *18*(1), 13-19. doi:10.1038/gim.2015.27 [doi]
- Haraldsdottir, S., Rafnar, T., Frankel, W. L., Einarsdottir, S., Sigurdsson, A., Hampel, H., . . . Stefansson, K. (2017). Comprehensive population-wide analysis of lynch syndrome in iceland reveals founder mutations in MSH6 and PMS2. *Nature Communications*, *8*, 14755. doi:10.1038/ncomms14755 [doi]

- Hendriks, Y. M., Jagmohan-Changur, S., van der Klift, H. M., Morreau, H., van Puijenbroek, M., Tops, C., . . . Wijnen, J. T. (2006). Heterozygous mutations in PMS2 cause hereditary nonpolyposis colorectal carcinoma (lynch syndrome). *Gastroenterology*, *130*(2), 312-322. doi:S0016-5085(05)02232-8 [pii]
- Holmes, J., Jr, Clark, S., & Modrich, P. (1990). Strand-specific mismatch correction in nuclear extracts of human and drosophila melanogaster cell lines. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(15), 5837-5841.
- Kansikas, M., Kariola, R., & Nystrom, M. (2011). Verification of the three-step model in assessing the pathogenicity of mismatch repair gene variants. *Human Mutation*, *32*(1), 107-115. doi:10.1002/humu.21409 [doi]
- Kansikas, M., Kasela, M., Kantelinen, J., & Nystrom, M. (2014). Assessing how reduced expression levels of the mismatch repair genes MLH1, MSH2, and MSH6 affect repair efficiency. *Human Mutation*, *35*(9), 1123-1127. doi:10.1002/humu.22605 [doi]
- Kantelinen, J., Kansikas, M., Korhonen, M. K., Ollila, S., Heinimann, K., Kariola, R., & Nystrom, M. (2010). MutSbeta exceeds MutSalpha in dinucleotide loop repair. *British Journal of Cancer*, *102*(6), 1068-1073. doi:10.1038/sj.bjc.6605531 [doi]
- Kondo, E., Horii, A., & Fukushige, S. (2001). The interacting domains of three MutL heterodimers in man: hMLH1 interacts with 36 homologous amino acid residues within hMLH3, hPMS1 and hPMS2. *Nucleic Acids Research*, *29*(8), 1695-1702.
- Korhonen, M. K., Raevaara, T. E., Lohi, H., & Nystrom, M. (2007). Conditional nuclear localization of hMLH3 suggests a minor activity in mismatch repair and

supports its role as a low-risk gene in HNPCC. *Oncology Reports*, 17(2), 351-354.

Kunkel, T. A., & Erie, D. A. (2005). DNA mismatch repair. *Annual Review of Biochemistry*, 74, 681-710. doi:10.1146/annurev.biochem.74.082803.133243 [doi]

Lynch, H. T., Snyder, C. L., Shaw, T. G., Heinen, C. D., & Hitchins, M. P. (2015). Milestones of lynch syndrome: 1895-2015. *Nature Reviews.Cancer*, 15(3), 181-194. doi:10.1038/nrc3878 [doi]

Niessen, R. C., Kleibeuker, J. H., Westers, H., Jager, P. O., Rozeveld, D., Bos, K. K., . . . Hofstra, R. M. (2009). PMS2 involvement in patients suspected of lynch syndrome. *Genes, Chromosomes & Cancer*, 48(4), 322-329. doi:10.1002/gcc.20642 [doi]

Nystrom-Lahti, M., Perrera, C., Raschle, M., Panyushkina-Seiler, E., Marra, G., Curci, A., . . . Jiricny, J. (2002). Functional analysis of MLH1 mutations linked to hereditary nonpolyposis colon cancer. *Genes, Chromosomes & Cancer*, 33(2), 160-167. doi:10.1002/gcc.1225 [pii]

Raevaara, T. E., Vaccaro, C., Abdel-Rahman, W. M., Mocetti, E., Bala, S., Lonnqvist, K. E., . . . Nystrom-Lahti, M. (2003). Pathogenicity of the hereditary colorectal cancer mutation hMLH1 del616 linked to shortage of the functional protein. *Gastroenterology*, 125(2), 501-509. doi:S0016508503009053 [pii]

Raschle, M., Marra, G., Nystrom-Lahti, M., Schar, P., & Jiricny, J. (1999). Identification of hMutLbeta, a heterodimer of hMLH1 and hPMS1. *The Journal of Biological Chemistry*, 274(45), 32368-32375.

- Roberts, M. E., Jackson, S. A., Susswein, L. R., Zeinomar, N., Ma, X., Marshall, M. L., . . . Chung, W. K. (2018). MSH6 and PMS2 germ-line pathogenic variants implicated in lynch syndrome are associated with breast cancer. *Genetics in Medicine : Official Journal of the American College of Medical Genetics*, doi:10.1038/gim.2017.254 [doi]
- Senter, L., Clendenning, M., Sotamaa, K., Hampel, H., Green, J., Potter, J. D., . . . de la Chapelle, A. (2008). The clinical phenotype of lynch syndrome due to germ-line PMS2 mutations. *Gastroenterology*, 135(2), 419-428. doi:10.1053/j.gastro.2008.04.026 [doi]
- ten Broeke, S. W., Brohet, R. M., Tops, C. M., van der Klift, H. M., Velthuisen, M. E., Bernstein, I., . . . Wijnen, J. T. (2015). Lynch syndrome caused by germline PMS2 mutations: Delineating the cancer risk. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, 33(4), 319-325. doi:10.1200/JCO.2014.57.8088 [doi]
- Ten Broeke, S. W., Suerink, M., & Nielsen, M. (2018). Response to roberts et al. 2018: Is breast cancer truly caused by MSH6 and PMS2 variants or is it simply due to a high prevalence of these variants in the population? *Genetics in Medicine : Official Journal of the American College of Medical Genetics*, doi:10.1038/s41436-018-0029-1 [doi]
- Ten Broeke, S. W., van der Klift, H. M., Tops, C. M. J., Aretz, S., Bernstein, I., Buchanan, D. D., . . . Win, A. K. (2018). Cancer risks for PMS2-associated lynch syndrome. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, 36(29), 2961-2968. doi:10.1200/JCO.2018.78.4777 [doi]

- Truninger, K., Menigatti, M., Luz, J., Russell, A., Haider, R., Gebbers, J. O., . . .
Marra, G. (2005). Immunohistochemical analysis reveals high frequency of
PMS2 defects in colorectal cancer. *Gastroenterology*, *128*(5), 1160-1171.
doi:S001650850500168X [pii]
- van der Klift, H. M., Tops, C. M., Bik, E. C., Boogaard, M. W., Borgstein, A. M.,
Hansson, K. B., . . . Wijnen, J. T. (2010). Quantification of sequence exchange
events between PMS2 and PMS2CL provides a basis for improved mutation
scanning of lynch syndrome patients. *Human Mutation*, *31*(5), 578-587.
doi:10.1002/humu.21229 [doi]
- Vaughn, C. P., Robles, J., Swensen, J. J., Miller, C. E., Lyon, E., Mao, R., . . .
Samowitz, W. S. (2010). Clinical analysis of PMS2: Mutation detection and
avoidance of pseudogenes. *Human Mutation*, *31*(5), 588-593.
doi:10.1002/humu.21230 [doi]
- Win, A. K., Jenkins, M. A., Dowty, J. G., Antoniou, A. C., Lee, A., Giles, G. G., . . .
MacInnis, R. J. (2017). Prevalence and penetrance of major genes and polygenes
for colorectal cancer. *Cancer Epidemiology, Biomarkers & Prevention : A
Publication of the American Association for Cancer Research, Cosponsored by
the American Society of Preventive Oncology*, *26*(3), 404-412. doi:10.1158/1055-
9965.EPI-16-0693 [doi]
- Yurgelun, M. B., & Hampel, H. (2018). Recent advances in lynch syndrome:
Diagnosis, treatment, and cancer prevention. *American Society of Clinical
Oncology Educational Book.American Society of Clinical Oncology.Annual
Meeting*, (38):101-109. doi(38), 101-109. doi:10.1200/EDBK_208341 [doi]

Figures

Figure 1. Western blot analysis of KD extracts with reduced *PMS2* mRNA expression

Western Blot analysis was used to evaluate PMS2 protein expression of KD extracts and their controls. Commercial HCT116 cell line (ATCC[®] CCL-247[™]) lacks both MLH1 and PMS2 protein and served as a negative control, α -tubulin was used as a loading control. The results demonstrated decreased PMS2 protein expression in KD extracts, while the amount of MLH1 remained normal. Moreover, PMS2 protein expression increased according to mRNA expression in the clones.

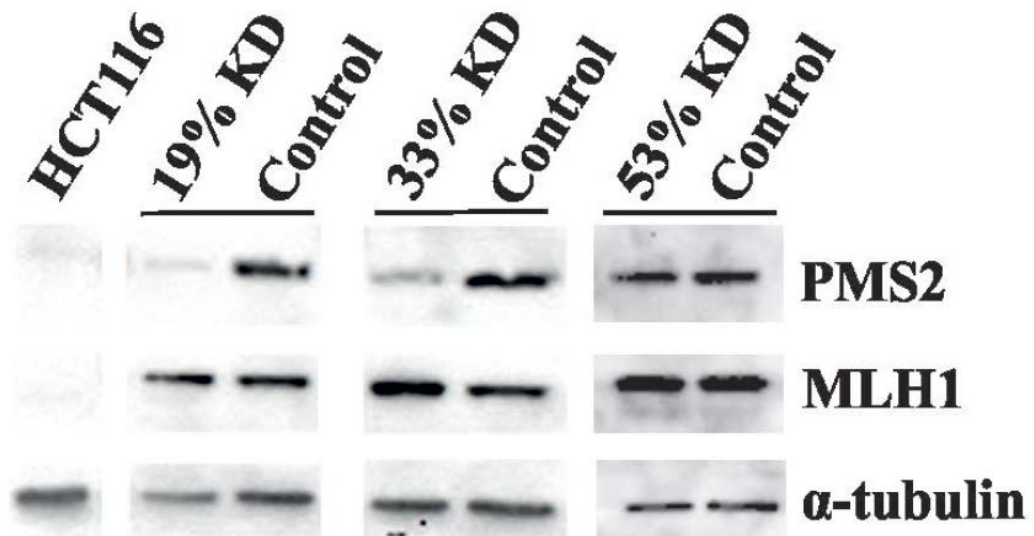


Figure 2. Functional MMR efficiency of KD extracts with reduced *PMS2* mRNA expression

a. In order to investigate the effects of reduced *PMS2* mRNA expression on the MMR capability, nuclear proteins extracted from KD cells were analyzed in the functional MMR assay. The average of absolute repair efficiencies (AR%_{n=3}) of KD extracts and their controls were calculated from three repetitions and are illustrated below the gel electrophoresis picture. A significant decrease in the MMR efficiency was observed in all extracts retaining 19%, 33% or 53% of *PMS2* mRNA expression compared to their respective controls ($p=0.002$; 0.001 ; 0.0002 , respectively).

b. Relative repair efficiencies (RR%) were calculated from the AR% values of the KD extracts relative to the AR% values of their respective controls (set to 100). These results further confirm the finding that the cells with 53% *PMS2* expression have the lowest repair capability.

