methylation (n = 47). There were significant differences between methylation-positive (n = 60) and -negative groups (n = 150) with respect to gender, tumor location, histologic type and tumor size (Table 1). Methylation-positive tumors showed a marked preponderance in females (p = 0.007) and the proximal colon (p = 0.004). The proportion of well-differentiated adenocarcinomas was lower in the methylation-positive group (p = 0.04). The average tumor size was significantly larger in methylation-positive tumors (60.0 ± 28.9 mm) than in methylation-negative ones (52.2 ± 22.4 mm) (p = 0.04). Regarding MSI status, the frequency of MSI-H was significantly higher in the methylation-positive group (p < 0.0001).

In the methylation-positive group, there were significant differences between patients showing full methylation (n = 13) and partial methylation (n = 47) with regard to age at onset, tumor location and MSI status. The average age at onset was significantly higher in patients with full methylation than in those with partial methylation (72.8 ± 10.6 y/o vs. 64.5 ± 10.1 y/o, p = 0.012). All tumors with full methylation developed in the proximal colon and showed MSI-H (13/13, 100%), while those with partial methylation were more prevalent in the distal colon (32/47, 68.1%) and MSI-H was observed less frequently than in those with full methylation (2/47, 4.3% vs. 13/13, 100%, p < 0.0001).

Comparison of *MLH1* genotypes with methylation status of the *MLH1* promoter region

Genotypes and allelic frequencies of the *MLH1*-93G/A SNP were examined in 210 sporadic CRC patients and 100 PBL samples from normal donors, and the results were compared with previous studies carried out in cohorts of different populations. The allelic frequency for each allele was nearly 50% in the normal donors, other Japanese and Korean cohorts, while the frequency of the G-allele (78–80%) was higher than that of the A-allele (20–22%) in Caucasians [12,16,20,25]. In each study, observed genotype frequencies were consistent with the expected frequencies according to the Hardy-Weinberg equilibrium.

In the 210 sporadic CRCs, the proportions of genotypes A/A, A/G and G/G were 26% (n = 54), 51% (n = 108) and 23% (n = 48), respectively (Table 2). There were no significant associations between genotypes and clinicopathological parameters such as age at onset, sex, tumor location, Dukes classification and histologic type.

Table 2 Association between methylation status of the *MLH1* promoter region and genotypes of the *MLH1*-93G/A SNP in sporadic colorectal cancers

<Total cases>	Genotype				Allelic frequency			
	A/A (n = 54)	A/G (n = 108)	G/G (n = 48)	p values	A	G	RR	p values
Methylation-positive	24	26	10	0.0103[1]	74	46	1.303	0.0094[1]
Full methylation	4	8	1		24	10		
Partial methylation	20	18	9	0.0229[2]	58	36		0.0044[2]
Methylation-negative (No methylation)	30	82	38		142	158		
<Female cases>	Genotype	Allelic frequency						
	A/A (n = 23)	A/G (n = 37)	G/G (n = 22)	p values	A	G	RR	p values
Methylation-positive	14	13	5	0.0259[1]	41	23	1.525	0.0067[1]
Full methylation	4	5	1		13	7		
Partial methylation	10	8	4	NS[2]	28	16		0.0223[2]
Methylation-negative (No methylation)	9	24	17		42	58		
<Male cases>	Genotype	Allelic frequency						
	A/A (n = 31)	A/G (n = 71)	G/G (n = 26)	p values	A	G	RR	p values
Methylation-positive	10	13	5	NS[1]	33	23	1.179	NS[1]
Full methylation	0	3	0		3	3		
Partial methylation	10	10	5	NS[2)]	30	20		NS[2)]
Methylation-negative (No methylation)	21	58	21		100	100		

[1]P values were analyzed using Pearson's chi-square test between methylation-positive (full + partial) and methylation-negative cases (no methylation). NS: not significant. P values of <0.05 were statistically significant.
[2]P values were analyzed using Pearson's chi-square test between full, partial and methylation-negative cases (no methylation). NS: not significant. P values of <0.05 were statistically significant.

The proportions of the *MLH1* genotypes were compared with methylation-positive and methylation-negative cases or cases of full methylation, partial methylation and no methylation of the *MLH1* promoter region (Table 2), and were significantly associated with methylation status (methylation-positive vs. methylation-negative, p = 0.0103; full methylation vs. partial methylation vs. no methylation, p = 0.0229). Allelic frequencies of the *MLH1*-93G/A SNP were also compared with methylation status of the *MLH1* promoter region. The A-allele was more common in methylation-positive cases (RR 1.303, p = 0.0094) and was also more common in subjects with full methylation and partial methylation than the G-allele (p = 0.0044). In an analysis of cases subdivided by gender, the proportions of the *MLH1* genotypes were significantly associated with methylation status in females (methylation-positive vs. methylation-negative, p = 0.0259), but not in males. Furthermore, allelic frequencies of the A-allele were also significantly associated with methylation status in females (RR 1.525, p = 0.0067).

The clinical characteristics, pathological features and MSI status based on methylation status (full, partial or none) were compared according to three genotypes of the *MLH1*-93G/A SNP (Additional file 1: Table S2). Patients who had tumors with full methylation were older and included a marked preponderance of females (compared to no methylation), right-sided lesions and MSI-H compared to those with partial and no methylation in genotype A/A. In contrast, patients who had tumors with full methylation were significantly different regarding tumor location, pathological type (compared to no methylation) and MSI status compared to those with partial and no methylation in genotype A/G. The distribution of mucinous and poorly differentiated adenocarcinomas is significantly higher in patients with full methylation compared to those with no methylation. In genotype G/G, there was no difference among the three methylation types except for MSI-H.

MLH1 genotypes and other risk factors contributing to *MLH1* promoter methylation in CRCs

In logistic regression analysis using the genotype of the *MLH1*-93G/A SNP (A/A vs. A/G + G/G), tumor location (right vs. left), gender (female vs. male) and age at onset (more than 70 vs. less than 70) as the independent variables, genotype was shown to be the most significant risk factor for hypermethylation of the *MLH1* promoter region (Table 3). The odds ratio (OR) in a multivariate analysis for genotype A/A was 2.82 (95% CI 1.42–5.62, p = 0.003), followed by tumor location (OR 2.4, 95% CI 1.23–4.70, p = 0.011) and gender (OR 2.13, 95% CI 1.08–3.92, p = 0.027), while age at onset was not a significant factor contributing to methylation status.

Table 3 Multivariate-adjusted odds ratio for *MLH1* promoter methylation with genotypes and other variables

Factors	OR[1]	95% CI[2]	p values[3]
Genotype of the *MLH1* (A/A vs. A/G, G/G)	2.82	1.42-5.62	0.003
Tumor location (right vs. left)	2.40	1.23-4.70	0.011
Gender (female vs. male)	2.06	1.08-3.92	0.027
Age of onset (more than 70 vs. less than 70)	1.05	0.53-2.07	0.885

[1]*OR*: Odds ratio, Odds ratio adjusted for other variables listed in the table.
[2]*CI*: Confidence interval.
[3]P values of <0.05 were statistically significant.

Analysis of genotype frequencies and haplotype of the SNPs surrounding the *MLH1*-93G/A SNP and their association with methylation status

We analyzed the genotype frequencies of six SNPs located −47 kb upstream to 6.4 kb downstream of the MLH1 translation start site, namely, rs2276807, rs4678922, rs6789043, rs1046512, rs3774343 and rs4647215, surrounding the *MLH1*-93G/A SNP (rs1800734) (Figure 2a), and their association with *MLH1* methylation status (methylation-positive vs. methylation-negative). The observed frequency of each genotype was consistent with the expected frequency according to the Hardy-Weinberg equilibrium. There were no significant associations between the frequency of each genotype and the methylation status in these SNPs, except for rs1800734. Furthermore, we analyzed the haplotypes of these seven SNPs and their association with *MLH1* methylation status (methylation-positive vs. methylation-negative). As a result, only the haplotype A-A-T-A-A-A-C (rs2276807-rs4678922-rs6789043-rs1046512-rs1800734-rs3774343-rs4647215) had a significant association with *MLH1* promoter hypermethylation in the analysis of all cases (p = 0.012), as well as in the female cases (p = 0.00038), but this was not significant in the male cases (Table 4).

EMSA

To elucidate whether the genotype of the *MLH1*-93G/A SNP affects the binding activity of nuclear transcription factors, each synthetic double-stranded DNA oligomer of the *MLH1*-93G/A SNP was subjected to EMSA (Figure 2b). First, in order to confirm the accuracy of this assay, a competition assay was performed using ^{32}P-labeled MLH-184 to −132 and unlabeled MLH1-184 to −132 oligomers as described previously [12] (Figure 2b, lanes 1–3). A shifted band was observed with HeLa nuclear extract, which was blocked completely in the presence of an excess of unlabeled competitor sequence, as reported previously. HeLa cell nuclear extract was then incubated with 20-bp DNA oligomers homologous to each genotype of the *MLH1*-93G/A SNP (Figure 2b, lanes 4–7, 8–11). When the ^{32}P-labeled MLH1-93G

Table 4 Association between haplotype of seven SNPs including *MLH1*-93G/A SNP and the methylation status of the *MLH1* promoter region

<Total cases > Haplotype[1]	Haplotype frequencies[2]			χ^2-value	p-value[3]
	Total	M-negative	M-positive		
C-A-T-A-**A**-A-C	0.3141	0.3031	0.3431	0.6274	0.4283
A-A-T-A-**G**-A-C	0.3116	0.3366	0.2499	2.9665	0.085
A-A-T-A-**A**-A-C	**0.198**	**0.1667**	**0.2755**	**6.3116**	**0.012**
C-T-C-C-**G**-G-A	0.149	0.1644	0.1102	1.9578	0.1618
C-A-T-A-**G**-A-C	0.0273	0.0291	0.0213	0.1968	0.6573
<Female cases > Haplotype[1)]		Haplotype frequencies[2]		χ^2-value	p-value[3]
	Total	M-negative	M-positive		
C-A-T-A-**A**-A-C	0.2983	0.3046	0.2955	0.0154	0.9014
A-A-T-A-**G**-A-C	0.2801	0.3346	0.2017	3.3986	0.0653
A-A-T-A-**A**-A-C	**0.2077**	**0.1154**	**0.3452**	**12.6416**	**0.00038**
C-T-C-C-**G**-G-A	0.1829	0.21	0.1406	1.2578	0.2621
C-A-T-A-**G**-A-C	0.0309	0.0354	0.017	0.4818	0.4876
<Male cases > Haplotype[1]		Haplotype frequencies[2]		χ^2-value	p-value[3]
	Total	M-negative	M-positive		
C-A-T-A-**A**-A-C	0.3323	0.3381	0.3105	0.1457	0.7027
A-A-T-A-**G**-A-C	0.3243	0.3028	0.4031	1.9442	0.1632
A-A-T-A-**A**-A-C	0.1916	0.1922	0.1895	2.00E-03	0.9643
C-T-C-C-**G**-G-A	0.127	0.1414	0.0741	1.7339	0.1879
C-A-T-A-**G**-A-C	0.0249	0.0255	0.0229	0.0117	0.9138

[1]Loci of SNPs examined were as follows: rs2276807 - rs4678922 - rs6789043 -rs1046512- rs1800734 (bold letter) - rs3774343 - rs4647215.
[2]Total cases, methylation-negative (M-negative) cases and methylation-positive (M-positive) cases of haplotype frequencies.
[3]P values of <0.05 were statistically significant.

probe was mixed with the nuclear extract, we detected a shifted band, which was blocked by the unlabeled MLH1-93G competitor (Figure 2b, lanes 5 and 6), but not by the unlabeled MLH1-93A competitor (Figure 2b, lane 7). However, a shifted band was not detected when ^{32}P-labeled MLH1-93A probe was mixed with the HeLa cell nuclear extract (Figure 2b, lanes 9–11).

Discussion

Methylation of the *MLH1* promoter region and subsequent transcriptional silencing have been shown to play a critical role in the development of MSI-positive CRCs [3]. In a previous study, we reported two types of methylation profile in the *MLH1* promoter region, namely, full methylation and partial methylation [6]. Only the former was associated with *MLH1* gene silencing followed by the MSI-H phenotype. Partial methylation was observed in CRCs that developed in both proximal and distal colon, while the majority of cases showing full methylation developed in the former. The average age at onset was 10 years earlier in CRCs with partial methylation than in cases with full methylation [7]. Partial methylation of the *MLH1* promoter region implies initiation of the methylation process, but only a subset of cases with partial methylation may eventually progress to full methylation and develop MSI-positive CRCs. It is therefore important to elucidate factors contributing to methylation of the *MLH1* promoter region in CRC tumorigenesis.

Recent studies have reported such a factor. The *MLH1*-93G/A SNP located in the promoter region is significantly associated with *MLH1* promoter methylation followed by loss of MLH1 protein expression and plays an important role in MSI-H colorectal tumorigenesis [14,16-21]. However, these results are derived mostly from Caucasian populations, in which the frequencies of the A-allele and A/A homozygosity are low, at 20–22% and 4–5%, compared with those in East Asians including the Japanese [12,16,20,25]. The allelic frequency of *MLH1*-93A in a Japanese cohort is as much as 50%; therefore, study on the Japanese would be important to validate the results previously reported for Caucasians. The present study confirms the association between the genotype of *MLH1*-93G/A and methylation status for sporadic CRCs in a Japanese population. The frequency of methylation of the *MLH1* promoter region in CRCs is significantly higher in those carrying the A-allele in

Japanese. The *MLH1*-93G/A SNP genotype is associated with the methylation status of the *MLH1* promoter region, with either full or partial methylation.

Previous studies reported a different affinity of the DNA binding proteins to the *MLH1*-93G/A SNP, suggesting that some transcription factors bind preferentially to the sequence of MLH1-93G but not to MLH1-93A [26,27]. In EMSA using HeLa cell nuclear extract, the band showing a mobility shift of the ^{32}P-labeled MLH1-93G probe had greater intensity than that of the MLH1-93A probe, suggesting that the affinity of DNA binding proteins to the MLH1-93A sequence is less than that to MLH1-93G (Figure 2b). These results suggest that individuals carrying the *MLH1*-93A allele are prone to transcriptional silencing due to the decreased affinity to allele-specific DNA binding proteins. However, the definitive mechanism of developing *MLH1* promoter methylation based on the different affinity of DNA binding proteins to the *MLH1*-93G/A SNP has not been elucidated yet. Furthermore, the frequencies of MSI-positive CRC were reported to be as high as 10–15% in Caucasians, compared with 9.05% (19/210) in this series and 13% in another Japanese cohort [28]. Assuming that the frequency of MSI in the Japanese is the same as that in Caucasians, carrying the A-allele at the *MLH1*-93G/A SNP is not the only risk factor for developing MSI-positive CRCs. Methylation of the *MLH1* promoter region was found in 28.6% (60/210) of patients and 78.3% (47/60) showed partial methylation, among which methylation was limited to the most upstream region of the *MLH1* promoter sequences that is distant from the *MLH1*-93G/A SNP. As shown in Table 2, the frequency of A/A homozygosity was significantly higher in cases with partial methylation (42.5%, 20/47) than in those with full methylation (30.8%, 4/13) or no methylation (20.0%, 30/150) ($p = 0.0229$). These data suggest that the *MLH1*-93G/A genotype was not the only determinant of the MSI phenotype, which was mostly associated with full methylation.

We further examined the association between the genotypes of the six other SNPs surrounding the *MLH1*-93G/A SNP (rs1800734) and methylation status in the *MLH1* promoter region. Rs1800734 was the only SNP showing a significant association with methylation. A haplotype-based case–control study showed a significant association of the haplotype comprising A-A-T-A-A-A-C alleles with *MLH1* promoter methylation (Table 4). The A allele of rs1800734 had only 2 haplotypes comprising either C-A-T-A-A-A-C or A-A-T-A-A-A-C, and the latter showed a significant association with *MLH1* promoter hypermethylation; that is, rs2276807 located in the most upstream region spanning −47 kb of rs1800734 was a determinant of the haplotype associated with *MLH1* promoter hypermethylation. These findings suggest that additional genetic and/or environmental factors may contribute to the progression of methylation status from partial to full methylation, eventually associated with the MSI phenotype. To our knowledge, this is the first report of an association between this haplotype and methylation status in the *MLH1* promoter region.

In an analysis of cases subdivided by gender, the frequency of the A-allele *MLH1*-93G/A SNP was significantly higher in females (RR 1.525, $p = 0.0067$), while this was not significant in males (RR 1.179) (Table 2). Subjects carrying the A-allele, particularly females, may harbor an increased risk of methylation of the *MLH1* promoter region. Furthermore, a haplotype associated with A-alleles of both rs2276807 and *MLH1*-93G/A (rs1800734) also had a significant association with *MLH1* promoter methylation in female subjects (Table 4). Slattery et al. suggested that estrogen exposure in women protects against MSI, whereas the lack of estrogen in older women increases the risk of MSI, as shown in a population-based case–control study [29]. In this series, the clinical characteristics of methylation-positive tumors, such as female predominance and late age at onset, are compatible with this epidemiological observation for MSI-positive tumors. Individuals carrying the A-allele of *MLH1*-93G/A (rs1800734), especially in association with the A-allele of rs2276807, may be susceptible to methylation, but hormonal status or other genetic factors may modify the age of onset of the cancer. As the present study size is relatively small, further studies are needed to elucidate the mechanism of methylation susceptibility of the *MLH1* promoter region defined by the genotype of *MLH1*-93G/A SNP, along with the relevant haplotype.

Conclusions

The *MLH1*-93G/A SNP (rs1800734) was significantly associated with methylation of the *MLH1* promoter region in a Japanese population. Furthermore, a haplotype comprising the A-allele of rs2276807 and the A-allele of *MLH1*-93G/A SNP showed a significant association with methylation of the *MLH1* promoter region. Individuals, particularly females, carrying the A-allele at the *MLH1*-93G/A SNP, especially in association with the A-allele of rs2276807, may harbor an increased risk of methylation of the *MLH1* promoter region.

Additional file

Additional file 1: Haplotype defined by the MLH1-93G/A polymorphism. Table S1. PCR primer sequences and PCR conditions of the six SNPs. **Table S2.** Association between clinicopathological features and methylation status according to the genotype of the *MLH1* promoter region.

Abbreviations

BiPS: Na-bisulfite PCR/single-strand conformation polymorphism; CRC: Colorectal cancer; EMSA: Electrophoretic mobility shift assay;

HNPCC: Hereditary nonpolyposis colorectal cancer; *MMR* gene: mismatch repair gene; MSI: Microsatellite instability; MSI-H: high-frequency MSI; MSI-L: low-frequency MSI; MSP: Methylation-specific PCR; PBL: Peripheral blood lymphocyte; SNP: Single-nucleotide polymorphism; SSCP: Single-strand conformation polymorphism.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
M.Y. designed the study, performed genetic analysis and wrote the manuscript; T.M. performed genetic analysis and revised the manuscript; S.K. designed the study, performed genetic analysis, revised the manuscript and gave final approval of the manuscript; L.A. performed the English editing and revised the manuscript; Y.Y. designed the study and revised the manuscript. All authors read and approved the final manuscript.

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Comparison of clinical features between suspected familial colorectal cancer type X and Lynch syndrome in Japanese patients with colorectal cancer: a cross-sectional study conducted by the Japanese Society for Cancer of the Colon and Rectum

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Abstract

Objective: The characteristics of familial colorectal cancer type X are poorly defined. Here we aimed to clarify the differences in clinical features between suspected familial colorectal cancer type X and Lynch syndrome in Japanese patients.

Methods: We performed germline mutation analyses of mismatch repair genes in 125 patients. Patients who met the Amsterdam Criteria I but lacked mismatch repair gene mutations were diagnosed with suspected familial colorectal cancer type X.

Results: We identified 69 patients with Lynch syndrome and 25 with suspected familial colorectal cancer type X. The frequencies of gastric and extracolonic Lynch syndrome-associated cancers were lower

with suspected familial colorectal cancer type X than with Lynch syndrome. The number of organs with Lynch syndrome-associated cancer was significantly lower with suspected familial colorectal cancer type X than with Lynch syndrome. The cumulative incidence of extracolonic Lynch syndrome-associated cancer was significantly lower with suspected familial colorectal cancer type X than with Lynch syndrome. We estimated that the median cancer risk in 60-year-old patients with Lynch syndrome was 89, 36 and 24% for colorectal, endometrial and gastric cancers, respectively. Analyses of family members, including probands, revealed that the median age at diagnosis of extracolonic Lynch syndrome-associated cancer was significantly older with suspected familial colorectal cancer type X than with Lynch syndrome. The frequency of extracolonic Lynch syndrome-associated cancer was significantly lower with suspected familial colorectal cancer type X than with Lynch syndrome.
Conclusion: A significant difference in extracolonic Lynch syndrome-associated cancer was evident between suspected familial colorectal cancer type X and Lynch syndrome.

Key words: colorectal cancer, familial colorectal cancer type X, Lynch syndrome

Introduction

A family history of colorectal cancer is found in ~15% of all patients with the disease (1). Lynch syndrome (LS), formerly termed hereditary nonpolyposis colorectal cancer (HNPCC), is the most common familial colorectal cancer, and accounts for 1–3% of all colorectal cancer (2). LS with autosomal dominant inheritance is caused by germline mutations in mismatch repair (MMR) genes, including the *MSH2* (3), *MLH1* (4) and *MSH6* (5) genes. Clinically, LS is characterized by high incidences of endometrial, small intestinal and urinary tract cancers, as well as an early-onset of colorectal cancer. Inactivation of MMR genes by germline and somatic mutations triggers LS-associated tumors. Consequently, a high frequency of replication errors results at both microsatellite regions and repetitive sequences in the coding regions of various growth-related target genes (6), creating a growth advantage for LS-associated tumors. However, some patients lack deficient mismatch repair (dMMR), regardless of a familial history of colorectal cancer. Recently, patients meeting the Amsterdam Criteria I (7) but lacking dMMR and/or microsatellite instability (MSI) in the tumor tissue were classified as having familial colorectal cancer type X (FCCTX), the cancer risk of which is lower than that of LS (8). Thus, the carcinogenesis of FCCTX is considered different from that of LS, because of the lack of dMMR. Even though the definition of FCCTX has not strictly been defined, but patients with dMMR in the germline but have no information about dMMR in the tumor tissue could be called 'suspected FCCTX'. To date, neither FCCTX nor suspected FCCTX (s-FCCTX) cases have been reported in Japanese patients. To clarify the characteristics of s-FCCTX in Japanese patients, we compared the clinical features of s-FCCTX with those of LS.

Patients and methods

Study design

This nationwide cross-sectional study was conducted by the HNPCC Registry and Genetic Testing Project of the Japanese Society for Cancer of the Colon and Rectum (JSCCR) between September 2002 and July 2010. In this study, we aimed to clarify the following characteristics of Japanese patients with LS:

(i) the organ(s) in which the cancer developed;
(ii) the penetration rate of the cancer;
(iii) the prognosis of the cancer;
(iv) the relationship between genotype and phenotype; and
(v) the causative genes.

Despite mutation analyses, we were unable to detect MMR gene mutations in some patients. Therefore, we defined patients meeting the Amsterdam Criteria I but lacking MMR gene mutations as having s-FCCTX, and planned this study to clarify the differences in clinical features, particularly in incidence, between s-FCCTX and LS in Japanese patients with colorectal cancer.

The inclusion criteria were: age ≥16 years; histologically confirmed adenocarcinoma of the colon and rectum; invasion of the submucosa or deeper; and fulfillment of the modified Amsterdam Criteria II, which includes gastric cancer, because gastric cancer is common in Asian patients with LS (9). We also included first-degree relatives of the patient with germline MMR gene mutations. The modified criteria did not require pathological confirmation of a family history of cancer. The exclusion criteria were: familial adenomatous polyposis to be included in the Amsterdam Criteria II (10) as well as another polyposis syndrome: Cowden disease, Peutz–Jeghers syndrome and Juvenile polyposis syndrome.

After genetic counseling and the provision of written informed consent, the patients were enrolled in this study and given an anonymous identifier. Blood samples and clinical information were collected either from medical records or directly from patients using a case report form. Clinical information included: sex; date of birth; histological type; location of colorectal cancer; occurrence of extracolonic cancer; date at diagnosis of cancer, including extracolonic cancer; final confirmed surviving date; distinction between life and death; date of death; and family history. The study protocol and informed consent form were approved by the institutional review board at each institution.

Follow-up survey

In the follow-up survey, a case report form was sent annually to the data manager of each institution, requesting the anonymous identifier, occurrence of cancer, date at diagnosis of cancer; distinction between life and death, final confirmed surviving date and date of death of patients and their relatives.

Mutation analysis

Genomic DNA was extracted from peripheral blood samples using the standard phenol extraction/purification procedure. Mutation analysis was performed by direct sequencing of the entire coding region of *MLH1*, *MSH2* and *MSH6*. The polymerase chain reaction (PCR) mixture (25 μl) contained 50 ng of genomic DNA, 0.4 μM of each

primer, 1.5 mM $MgCl_2$, 0.2 mM of each NTP, 1× PCR buffer and 0.5 μl of KOD Plus DNA Polymerase (Toyobo Co., Ltd, Osaka, Japan). PCR comprised: initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 95°C for 10 s; annealing at 57°C for 30 s; extension at 68°C for 1 min; and final extension at 68°C for 5 min. The PCR products were purified with ExoSAP-IT (Affymetrix Inc., Santa Clara, CA, USA) and underwent capillary sequencing using a BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's recommendations. The list of primer sequences used for PCR and sequencing are listed in Supplementary Table S1. The products were purified using a BigDye XTerminator Purification Kit (Applied Biosystems Inc.,) and loaded into a 3730xl Genetic Analyzer (Applied Biosystems Inc.).

For samples without deleterious mutations in the three genes, multiplex ligation of probe amplification was performed using a SALSA MLPA MLH1/MSH2 Kit (MRC-Holland, Amsterdam, The Netherlands) according to the supplier's protocol (11). Because tumor tissue samples were not collected in this study, microsatellite instability was not analyzed.

Statistical analysis

The right colon comprises the cecum, ascending colon and transverse colon. We endeavored to collect clinical information from all patients; however, the pathological type of eight of 94 patients remains unknown.

Data are presented as totals, medians (range), means (95% confidence intervals) or percentages (95% CIs). Statistical analysis was performed using Fisher's exact test and the Mann–Whitney *U*-test. Cumulative cancer risks were calculated using the Kaplan–Meier method, and the log-rank test was used to compare risks between s-FCCTX and LS. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html), a graphical user interface for R version 3.1.0 (The R Foundation for Statistical Computing, Vienna, Austria) (12). This interface is a modified version of R Commander version 2.0–4, which was designed to add statistical functions frequently used in biostatistics.

Results

Patients

Between September 2002 and July 2010, 142 patients were registered to the HNPCC Registry and Genetic Testing Project of the JSCCR with suspected LS. Of them, 123 were eligible for inclusion in this study, and 19 did not meet the inclusion criteria. However, we performed MMR gene mutation analyses on patients who did not meet the eligibility criteria: seven patients with a germline MMR gene mutation were included with the patients with LS. Among 142 patients, 69 met the Amsterdam Criteria I and 73 did not meet the Amsterdam Criteria I. Mutation analyses on the 137 patient's enrolled detected 69 patients with germline MMR gene mutations. Of them, 36 had *MLH1* mutations, 31 had *MSH2* mutations and 2 had *MSH6* mutations. Of the 142 patients, 25 were classified as having s-FCCTX, because they fulfilled the Amsterdam Criteria I without dMMR. We compared the 25 patients with s-FCCTX with the 69 patients with LS (Fig. 1).

Differences in characteristics between patients with s-FCCTX and patients with LS patients (proband)

Clinical characteristics are summarized in Table 1. The analyses of probands identified no significant difference in age at diagnosis of

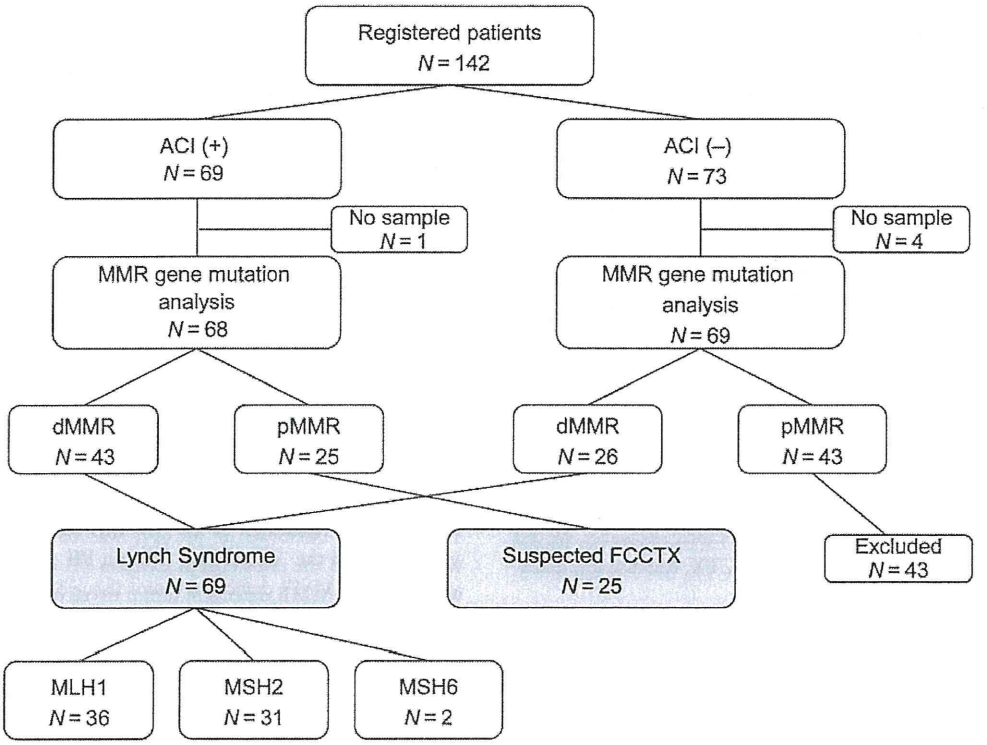

Figure 1. Consolidated standards of reporting trials diagram. MMR, mismatch repair; dMMR, deficient mismatch repair; pMMR, proficient mismatch repair; ACI, Amsterdam Criteria I.

Table 1. Characteristics of suspected familial colorectal cancer type X and Lynch syndrome

	s-FCCTX ($n = 25$)	LS ($n = 69$)	P value
Patients (proband) analysis			
Male	16/25	33/69	0.24
Median age at diagnosis of colorectal cancer (range)	48 (21–74)	45 (24–70)	0.39
Median age at diagnosis of LS-associated cancer (range)	48 (21–74)	44 (24–70)	0.27
Median age at diagnosis of extracolonic LS-associated cancer (range)	54 (24–83)	52 (23–82)	0.36
Median number of colorectal cancer (range)	1 (1–3)	1 (1–4)	0.78
Right colon cancer	14/25	43/69	0.64
Poorly differentiated adenocarcinoma or mucinous carcinoma	6/24	15/62	>0.99
Endometrial cancer	0/9	12/36	0.086
Small intestine cancer	1/25	4/69	>0.99
Urinary tract cancer	0/25	7/69	0.18
Gastric cancer	0/25	11/69	0.033
Median number of LS-associated cancer organ (range)	1 (1–2)	1 (1–4)	0.003
Extracolonic LS-associated cancer	1/25	26/69	0.001
Familial member analysis			
Median number of first-degree relatives (range)	4 (2–13)	5 (2–12)	0.96
Median number of second-degree relatives (range)	1 (0–17)	1 (0–11)	0.31
Median age at diagnosis of colorectal cancer (range)	42 (21–55)	37 (22–60)	0.11
Median age at diagnosis of LS-associated cancer (range)	42 (21–55)	36 (20–60)	0.043
Median age at diagnosis of extracolonic LS-associated cancer (range)	67 (44–86)	49 (20–80)	0.007
Colorectal cancer	25/25	64/69	0.32
Endometrial cancer	1/25	28/69	<0.001
Small intestine cancer	1/25	5/69	>0.99
Urinary tract cancer	0/25	7/69	0.18
Gastric cancer	7/25	43/69	0.005
Extracolonic LS-associated cancer	8/25	51/69	<0.001

s-FCCTX, suspected familial colorectal cancer type X; LS, Lynch syndrome.

colorectal cancer and LS-associated cancer between patients with s-FCCTX and patients with LS.

The frequency of gastric cancer in patients with s-FCCTX was significantly lower than that in patients with LS ($P = 0.033$), and the frequency of endometrial cancer in patients with s-FCCTX tended to be lower than that in patients with LS ($P = 0.086$). The frequency of extracolonic LS-associated cancer in patients with s-FCCTX was significantly lower than that in patients with LS ($P = 0.001$). Whereas the median number of organs with LS-associated cancer was one for both patients with s-FCCTX and patients with LS, there was a significant difference between patients with s-FCCTX and patients with LS ($P = 0.003$). The mean numbers of organs with LS-associated cancer were 1.04 (0.96–1.12) in patients with s-FCCTX and 1.43 (1.27–1.60) in patients with LS ($P = 0.003$; Mann–Whitney U-test).

The cumulative extracolonic LS-associated cancer and colorectal cancer risks in patients with s-FCCTX were similar to those in patients with LS; however, the cumulative extracolonic LS-associated, endometrial and gastric cancer risks were significantly lower in patients with s-FCCTX than in patients with LS (Fig. 2). In this study, we estimated that the median cancer risk of 60-year-old patients with LS was 86% (77–93%) for colorectal cancer, 36% (20–59%) for endometrial cancer and 28% (16–46%) for gastric cancer; however, we did not estimate cancer risk in patients with s-FCCTX, because few extracolonic cancers occur.

Differences in characteristics between family members with s-FCCTX and LS

In the analyses of family members, including probands, the median age at diagnosis of LS-associated cancer was significantly greater in family members of patients with s-FCCTX than in family members of patients with LS ($P = 0.043$). When colon cancer was excluded, the tendency was stronger ($P = 0.007$): median age at diagnosis of colorectal cancer in family members of patients with s-FCCTX was similar to that in family members of patients with LS.

The frequencies of gastric cancer ($P = 0.005$) and endometrial cancer ($P < 0.001$) in family members of patients with s-FCCTX were significantly lower than those in family members of patients with LS. Moreover, the frequency of extracolonic LS-associated cancer in family members of patients with s-FCCTX was significantly lower than that in family members of patients with LS ($P < 0.001$).

Discussion

We defined patients meeting the Amsterdam Criteria I but lacking MMR gene mutations as having s-FCCTX. To date, the definition of FCCTX is still controversial. Originally, FCCTX collectively describes cases of colorectal cancer that meet clinical Amsterdam Criteria I, but whose tumors are DNA MMR proficient as assessed by MSI testing (8), however, some studies of FCCTX have also included Amsterdam Criteria II families with MMR-stable tumors (13–16). The study by Nieminen et al. (17) was based on FCCX families, which fulfilled the Amsterdam Criteria I/II or the Bethesda criteria without DNA MMR defects in tumor tissue or in the germline. Moreover, there is the problem with MSI testing. It has been reported that frequency of MSI-H is 69% in LS with *MSH6* mutation (18). Therefore, LS with *MSH6* mutation may be included in the patients with Amsterdam Criteria who does not show MSI. In addition, the term 'Lynch-like syndrome' has recently been proposed (19). This indicates that dMMR (without hypermethylation of *MLH1* promotor) without germline mutation of the MMR genes, a part of which may be