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Identification of germline *MLH1* alterations in familial prostate cancer

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ABSTRACT

Several linkage and loss of heterozygosity (LOH) analyses suggest that the region 3p21-p26, which is a chromosomal location of *MLH1*, could harbour a susceptibility gene for prostate cancer (PRCA). Furthermore, in a recent candidate single nucleotide polymorphism (SNP) analysis the I219V variation of the *MLH1* gene was associated with PRCA. Microsatellite instability (MSI) and germ-line *MLH1* mutations were originally demonstrated in hereditary non-polyposis colorectal cancer (HNPCC) but MSI and loss of *MLH1* function have also been detected in PRCA. To assess the contribution of *MLH1* germline mutations to the development of PRCA in Finland different approaches were used. First, the samples from 11 PRCA-colon cancer patients were screened for MLH1, MSH2 and MSH6 protein expression by immunohistochemistry (IHC). IHC revealed one patient with a putative *MLH1* aberration and sequencing of this sample revealed five sequence variants including two missense variants P434L and I219V. Second, the samples from Finnish hereditary prostate cancer (HPC) families were used for the screening of *MLH1* mutations which produced twelve *MLH1* sequence variants including two missense mutations, I219V, as in the PRCA-colon cancer patient, and V647M. P434L and V647 were both novel, rare variants. Carrier frequencies of the I219V mutation were compared between hereditary prostate cancer (HPC) patients, unselected PRCA cases, patients with benign prostate hyperplasia and controls, but no differences between the sample groups were found. P434L was not present in this study population and V647M was a very rare variant found only in one HPC family. According to the present results, *MLH1* does not have a major role in PRCA causation in Finland.

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1. Introduction

Numerous factors such as race, diet, environmental factors and family history have been considered to be risk factors for prostate cancer (PRCA).[1] Lichtenstein and colleagues[2] claim that approximately 42% of the risk of PRCA could be due to hereditary genetic factors. Despite the tremendous effort that has been put into hereditary prostate cancer (HPC) linkage studies to identify risk genes, only three susceptibility genes have been identified from the chromosomal regions mapped through these analyses: *ELAC2* at 17p11, *RNASEL* at 1q24-q25, and *MSR1* at 8p22-p23.[3–5] Although multiple chromosomal regions have been reported, only few regions have been confirmed in other independent studies. Very recently, three linkage analyses, including the one carried out on Finnish prostate cancer families, have shown positive linkage near the *MLH1* locus on 3p.[6–8] As *MLH1* is a known tumour suppressor gene, it therefore represents an immediate candidate gene for the detected locus. Initially, *MLH1* was associated with hereditary nonpolyposis colorectal cancer (HNPCC), where microsatellite instability (MSI) was observed with defects in the mismatch repair pathway (MMR). Inactivation of the MMR pathway in HNPCC patients is caused by germline mutation in one of the MMR genes, most often in *MLH1*[9] indicating its major role in guarding the genome integrity. Varying degrees of MSI (20–65%) and loss of the MMR proteins and down-regulation of MMR enzyme activityhave also been detected in PRCA,[10–14] suggesting a possible role for *MLH1* in prostatic carcinogenesis. *MLH1* maps to 3p22, a region also showing loss of heterozygosity (LOH) in human PRCA cell line [15] and clinical samples.[16] Furthermore, in a recent candidate single nucleotide polymorphism (SNP) analysis the variant I219V in *MLH1* showed an association with prostate cancer supporting the role for *MLH1* in prostate cancer predisposition.[17] Here, we report a study to evaluate the role of *MLH1* in PRCA predisposition in Finland.

2. Materials and methods

2.1. Patients with PRCA and colon cancer

A search of the discharge registry of the Tampere University Hospital revealed 355 PRCA patients with additional solid primary tumour (excluding skin malignancies except melanoma) from 1 January 1970 until 31 December 1999. Fifteen of the patients had both PRCA and colon cancer. Paraffin embedded prostate and/or colon cancer samples were available for analyses from 11 of them.

2.2. HPC families, patients with unselected PRCA and controls

Identification and collection of the Finnish HPC families have been described elsewhere.[6] For single-strand conformation polymorphism (SSCP) and minisequencing analyses samples from the youngest affected patient available in each of 121 families with HPC were used for the analysis. The families had either two or more affected members who were first- or second-degree relatives. The mean age at diagnosis for the index patients was 64.8 years (range 44–86 years), and the mean number of affected family members was 2.8 (range 2–7). For *MLH1* direct sequencing, genomic DNA samples from altogether 18 affected persons were selected, representing the six families with the best multipoint heterogeneity LOD scores (HLOD) (> 0.5) per family at 3p.[18]

MLH1 mutation frequencies were analysed among 200 unselected, consecutive patients with PRCA, 200 healthy male blood donors, and 202 patients diagnosed with benign prostate hyperplasia (BPH). The mean age at diagnosis for the unselected PRCA patients was 67.1 years (range 47–88). The DNA samples from unselected consecutive PRCA patients were collected from patients diagnosed with PRCA in 1999 in the Tampere University Hospital. Tampere University Hospital is a regional referral centre in the area for all patients with PRCA, which results in an unselected, population-based collection of patients. The population controls consisted of DNA samples from anonymous, voluntary and healthy male blood donors obtained from the Blood Center of the Finnish Red Cross in Tampere. The blood donor must be 18 to 65 years of age, which means that on average 8.5% of them will have a PRCA diagnosis later in life given the current incidence rates according to the Finnish Cancer Registry. The age-matched control group consisted of patients diagnosed with BPH in the Tampere University Hospital. The mean age for the BPH patients was 72.5 (range 48–93), which correlates with the mean age of diagnosis for the PRCA patients (67.1 years; range 47–88). The diagnosis of BPH was based on lower-urinary tract symptoms, free uroflowmetry, and evidence, by palpation or transrectal ultrasound, of increased prostate size. If PSA was elevated or digital rectal examination showed any abnormality indicative of PRCA the patients underwent biopsies to exclude diagnoses of PRCA, high-grade prostate intraepithelial neoplasia (PIN), atypical small acinar cell proliferation (ASAP), or suspicions of malignancy. Written informed consent was obtained from all living patients and also, for families with HPC, from the unaffected members.

2.3. Immunohistochemistry

MLH1-, MSH2-, and MSH6-immunohistochemistry was performed on paraffin sections in a LabVision Autostainer instrument (LabVision Corporation, Fremont, CA, USA). For epitope retrieval, the sections were subjected to four cycles (7 min + 3 × 5 min) of heating in a microwave oven at 850 W in Tris-EDTA buffer, pH 9.0. The following primary antibodies were used: for MLH1 clone G168-15 (BD Biosciences Pharmingen) at 1:25; for MSH-2 clone FE11 (Oncogene Sciences) at 1:150; for MSH-6 clone 44 (Transduction Laboratories) at 1:200. Visualisation of the primary antibody was done with the two-step Envision™ polymer kit (DakoCytomation Denmark A/S, Glostrup, Denmark) using diaminobenzidine as chromogen. Microscopic evaluation followed the recommendation of the International Collaborative Group of HNPCC.[19]

2.4. SSCP, resequencing and minisequencing

Mutation screening of the coding region of *MLH1* on 121 HPC cases was performed using SSCP analysis as described

previously.[20] For sequencing analysis PCR products were purified in 96-format Acro Prep Filter Plates (Pall Life Sciences, Ann Arbor, MI, USA) using the Perfect Vac Manifold vacuum machine (Eppendorf AG, Hamburg, Germany). Sequencing was performed according to the instructions of the manufacturer using BigDye Terminator v.3.1 Cycle Sequencing Kit and automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed with Sequencher 4.2.2 software (Gene Codes Corporation, Ann Arbor, MI, USA). The frequencies of the three *MLH1* variants were determined in the sample sets of patients and controls described above by minisequencing[21] or by direct sequencing. Primer sequences are available upon request from the authors.

2.5. *Statistical analyses*

Association of the I219V variant with unselected PRCA and HPC was tested by logistic regression analysis using SPSS statistical software package (SPSS 12.0). Association with clinical and pathological features of the disease (age at onset, PSA value at diagnosis, T-stage, WHO grade and Gleason score) was tested among unselected PRCA cases by the Pearson χ^2-test, Kruskal–Wallis test, Fisher's exact test, and *t*-test included in the SPSS statistical software package (SPSS 12.0).

3. Results

Immunohistochemistry (IHC) analysis of MLH1, MSH2 and MSH6 proteins among 11 PRCA-colon cancer patients revealed abnormal staining patterns in two patients; patient A with locally advanced PRCA and Dukes B mucinous carcinoma in descending colon pointing to MLH1 alteration and patient B with PRCA and mucinous carcinoma of the cecum revealed MSH2 abnormality. Enough tissue material for DNA extraction was available from patient A only. Sequencing the *MLH1* coding region from this patient's sample revealed two missense mutations: P434L in exon 12 and I219V in exon 8. Two silent mutations and one intronic variant were also found. SSCP analysis of the probands from 121 HPC families and direct sequencing of the affected cases from the six chromosome 3p-linked families revealed a total of 12 *MLH1* sequence variants (Table 1). None of the variants were truncating mutations. Three of the changes were located in exonic regions, I219V and V647M were missense variants and L653 was a silent change. Three of the variants were located in the 5'UTR or 3'UTR regions, and six of the changes took place in introns. The exonic changes I219V and L653 and intronic variants 453 + 79 A > G, 1558 + 14 G > A, and 1668-19 A > G have been previously reported.[22–25]

The frequencies of the three missense mutations of the *MLH1* gene were determined by minisequencing or direct sequencing among patients with unselected PRCA or HPC and in the two control groups. The P434L missense mutation found in the PRCA-colon cancer patient was not detected in any of those sample groups. The novel missense mutation V647M in exon 17 was found in only one HPC proband. In this family there was no sample from the second affected family member (deceased). V647M was neither detected among patients with unselected PRCA nor among population controls (Table 2). The carrier frequencies for I219V were 54.5%, 54.0%, 55.0%, and 54.0% in the probands with HPC, unselected PRCA cases, population controls and BPH cases respectively. The frequency of I219V was found to be in Hardy–Weinberg equilibrium. No statistically significant differences were observed in the carrier frequencies of I219V between the sample groups (Pearson χ^2 test, $P = 0.996$) and subsequently no association was seen between the variant I219V and HPC (odds ratio (OR), 0.98; 95% confidence interval (CI), 0.62–1.55) or unselected PRCA (OR, 0.96; 95% CI, 0.65–1.42) (Table 2). No association was detected even when the age-matched group of BPH cases was used as a control group (HPC; OR, 1.02; 95% CI, 0.65–1.61 and unselected PRCA; OR, 1.00; 95% CI, 0.68–1.48) (Table 2). The mean age at diagnosis of the I219V variant carriers among patients with unselected PRCA was statistically higher compared with non-carriers (68.1 *versus* 65.9 years; *t*-test, $P = 0.03$). No other statistically significant associations of the I219V variant with clinical or pathological features of the disease were observed (PSA value at diagnosis, T-stage, WHO grade and Gleason score, data not shown).

Table 1 – Summary of MLH1 variations found in Finnish colon-PRCA patients or HPC families

Mutation[a]	Amino acid change	Exon/Intron
−28 A > G[b]		5'UTR
−7 C > T[b]		5'UTR
453 + 79 A > G[c]		Intron 5
454 − 51 T > C[b]		Intron 5
655 A > G[b,c,d]	I219V	Exon 8
885 − 24 T > A[b]		Intron 10
1304 C > T[d]	P434L	Exon 12
1413 G > A[d]	K471	Exon 13
1558 + 14 G > A[b,c]		Intron 13
1558 + 58 G > A[d]		Intron 13
1668 − 19 A > G[b,c]		Intron 14
1878 C > T[d]	F626	Exon 16
1939 G > A[b]	V647M	Exon 17
1959 G > T[c]	L653	Exon 17
1990 − 121 C > T[c]		Intron 17
2271*35_37delCTT[b]		3'UTR

a Numbering is according to the cDNA (NM_000249) starting at the A in the start codon.
b A sequence variant was found by SSCP analysis of 121 HPC families.
c A sequence variant was found by direct sequencing of six 3p-positive HPC families.
d A sequence variant was found by sequencing colon-PRCA patient.

4. Discussion

Chen and colleagues[26] and Yeh and colleagues[14] have reported decreased expression of *MLH1* in PRCA cell lines and primary tumours. Subsequently, it was also shown that loss of the MMR function may result in MSI in secondary genes, like *BAX*, containing microsatellites in their coding regions[13] indicating that the MMR pathway may play an important role in the development of PRCA. Another reason for our interest in *MLH1* is based on the results of our recent genome-wide linkage study, which suggested a Finnish PRCA susceptibility locus

Table 2 – Association of the I219V MLH1 gene mutation with unselected PRCA or HPC

Sample	Carrier frequency (%)	OR (95 % CI)	P
Population controls	110/200 (55.0 %)	1.00	
Patients with unselected PRCA	108/200 (54.0 %)	0.96 (0.65 – 1.42)[a]	0.84[a]
		1.00 (0.68 – 1.48)[b]	0.99[b]
Patients with HPC	66/121 (54.5 %)	0.98 (0.62 – 1.55)[a]	0.94[a]
		1.02 (0.65 – 1.61)[b]	0.92[b]
Patients with BPH	109/202 (54.0 %)		

a Population controls (blood donors) used as a control group.
b BPH cases used as a control group.

to reside on 3p25-p26,[27] near the *MLH1* locus on 3p22. Recently this region has also been suggested to be a susceptibility locus for general cancer susceptibility genes among prostate cancer families[7] and for clinically significant prostate cancer.[8] Furthermore, in candidate SNP analysis from genomic regions that show linkage to prostate cancer susceptibility the I219V variation (rs1799977) of the *MLH1* gene was associated with PRCA.[17] These multiple pieces of evidence support the 3p-area harbouring a tumour suppressor gene.

Although PRCA is not considered to be a common feature of HNPCC, a combination of colorectal and prostate carcinomas has frequently been observed in the same patient[28–30] which could be partly explained by genetic influence. We screened 11 PRCA-colon cancer patients for MLH1, MSH2 and MSH6 protein expression using immunohistochemistry. SSCP analysis of the youngest affected patient available in each of the 121 families with HPC enabled us to search for the mutations from all the Finnish HPC families. Being aware of the decreased sensitivity of the SSCP method to reveal sequence variations we used direct sequencing to study all the affected cases available from six HPC families having positive linkage in 3p25-p26. These three approaches failed to detect any truncating mutations that would indicate a direct causative role for *MLH1* in HPC. Yet, three missense variants were detected. The novel variation P434L found in a PRCA-colon cancer patient was not detected in PRCA patients or controls. The novel missense mutation V647M in exon 17 proved to be extremely rare, as it was found only in one HPC family. The variant I219V behaved more like a polymorphism. Neither of these changes affected the charge status of the amino acid because all of them have nonpolar side chains. In addition, no co-segregation of the variants with the disease was seen in the HPC-families. Although the missense variant I219V found was frequently observed in PRCA patients, we failed to see any association between the variant I219V and HPC or unselected PRCA. In contrast to our results, Burmester and colleagues [17] reported a significant difference in allele frequency between the prostate cancer cases and controls for the I219V variant. The difference remained significant in an age-matched subsample. However, we could not detect any difference in carrier frequency even between the age-matched control group of BPH cases and patients with HPC or unselected PRCA. Interestingly, in the US study the major allele frequency was 0.728 in controls and 0.629 in PRCA cases, while in our study the major allele frequency was 0.661, 0.678, 0.67 and 0.678 in HPC cases, unselected PRCA cases, population controls and BPH cases respectively. Finland has a known genetically homogeneous founder population where ethnic differences in samples are minimised and therefore the allele frequencies should not be distorted due to admixture problems. Our result stands in line with the study by Liu and colleagues [22] that initially identified the changed I219V among colorectal cancer patients and reported it as a polymorphism having no causative role in the disease. On the other hand, Bagnoli and colleagues [31] recently reported an association between the variant I219V and refractory ulcerative colitis. Interestingly, among our unselected PRCA cases the carriers of the variant had a statistically significant tendency ($p = 0.03$) to be diagnosed at older age compared to non-carriers, suggesting that the polymorphism may influence the disease onset. This, however, would warrant further studies on other sample sets. Finally, even though the functional studies of *MLH1* variants by Trojan and colleagues [32] did not reveal any effect for I219V on MMR function, the possibility for that and subsequent PRCA involvement cannot be totally excluded. No such data are available for the rare novel mutations V647M and P434L found in this study.

In summary, no truncating or otherwise clearly deleterious mutations were observed in *MLH1* mutation analysis nor could we associate the I219V, P434L or V647M variants with PRCA in the Finnish population. Therefore, our results do not indicate a significant role for the *MLH1* gene in the causation of PRCA and therefore *MLH1* can most likely be excluded as a candidate gene for the 3p-linkage area, at least in the Finnish population.

Conflict of interest statement

None declared.

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REFERENCES

1. Crawford ED. Epidemiology of prostate cancer. *Urology* 2003;**62**:3–12.

2. Lichtenstein P, Holm NV, Verkasalo PK, et al. Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med* 2000;**343**:78–85.
3. Tavtigian SV, Simard J, Teng DH, et al. A candidate prostate cancer susceptibility gene at chromosome 17p. *Nat Genet* 2001;**27**:172–80.
4. Carpten J, Nupponen N, Isaacs S, et al. Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. *Nat Genet* 2002;**30**:181–4.
5. Xu J, Zheng SL, Komiya A, et al. Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. *Nat Genet* 2002;**32**:321–5.
6. Schleutker J, Matikainen M, Smith J, et al. A genetic epidemiological study of hereditary prostate cancer (HPC) in Finland: frequent HPCX linkage in families with late-onset disease. *Clin Cancer Res* 2000;**6**:4810–5.
7. Chang BL, Gillanders EM, Isaacs SD, et al. Evidence for a general cancer susceptibility locus at 3p24 in families with hereditary prostate cancer. *Cancer Lett* 2005;**219**:177–82.
8. Chang BL, Isaacs SD, Wiley KE, et al. Genome-wide screen for prostate cancer susceptibility genes in men with clinically significant disease. *Prostate* 2005;**64**:356–61.
9. Peltomaki P, Vasen HF. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. *The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. Gastroenterology* 1997;**113**:1146–58.
10. Egawa S, Uchida T, Suyama K, et al. Genomic instability of microsatellite repeats in prostate cancer: relationship to clinicopathological variables. *Cancer Res* 1994;**55**:2415–21.
11. Gao X, Wu N, Grignon D, et al. High frequency of mutator phenotype in human prostatic adenocarcinoma. *Oncogene* 1994;**9**:2999–3003.
12. Watanabe M, Imai H, Shiraishi T, Shimazaki J, Kotake T, Yatani R. Microsatellite instability in human prostate cancer. *Br J Cancer* 1995;**72**:562–4.
13. Chen Y, Wang J, Fraig MM, et al. Alterations in PMS2, MSH2 and MLH1 expression in human prostate cancer. *Int J Oncol* 2003;**22**:1033–43.
14. Yeh CC, Lee C, Dahiya R. DNA mismatch repair enzyme activity and gene expression in prostate cancer. *Biochem Biophys Res Commun* 2001;**285**:409–13.
15. Dumur CI, Dechsukhum C, Ware JL, et al. Genome-wide detection of LOH in prostate cancer using human SNP microarray technology. *Genomics* 2003;**81**:260–9.
16. Dahiya R, McCarville J, Hu W, et al. Chromosome 3p24-26 and 3p22-12 loss in human prostatic adenocarcinoma. *Int J Cancer* 1997;**71**:20–5.
17. Burmester JK, Suarez BK, Lin JH, et al. Analysis of candidate genes for prostate cancer. *Hum Hered* 2004;**57**:172–8.
18. Rokman A, Baffoe-Bonnie AB, Gillanders E, et al. Hereditary prostate cancer in Finland: fine-mapping validates 3p26 as a major predisposition locus. *Hum Genet* 2005;**116**:43–50.
19. Muller W, Burgart LJ, Krause-Paulus R, et al. The reliability of immunohistochemistry as a prescreening method for the diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC)—results of an international collaborative study. *Fam Cancer* 2001;**1**:87–92.
20. Seppala EH, Ikonen T, Autio V, et al. Germ-line alterations in MSR1 gene and prostate cancer risk. *Clin Cancer Res* 2003;**9**:5252–6.
21. Syvänen AC. Solid-phase minisequencing as a tool to detect DNA polymorphism. *Methods Mol Biol* 1998;**98**:291–8.
22. Liu B, Nicolaides NC, Markowitz S, et al. Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nat Genet* 1995;**9**:48–55.
23. Tannergard P, Lipford JR, Kolodner R, Frodin JE, Nordenskjold A, Lindblom A. Mutation screening in the hMLH1 gene in Swedish hereditary nonpolyposis colon cancer families. *Cancer Res* 1995;**55**:6092–6.
24. Percesepe A, Borghi F, Menigatti M, et al. Molecular screening for hereditary nonpolyposis colorectal cancer: a prospective, population-based study. *J Clin Oncol* 2001;**19**:3944–50.
25. Hudler P, Vouk K, Liovic M, Repse S, Juvan R, Komel R. Mutations in the hMLH1 gene in Slovenian patients with gastric carcinoma. *Clin Genet* 2004;**65**:405–11.
26. Chen Y, Wang J, Fraig MM, et al. Defects of DNA mismatch repair in human prostate cancer. *Cancer Res* 2001;**61**:4112–21.
27. Schleutker J, Baffoe-Bonnie AB, Gillanders E, et al. Genome-wide scan for linkage in finnish hereditary prostate cancer (HPC) families identifies novel susceptibility loci at 11q14 and 3p25-26. *Prostate* 2003;**57**:280–9.
28. Schatzkin A, Baranovsky A, Kessler LG. Diet and cancer. Evidence from associations of multiple primary cancers in the SEER program. *Cancer* 1988;**62**:1451–7.
29. Enblad P, Adami HO, Glimelius B, Krusemo U, Pahlman L. The risk of subsequent primary malignant diseases after cancers of the colon and rectum. A nationwide cohort study. *Cancer* 1990;**65**:2091–100.
30. Bittorf B, Kessler H, Merkel S, et al. Multiple primary malignancies: An epidemiological and pedigree analysis of 57 patients with at least three tumours. *Eur J Surg Oncol* 2001;**27**:302–13.
31. Bagnoli S, Putignano AL, Melean G, et al. Susceptibility to refractory ulcerative colitis is associated with polymorphism in the hMLH1 mismatch repair gene. *Inflamm Bowel Dis* 2004;**10**:705–8.
32. Trojan J, Zeuzem S, Randolph A, et al. Functional analysis of hMLH1 variants and HNPCC-related mutations using a human expression system. *Gastroenterology* 2002;**122**:211–9.