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Epidemiological, clinical and molecular characterization of Lynch-like syndrome: A population-based study

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Colorectal carcinomas that are mismatch repair (MMR)-deficient in the absence of *MLH1* promoter methylation or germline mutations represent Lynch-like syndrome (LLS). Double somatic events inactivating MMR genes are involved in the etiology of LLS tumors. Our purpose was to define the clinical and broader molecular hallmarks of LLS tumors and the population incidence of LLS, which remain poorly characterized. We investigated 762 consecutive colorectal carcinomas operated in Central Finland in 2000–2010. LLS cases were identified by a stepwise protocol based on MMR protein expression, *MLH1* methylation and MMR gene mutation status. LLS tumors were profiled for CpG Island Methylator Phenotype (CIMP) and somatic mutations in 578 cancer-relevant genes. Among 107 MMR-deficient tumors, 81 (76%) were attributable to *MLH1* promoter methylation and 9 (8%) to germline mutations (Lynch syndrome, LS), leaving 14 LLS cases (13%) (3 remained unclassified). LLS carcinomas were diagnosed at a mean age of 65 years (*vs.* 44 years in LS, $p < 0.001$), had a proximal to distal ratio of 1:1, and all were *BRAF* V600E-negative. Two somatic events in MMR genes were identifiable in 11 tumors (79%). As novel findings, the tumors contained an average of 31 nonsynonymous somatic mutations/Mb and 13/14 were CIMP-positive. In conclusion, we establish the epidemiological, clinical and molecular characteristics of LLS in a population-based study design. Significantly more frequent CIMP-positivity and lower rates of somatic mutations make a distinction to LS. The absence of *BRAF* V600E mutation separates LLS colorectal carcinomas from *MLH1*-methylated colorectal carcinomas with CIMP-positive phenotype.

Key words: lynch syndrome, lynch-like syndrome, colorectal carcinoma, MSI, DNA mismatch repair, CpG Island Methylator phenotype

Abbreviations: CCP: comprehensive cancer panel; CIMP: CpG island methylator phenotype; Dm: methylation dosage ratio; dMMR: MMR deficient; FFPE: formalin-fixed paraffin embedded; IHC: immunohistochemistry; LLS: Lynch-like syndrome; LOH: Loss of heterozygosity; LS: Lynch syndrome; LSRFi: the National LS registry of Finland; MethyQESD: methylation-quantification of endonuclease-resistant DNA; MMR: DNA mismatch repair; MSI-H: high-degree microsatellite instability; MS-MLPA: methylation-specific multiplex ligation-dependent probe amplification; MSS: microsatellite-stable; pMMR: MMR proficient; TMA: tissue microarray; VCP: variant calling pipeline

Additional Supporting Information may be found in the online version of this article.

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What's new?

Lynch-like syndrome (LLS), characterized by mismatch repair (MMR)-deficient colorectal tumors that lack *MLH1* promoter methylation and germline mutations, remains a diagnostic challenge. Here, LLS was found to account for about 13 percent of MMR-deficient colorectal carcinomas in patients diagnosed in Central Finland between 2000 and 2010. While LLS tumors could not be reliably distinguished from Lynch syndrome (LS) tumors based on clinical or histological factors, LLS tumors differed significantly from sporadic *MLH1*-methylated and LS tumors DNA methylation and somatic mutation profiles. The findings provide valuable insight into LLS and could facilitate advances in LLS diagnosis and treatment.

Introduction

Some 16% of colorectal carcinomas are hypermutated and most of the latter show high-degree microsatellite instability (MSI-H).[1] MSI-H is a favorable prognostic factor and may have predictive value as well, although the latter is controversial and may depend on the molecular basis of MSI.[2] The most common mechanism is biallelic promoter methylation of the DNA mismatch repair (MMR) gene *MLH1* that accounts for two-thirds of unselected cases with MSI-H.[3–5] Some 12–16% of MMR-deficient colorectal cancers are attributable to an inherited germline mutation of *MLH1*, *MSH2*, *MSH6*, or *PMS2* combined with an inactivating somatic event (Lynch syndrome, LS).[4–6]

Recent molecular advances have made it possible to distinguish another group of MSI-H cancers designated as Lynch-like syndrome (LLS).[7] Neither *MLH1* promoter methylation nor germline mutations (LS) explain LLS tumors; instead, two somatic mutational events inactivating a given MMR gene are detectable in more than half of LLS tumors.[4,8–10] Apart from MMR genes, the broader somatic mutational profiles of LLS tumors are unknown and only limited information is available of the clinicopathological characteristics and the incidence of LLS in the population.[4,8–10]

To shed light to some of these open questions, we conducted a molecular study on 762 consecutive colorectal cancers operated in the Jyväskylä Central Hospital district of Finland in 2000–2010.[11] The relative shares of LS, *MLH1*-hypermethylated and LLS fractions of colorectal cancers were determined. For the LLS tumors, the somatic mutation profiles of 578 cancer-associated genes were established and compared to those of LS colorectal tumors from our previous investigation.[12] Our study provides important new information of the molecular epidemiology and underlying developmental mechanisms of LLS.

Materials and Methods

Patients and samples

We conducted a retrospective study on consecutive colorectal carcinomas (n = 762) diagnosed in the hospital region of Central Finland during years 2000–2010. The tumors were histopathologically classified by stage and grade, after the UICC guidelines (6th edition), as described.[11] All samples used for molecular studies were formalin-fixed paraffin embedded (FFPE) specimens. Representative areas of normal and tumor tissue were selected based on histological evaluation and used for DNA isolation by a non-enzymatic protocol.[13]

Our study was approved by the Institutional Review Board of the Helsinki University Central Hospital (466/E6/01) and the ethics committee of Jyväskylä Central Hospital (Dnro 13 U/2011). The National Supervisory Authority for Welfare and Health (Dnro 1272/04/044/07 and Dnro 10,741/06.01.03.01/2015) approved the use of the patient registry and collection of archival specimens.

Immunohistochemistry

Tumor representative TMA-blocks (tissue microarray) were prepared from FFPE samples. Immunohistochemistry (IHC) staining was performed using the LabVision Autostainer 480 (Thermo Fisher Scientific, Fremont, CA, USA) and BrightVisionþ polymer detection kit (ImmunoLogic BV, Duiven, The Netherlands) to determine the expression of MMR genes MLH1, MSH2, MSH6 and PMS2 according to a standard protocol.[11] Samples with deficient expression of one or more of the MMR proteins compared to positive controls were considered MMR deficient (dMMR) whereas tumors exhibiting normal positive staining for all four genes were MMR proficient (pMMR).[14] If the MMR status remained unclear after IHC from TMA blocks, we performed re-analysis using sections of whole FFPE blocks. Cases with *BRAF* V600E hotspot mutation were also identified by IHC,[11] which has been validated against qPCR as a reliable method for *BRAF* V600E detection.[15]

Microsatellite instability analysis

BAT25 and *BAT26* mononucleotide repeat markers, specific and sensitive indicators of MSI-H, were used to determine the MSI status[16,17] for those samples for which MMR proficiency/-deficiency could not be determined by IHC. Tumors unstable for *BAT25* and/or *BAT26* were considered as MSI, and tumors stable for both markers were considered microsatellite-stable (MSS).

Exclusion of the Finnish founder mutation 1

The most prevalent LS predisposing mutation in the Finnish population, a 3.5-kb genomic deletion of *MLH1* exon 16 and flanking introns ("Mutation 1"), is not detectable by standard sequencing and was excluded by a mutation-specific test[18] in suspected LLS cases with absent MLH1 in tumor tissue.

Comprehensive Cancer Panel sequencing

Sixteen colorectal cancer samples in which *MLH1* methylation or LS registry information did not explain the dMMR phenotype, and their corresponding normal samples were included in CCP sequencing. Sequencing was conducted in the Institute for Molecular Medicine Finland (FIMM; Helsinki, Finland), as described.[12] Nimblegen Comprehensive Cancer Panel (Roche Diagnostics) was used to target 578 cancer-related genes with a 4 Mb design compiled from the Sanger Institute Cancer Gene Census Database and the NCBI Gene tests databases. The mean target coverage was 160-fold (Supporting Information Table 1). The variant calling pipeline (VCP 3.6) is described in Sulonen *et al.*[19] Alignment against the human genome GRCh37 reference-genome primary assembly was as described.[12]

Somatic mutation analysis of CCP data

From the panel sequencing data of paired normal and tumor samples, VarScan 2 mutation detection algorithm version 2.3.2[20] was used to identify non-synonymous somatic mutations (missense, nonsense, frameshift, in-frame coding deletion/insertion and splice site mutations), as described.[12] Variants with VarScan somatic *p*-value below 0.01 were considered in subsequent analyses. All non-synonymous sequence changes with the possibility of being pathogenic (pathogenicity classes 3–5) are called mutations throughout this paper.

Analysis of LLS tumors for two-hit inactivation of MMR genes

Data on MMR gene(s) pinpointed by IHC results in each LLS tumor were retrieved from CCP sequencing and examined for somatic mutations. Loss of heterozygosity (LOH) analysis was based on the comparison of paired tumor and normal samples[12] was performed by VarSeq (GoldenHelix®) using VCP 3.6 filtered sequencing data (.vcf-files). We followed the thresholds set for strict and putative LOH in Ollikainen *et al.*[21] Putative and strict LOH are called LOH throughout this paper.

Characterization of top-mutated genes

For each of the 578 genes targeted, we determined the proportion of tumors in which a particular gene was mutant according to our criteria for non-synonymous somatic mutations described above. To identify top mutated genes, the cut-off method developed earlier was applied.[12] Based on the distribution of mutated genes across the tumors, a cut-off of 29% (LLS) or 28% (LS) was set, as described in detail in Supporting Information Table 3 below.

IonTorrent sequencing

To verify CCP sequencing results for MMR genes, an Ion Ampliseq™ (Thermo Fisher Scientific) custom panel was designed to cover MLH1, MSH2, MSH6 and PMS2 coding and untranslated regions. The panel was designed for 125–175 bp amplicon range and comprised 163 amplicons covering 92.36% of the target areas. The libraries were prepared according to the standard Ion Ampliseq™ protocol supplied by the manufacturer. Sequencing was performed with Ion torrent PGM (Thermo Fisher Scientific). Primary data processing was performed on Torrent Suite™ Software (Thermo Fisher Scientific). Variant calling was performed on Ion reporter version 4.2 (Thermo Fisher Scientific). Variants with less than 20× coverage were filtered.

CpG island methylator phenotype status

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) was applied to determine the CpG island methylator phenotype (CIMP) status of LLS tumors. We focused on promoter methylation of the *CACNA1G, IGF2, NEUROG1, RUNX3* and *SOCS1* genes, for which SALSA MS-MLPA probemix ME042-B2 (MRC Holland, Amsterdam, The Netherlands[22] was used. The cut-offs for probe-specific hypermethylation in tumor tissue were determined against normal mucosa DNAs as described in Valo *et al.*[23] The hypermethylation threshold was defined as the mean methylation dosage ratio (Dm) in normal mucosa plus two standard deviations (if the mean Dm plus two standard deviations was lower than 0.15, the technical threshold of 0.15 was used as a cut-off). The probemix contains 3–5 probes per gene, of which methylation of ≥25% indicated methylation of the gene in question.[24] According to Weisenberger *et al.*,[25] a sample was considered CIMP positive when three or more out of the five genes showed methylation. The Ogino 5/8 definition for CIMP[26] which includes three additional marker genes (*CDKN2A*, *MLH1* and *CRABP1*) was tested as an alternative. The ME042-B2 assay also includes probes for the *BRAF* V600E mutation and was used to verify the BRAF-IHC data.

MLH1 methylation analysis

MLH1 promoter methylation was studied in samples with deficient MLH1 expression by IHC. The Methylation-quantification of endonuclease-resistant DNA (MethyQESD) was the primary method used. The analysis method is quantitative combining methylation-sensitive digestion with real-time PCR.[27] The methylation-sensitive endonuclease *Hin*6I only recognizes and cuts unmethylated CGCG sites, after which the proportions of un-cut (methylated) and cut (unmethylated) DNAs are determined by real-time PCR.[27] As a rule, samples with methylation percentage of 16.5% are considered methylated.[27] Empirically, for the present purposes a somewhat lower percentage (≥ 11.7%) was used since it was supported by other data, such as parallel *MLH1* methylation analysis by MS-MLPA (*MLH1* is included in the SALSA MS-MLPA probemix ME042-B2, see above).

Statistical analyses

Statistical analyses were performed using the SPSS software, version 24.0 (IBM SPSS Inc., Chicago, IL, USA). The applicability of parametric *vs.* non-parametric tests was first investigated. Statistical significance for the differences in mean ages

of onset and the distributions of mutations or mutant genes between two independent groups were analyzed by the Mann–Whitney U test. Differences between groups of >2 were analyzed by the Chi-square test. For pairwise comparisons of frequency data the Fisher's exact test was used. Two-tailed *p*-values <0.05 were considered significant.

Results

Molecular stratification of colorectal carcinomas from a population-based cohort

Among the 762 colorectal carcinomas examined, 107 showed immunohistochemical absence of one or more MMR proteins and were classified MMR-deficient (dMMR) (Fig. 1). *MLH1* promoter methylation analysis of tumors lacking MLH1 protein identified 81 cases with *MLH1* methylation (76% of all dMMR tumors). All dMMR cases, especially those with absent MLH1 not explained by *MLH1* methylation and those with absent MSH2, MSH6, or PMS2 protein(s), were checked against the National LS registry of Finland (LSRFi), resulting in the diagnosis of Lynch syndrome (LS) in eight. An additional LS case not belonging to any existing LS families (case K14915Ca in Supporting Information Table 2) was subsequently found by sequencing of normal and tumor tissues of the remaining cases (Fig. 1). Thus, LS accounted for 9 of 107 dMMR cases (8%).

Of 18 cases left when *MLH1*-hypermethylated cases and LSRFi-associated LS cases were excluded, sufficient biological material was available from 16 cases to be included in targeted sequencing. Sequencing was unsuccessful in one; thus, a total of three cases remained unclassified because their *MLH1* methylation status or germline mutation status could not be determined. After exclusion of case K14915Ca that revealed a germline mutation in *MLH1* (see above), fourteen cases remained with neither hypermethylation of *MLH1* nor germline mutations in MMR genes, and were considered to represent Lynch-like syndrome (LLS). The LLS group constituted 13% of all dMMR cases and 2% of the entire population-based cohort.

Mechanisms of MMR gene inactivation in LLS tumors

Immunohistochemical pattern suggested *MLH1* inactivation (absent MLH1 and PMS2) in four tumors, *MSH2* inactivation (absent MSH2 and MSH6) in six tumors, and *MSH6* inactivation (selective absence of MSH6) and *PMS2* inactivation (selective absence of PMS2) in two tumors each (Table 1). To address the molecular mechanisms behind MMR gene inactivation, panel sequencing data of normal and tumor tissues were analyzed for somatic mutations and loss of heterozygosity (LOH) of the relevant genes. Two somatic events in MMR genes likely to underlie MMR deficiency were identified in 11 of 14 tumors (79%) (Tables 1 and 2). Somatic mutation accompanied by LOH occurred in 10 tumors and two somatic mutations in one tumor (91% and 9%, respectively, of the 11 two-hit-associated tumors). Two tumors bore two somatic mutations and LOH, simultaneously. Comprehensive Cancer Panel (CCP) and IonTorrent sequencing results were highly concordant: somatic mutations discovered by one method could be confirmed by the other method with the exception of three tumors, in which one of the hits was detected by CCP alone.

Mutation profiles of 578 cancer-relevant genes in LLS tumors

CCP sequencing revealed an average of 124 nonsynonymous somatic mutations per LLS tumor (31/Mb on average, range 2–154 mutations/Mb). Eleven of fourteen LLS tumors (79%) could be classified as hypermutated with the commonly used threshold of over 10 mutations per Mb (Table 1).[28] An average of 82 of 578 genes were mutant per tumor; the mean was 25, if only high-frequency mutations (allele-frequency 25% or higher) were considered. The present set of 9 LS tumors were not available for comparison, since (with one exception) these tumors did not enter the panel sequencing step (Fig. 1). We previously profiled 18 LS tumors from the national LS registry on the same sequencing platform as the present LLS tumors.[12] These tumors were now re-analyzed by the same bioinformatics pipeline as our LLS tumors and used for comparison. The average number of nonsynonymous somatic mutations was 617 per LS tumor, whereas the mean number of mutant genes was 255 (and 55 if only high-frequency mutations were considered). The corresponding figures in LLS tumors were all significantly lower (124, $p < 0.0001$, 82, $p < 0.0001$; and 25, $p = 0.010$, respectively) (Table 1). The mean number of nonsynonymous mutations in LS tumors (617) is equivalent to 154 mutations/Mb, and all 18 LS tumors fulfilled the definition of "hypermutated" (range 38–374 mutations/Mb).

We next plotted each of the 578 genes against the number of tumors in which it was mutant (only mutations with allele frequencies of 25% or higher were considered). The distribution ranged from one gene (*MAML2*) mutant in six samples (43%) to 354 genes without a single mutation in any of the 14 LLS samples (Supporting Information Table 3). Setting the cut-off at 4 of 14 tumors (29%) mutant for a given gene (in analogy to Ref. 12), 13 top mutant genes were detected in LLS tumors (Fig. 2*a*). The same procedure identified 39 top mutated genes in the 18 LS colorectal carcinomas used for comparison (Fig. 2*b*). LLS and LS tumors shared four top mutant genes (*PRKDC*, *TCF7L2*, *ARID1A* and *RPL22*). *TCF7L2*[29] and *RPL22*[30] contain mononucleotide repeats as part of their transcripts and are therefore susceptible to truncating mutations in MSI tumors (Supporting Information Fig. S1 and Supporting Information Table S4). Eleven of 13 LLS-associated genes were mutant with higher frequencies in LLS *vs.* LS tumors (Fig. 2*a*), and the difference reached statistical significance for *BCOR* (4/14 *vs.* 0/18, $p = 0.028$). *MAML2,* which encodes a member of the Mastermind-like family of proteins and positively regulates Notch signaling,[31] showed the highest involvement in LLS tumors (6/14, 43%); this clearly exceeded the corresponding frequency in LS tumors 2/18 (11%). Four of six mutant tumors shared a

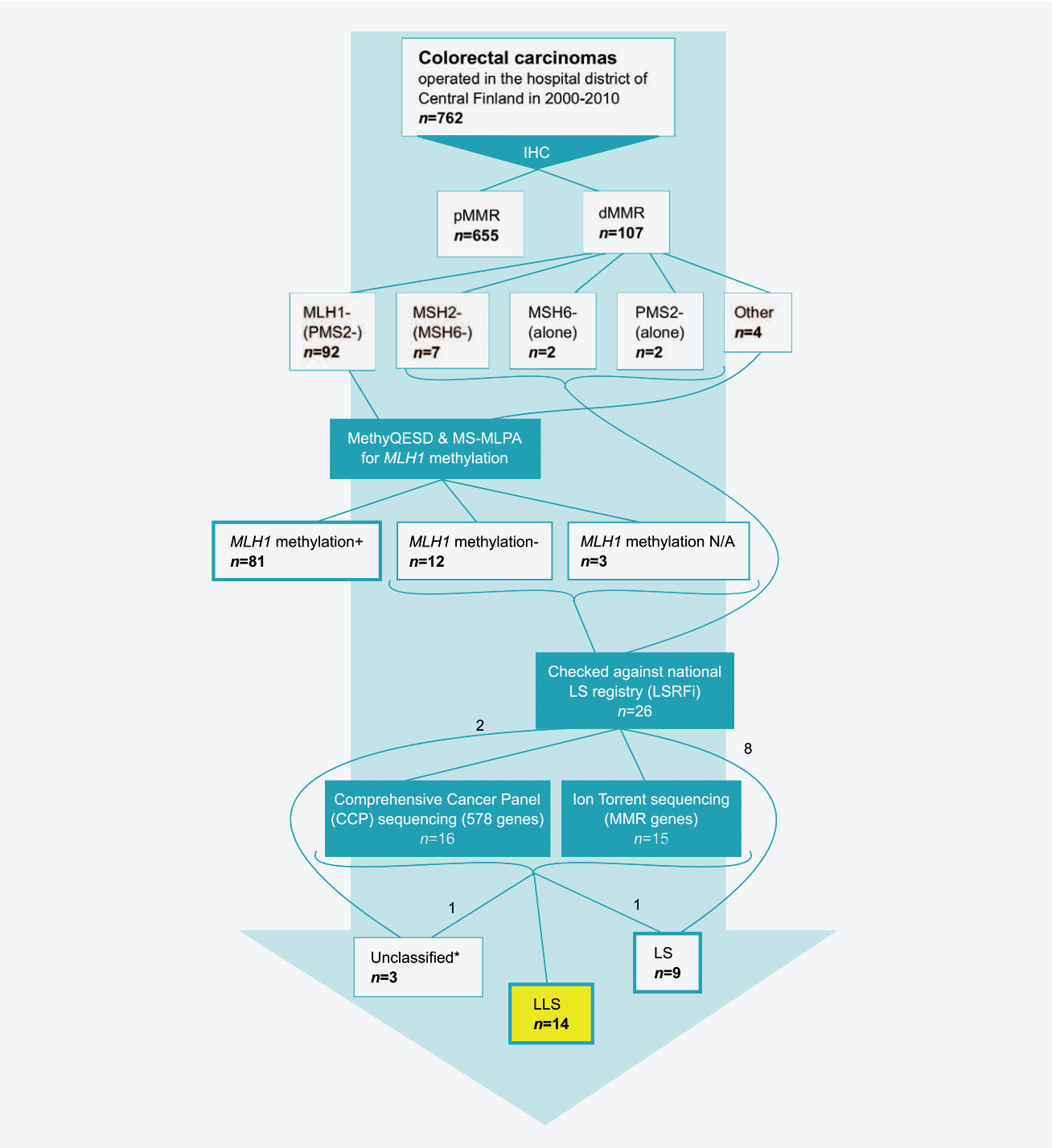

Figure 1. A step-wise protocol to stratify 762 consecutive colorectal carcinomas first into pMMR and dMMR groups and the latter further into *MLH1*-methylation positive, LS, and LLS subgroups. Four tumors did not comply with any of the four main IHC patterns and are referred to as "Other." Since absent MLH1 was part of the abnormal IHC phenotype in all four cases, the tumors were primarily tested for *MLH1* methylation and in the absence of demonstrable *MLH1* methylation, submitted to downstream steps. Asterisk (*) indicates those three dMMR cases to which a specific molecular subgroup could not be assigned because their *MLH1* methylation status or panel sequencing status (to exclude LS) could not be determined. [Color figure can be viewed at wileyonlinelibrary.com]

recurrent 9-bp deletion that affects a polyglutamine repeat of *MAML2* and is present in the COSMIC database (Supporting Information Table 4). Reflecting the relatively small total numbers of cases in the LLS and LS groups, the p values for differences were modest overall (Figs. 2*a* and *b*). Although not reaching statistical significance, our study recapitulated the

Table 1. Clinicopathological features of LLS colorectal carcinomas

Sample	Age of onset (years)	tumor %	Tumor location	Tumor grade	Tumor stage	MSI status	CIMP status	Pattern of MMR-loss[1]	*MLH1* methylation (%)	Mechanism of two-hit inactivation of MMR genes[2]	No. of non-synonymous somatic mutations ($p < 0.01$)	No. of genes mutated ($p < 0.01$)	No. of genes with high-frequency[3] 1mutations ($p < 0.01$)
K17631Ca	60	N/A	descending colon	1	1	MSI	positive	MLH1/PMS2	1	m + L	267 (67/Mb)	166	49
K11462Ca	70	50	descending colon	2	2a	MSI	positive	MLH1/PMS2	0.4	m + L	105 (26/Mb)	90	9
K1604Ca	67	80	caecum	mucinous	2a	MSI	negative	MLH1/PMS2	0.6	m + L	13 (3/Mb)	13	7
K15481Ca	74	50	descending colon	2	2a	MSI	positive	MLH1/PMS2	0.04	m + L	72 (18/Mb)	64	39
K7047Ca	73		rectum	1	2a	MSI	positive	MSH2/MSH6	NA	m + m/m + L	26 (7/Mb)	23	19
K11334Ca	71	60	sigmoid colon	2	3c	MSS	positive	MSH2/MSH6	NA	none	131 (33/Mb)	101	75
K3364Ca	62	40	transverse colon	1	3b	MSI	positive	MSH2/MSH6	NA	m + L	54 (14/Mb)	51	5
K9148Ca	38	80	transverse colon	1	2a	MSI	positive	MSH2/MSH6	NA	m + L	75 (19/Mb)	64	10
K14340Ca	70	60	sigmoid colon	2	2b	MSI	positive	MSH2/MSH6	NA	m + m/m + L	71 (18/Mb)	61	19
K8130Ca	75	70	caecum	1	1	MSI	positive	MSH2/MSH6	NA	m + L	133 (33/Mb)	104	39
K13654Ca	55	40	sigmoid colon	2	2a	MSI	positive	MSH6	NA	m + m	614 (154/Mb)	274	48
K2426Ca	89	40	ascending colon	1	3b	MSS	positive	MSH6	NA	none	8 (2/Mb)	6	1
K13040Ca	41	60	caecum	mucinous	2a	MSI	positive	PMS2	NA	none	42 (11/Mb)	39	5
K18762Ca	71	80	ascending colon	mucinous	4	MSI	positive	PMS2	NA	m + L	120 (30/Mb)	87	29
											Mean, 124 (31/Mb)	Mean, 82	Mean, 25

Abbreviation: N/A, not available.
[1]Pattern of MMR protein loss of expression by IHC.
[2]m, somatic mutation; L, LOH (see Supporting Information Table 3 for details).
[3]Mutant allele frequency 25% or higher.

Table 2. Evaluation of LLS tumors for somatic two-hit inactivation of MMR genes implicated by IHC results

	Sample	Hit one	Clinical significance[1]	Allele frequency (%)[2]	Hit two	Clinical significance[1]	Allele frequency (%)[2]
MLH1 (PMS2)	K17631Ca	*MLH1* 3:37042546T>G	Possibly pathogenic (splice site)	32	Strict LOH (*MLH1*)		
	K11462Ca	*MLH1* 3:37089123delGAA	Unknown (codon deletion, LK615L)	16	Putative LOH (*MLH1*)		
		PMS2 7:6026565insT	Pathogenic (frameshift)	17	Putative LOH (*PMS2*)		
	K1604Ca	*MLH1* 3:37070338delTGCAGCT	Pathogenic (frameshift)	38	Strict LOH (*MLH1*)		
	K15481Ca	*MLH1* 3:37067374delG	Pathogenic (Frameshift)	66	Strict LOH (*MLH1*)		
MSH2 (MSH6)	K7047Ca	*MSH2* 2:47702410G>A	Possibly pathogenic (splice site)	30	*MSH2* 2:47707898insA	Pathogenic (frameshift)	26
					Strict LOH (*MSH2*)		
	K11334Ca	None			None		
	K3364Ca	*MSH2* 2:47693838C>T	Pathogenic (nonsense)	22	Strict LOH (*MSH2*)		
	K9148Ca	*MSH2* 2:47641557G>C	Possibly pathogenic (splice site)	41	Strict LOH (*MSH2*)		
	K14340Ca	*MSH2* 2:47643434G>C	Possibly pathogenic (splice site)	18	*MSH2* 2:47656915G>T	Pathogenic (nonsense)	35
					Strict LOH (*MSH2*)		
		MSH6 2:48030639delC	Pathogenic (frameshift)	28	Strict LOH (*MSH6*)		
	K8130Ca	*MSH2* 2:47637395G>A	Unknown (missense, E177K)[3]	8	Putative LOH (*MSH2*)		
		MSH6 2:48026021G>A	Unknown (missense, R300Q)	6	Putative LOH (*MSH6*)		
MSH6	K13654Ca	*MSH6* 2:48032116C>T	Unknown (missense, P1169L)	14	*MSH6* 2:48027142G>A	Unknown (missense, G674R)	18
	K2426Ca	None			None		
PMS2	K13040Ca	None			None		
	K18762Ca	*PMS2* 7:6018283A>T	Unknown (missense, I740K)	11	Putative LOH (*PMS2*)		

IHC pattern of loss of MMR proteins is indicated on the left.
[1]Frameshift and nonsense changes were considered pathogenic and splice site changes as possibly pathogenic *a priori*.
[2]Based on Comprehensive Cancer Panel sequencing.
[3]Class 3 in the InSiGHT database.

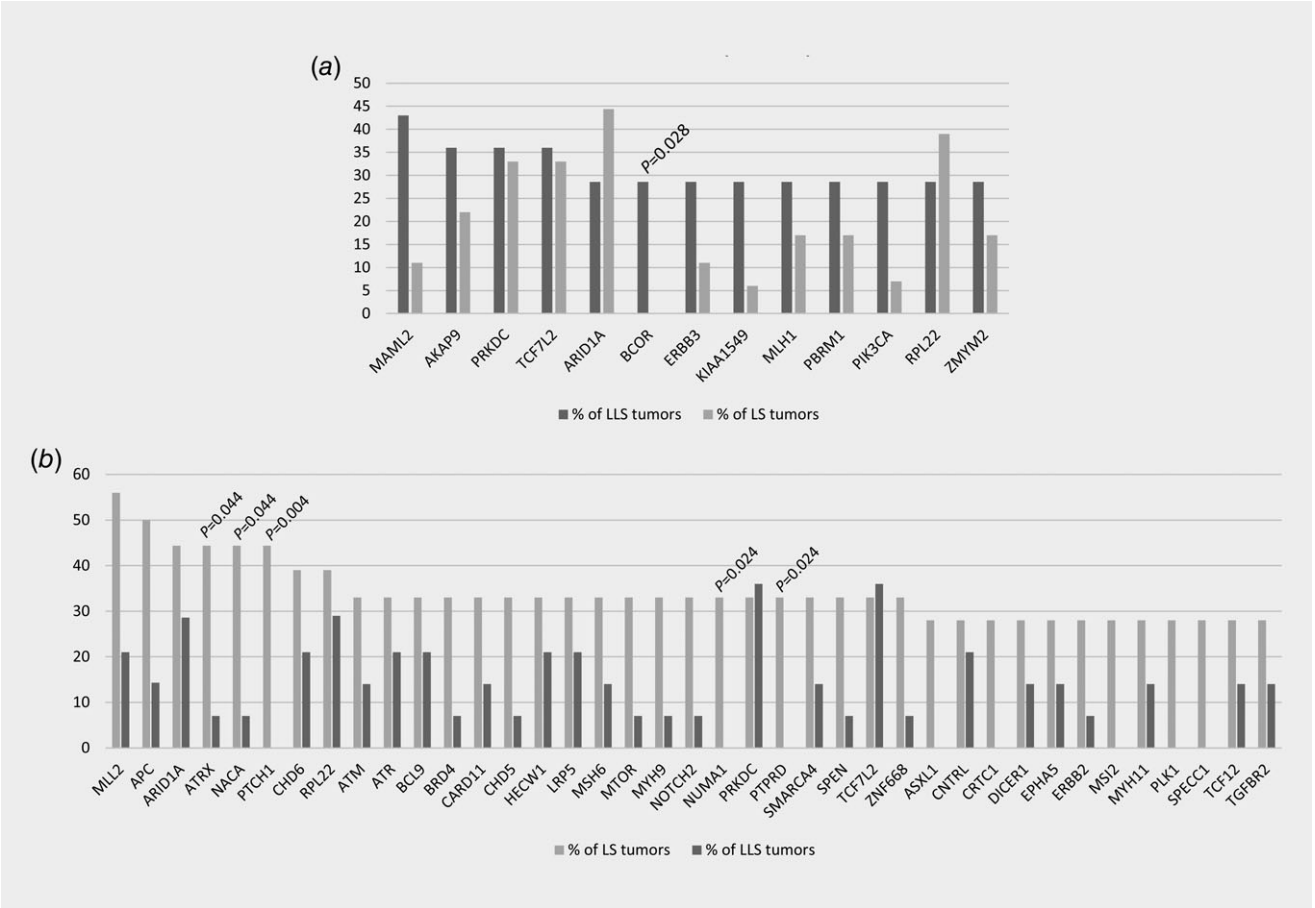

Figure 2. (*a*) Top **13** mutated genes in LLS-colorectal carcinomas. Genes affected with high-frequency mutations (mutant allele frequency ≥ **25**%) in at least **29**% (**4**/**14**) of LLS tumors are shown. Mutation percentages of the same genes in LS-colorectal carcinomas are depicted for comparison. The difference between LLS and LS was statistically significant for *BCOR* and non-significant for the remaining genes by Fisher's exact test. (*b*) Top **39** mutated genes in LS colorectal carcinomas. Genes affected with high-frequency mutations (mutant allele frequency ≥ **25**%) in at least **28**% (**5**/**18**) of LS tumors (from Ref. **12**) are shown. Mutation percentages of the same genes in LLS-colorectal carcinomas are displayed for comparison. Exact *p*-values for statistically significant differences are given above the frequency bars.

observation by Cohen *et al.*[32] of more frequent *PIK3CA* mutations in LLS (4/14, 29%) than LS tumors (1/18, 7%). Detailed mutation information for all 13 LLS-associated genes is available in Supporting Information Fig. S1 and Supporting Information Table S4.

CpG Island Methylator phenotype status of LLS tumors

Previous studies have linked CIMP-positivity to *MLH1* methylated sporadic tumors and a proportion of LS tumors,[33,34] whereas no information is available for LLS tumors. Therefore, it was of particular interest to determine the CIMP status of the present LLS tumors. By the Weisenberger criteria,[25] all but one tumor (13/14, 93%) were CIMP-positive (Table 1, Supporting Information Table 5). This frequency was significantly higher compared to that for LS colorectal tumors previously evaluated by the same criteria (9/18, 50%)[12] (p = 0.019). In LLS tumors, CIMP did not affect *MLH1* promoter, and IHC-analysis showed that all LLS tumors were negative regarding *BRAF* V600E mutation (see Table 3 below).

Clinicopathological characteristics of colorectal carcinoma subgroups

The basic clinicopathological characteristics of the different categories of colorectal carcinomas identified by molecular stratification (Fig. 1) are shown in Table 3. LLS colorectal carcinomas were diagnosed at the age of 65 years on the average *vs.* 44 years in the LS cases ($p < 0.001$), and 76 years in the *MLH1*-methylated group ($p < 0.001$) (Table 3). LLS tumors had a proximal to distal ratio of 1:1, whereas LS tumors occurred primarily (89%) in the proximal part of the colon ($p = 0.086$). Well-differentiated (grade 1) tumors accounted for the majority (43%) in the LLS group, whereas poorly differentiated (grade 3) tumors predominated with 59% frequency in the LS group ($p = 0.009$). LLS tumors revealed the highest frequency of mucinous tumors (21%) among the different tumor categories. Information of synchronous/metachronous tumors

Table 3. Summary of clinicopathological data of all samples[1]

	Lynch-like cases	Lynch cases	*p*-value (*vs.* LLS)	*MLH1* methylated cases	*p*-value (*vs.* LLS)	pMMR cases	*p*-value (*vs.* LLS)
Average age of onset (years)	65	44	< 0.001[2]	76	< 0.001[2]	70	0.123[2]
Tumor location[3]							
Proximal	7/14 (50%)	8/9 (89%)	0.086[4]	60/80 (74%)	0.105[4]	229/653 (35%)	0.267[4]
Distal	7/14 (50%)	1/9 (11%)		20/80 (25%)		424/653 (65%)	
Tumor grade[5]							
1	6/14 (43%)	1/9 (11%)	0.009[6]	17/81 (21%)	0.181[6]	221/654 (43%)	0.023[6]
2	5/14 (36%)	3/9 (33%)		34/81 (42%)		363/654 (55%)	
3	0/14 (0%)	5/9 (56%)		18/81 (22%)		42/654 (6%)	
4	0/14 (0%)	0/9 (0%)		1/81 (1%)		1/654 (0.15%)	
mucinous	3/14 (21%)	0/9 (0%)		11/81 (14%)		27/654 (4%)	
Tumor stage[5]							
1	2/14 (14%)	2/9 (22%)	0.702[6]	12/81 (15%)	0.745[6]	131/645 (20%)	0.717[6]
2a	7/14 (50%)	3/9 (33%)		31(81 (38%)		194/645 (30%)	
2b	1/14 (7%)	2/9 (22%)		9/81 (11%)		24/645 (4%)	
3a	0/14 (0%)	0/9 (0%)		2/81 (2%)		23/645 (4%)	
3b	2/14 (14%)	2/9 (22%)		14/81 (17%)		116/645 (18%)	
3c	1/14 (7%)	0/9 (0%)		12/81 (15%)		60/645 (9%)	
4	1/14 (7%)	0/9 (0%)		1/81 (1%)		97/645 (15%)	
BRAF V*600E status*							
Negative	12/12 (86%)	9/9 (100%)	1[4]	18/81 (22%)	< 0.0001[4]	604/638 (95%)	1[4]
Positive	0	0		75/81 (70%)		34/638 (5%)	
Presence of synchronous/ metachronous tumor(s)							
Colorectal adenoma	6/13 (46%)	5/9 (56%)	1[4]	24/81 (30%)	0.336[4]	221/634 (35%)	0.394[4]
Colorectal carcinoma	0/13 (0%)	2/9 (22%)	0.156[4]	5/81 (6%)	1[4]	32/634 (5%)	1[4]
Cancer of any organ	2/13 (15%)	5/9 (56%)	0.074[4]	NA		NA	

NA, Data not available.
[1]Three of 762 samples could not be categorized into any of the four subgroups (LLS, LS, *MLH1* methylated, and pMMR).
[2]Calculated by Mann–Whitney U-test.
[3]Caecum, ascending colon and transverse colon were counted as proximal and descending colon, sigmoid colon and rectum as distal.
[4]Calculated by Fisher's exact test.
[5]According to the UICC guidelines (6th edition).
[6]Calculated by Chi-square test.

was available from 13 LLS cases. With respect to cancers of any organs, one LLS patient was diagnosed with transitiocellular carcinoma of the bladder and another one with tonsillar diffuse large-cell B-cell lymphoma; the difference relative to LS patients was borderline significant (15% *vs.* 56%, $p = 0.074$).

Discussion

Our population-based investigation of 762 colorectal carcinomas operated in the hospital region of Central Finland during 2000–2010 identified 107 MMR-deficient cases (14% out of 762). Tumors with *MLH1* promoter methylation was the largest subgroup among MMR-deficient colorectal cancers (69%), followed by LLS (13%) and LS (8%) (Fig. 1). LLS cases were diagnosed at a mean age of 65 years, which differed significantly from both LS (44 years) and *MLH1*-methylated cases (76 years) (Table 3). Published reports have found lower mean or median ages at onset for LLS colorectal cancers compared to *MLH1*-hypermethylated colorectal cancers, and roughly comparable ages at onset for LLS *vs.* LS.[5,35] Our LLS colorectal carcinomas were equally often proximal and distal; some studies have found predilection to proximal colon in LLS.[35] Grade 1 tumors predominated in the LLS group, as opposed to grade 3 in LS (Table 3), in agreement with Rodriguez-Soler *et al.*[5] Synchronous or metachronous cancers are less common in LLS than LS patients (Table 3 and Ref. 35), emphasizing a sporadic non-syndromic nature of LLS.

To our knowledge, our study is the first to show a frequent methylator phenotype (not affecting *MLH1*) as a feature of LLS colorectal carcinomas. All LLS tumors except one (93%) exhibited CIMP positivity (Table 1), compared to 50% of LS tumors[12] ($p = 0.019$). At present, no universal consensus exists regarding the laboratory method, marker panel, or marker threshold values for CIMP definition.[24] We studied our samples with an alternative panel (Ogino 5/8, see Materials and Methods) as well. The same relative trend as with the original Weisenberger 3/5 panel was evident, but with somewhat lower frequencies for CIMP (11/14, 79% for LLS and 7/18, 39% for

LS, $p = 0.036$). Both LLS and LS colorectal carcinomas show wild-type *BRAF* V600E as a marked distinction to sporadic *MLH1*-methylated colorectal carcinomas (Table 3; Ref. 36). In sporadic colorectal carcinomas, *MLH1* methylation, CIMP-positivity and *BRAF* V600E mutation are tightly connected.[25] LLS colorectal carcinomas thus exhibit a unique tumor phenotype that distinguishes this group from LS (CIMP-status) and sporadic *MLH1*-methylated tumors (*BRAF* V600E status).

All LLS colorectal carcinomas except K11334Ca and K2426Ca showed MSI (Table 1). Stable microsatellites in the presence of abnormal MMR protein expression in the two cases might reflect the higher sensitivity of IHC to detect small subpopulations of tumor cells compared to MSI analysis.[37] All LS colorectal carcinomas had MSI-high phenotype as shown previously.[12] Despite proven MSI, targeted sequencing of 578 cancer-relevant genes revealed a significantly lower propensity for LLS tumors to acquire somatic nonsynonymous mutations compared to LS tumors (see Results). No obvious technical reason to explain the difference exists. As the *RPL22* and *TCF7L2* examples suggest (see Results), our somatic mutation analysis is expected to correctly identify a majority of small insertions and deletions, too, in addition to single nucleotide variants effectively identified by the available pipelines. Moreover, the average tumor percentages were even higher for LLS than LS tumors (59% *vs.* 49%, respectively). The distinct epigenetic patterns (see above) and mutational profiles of cancer-relevant genes (Figs. 2*a* and 2*b*) support intrinsic biological differences between the two tumor groups.

The unique molecular profile of CIMP without *BRAF*-V600E mutation raises the question about the pathway of colorectal tumorigenesis in LLS. Most colorectal carcinomas, including those occurring in LS, are thought to develop *via* adenomatous polyps, whereas *MLH1* hypermethylated carcinomas often arise from serrated polyps.[38,39] According to histopathological review, precursor lesions were not identified in any of our 14 LLS carcinomas. However, 6 cases showed serrated adenocarcinoma features (epithelial serration, clear or eosinophilic cytoplasm, abundant cytoplasm, vesicular nuclei, absence of or less than 10% necrosis of total area, mucin production and cell balls or papillary fronds in mucinous areas of the tumor).[40] Six carcinomas did not have serrated histology. Two carcinomas had some serrated features but were not considered to represent serrated adenocarcinoma. In the series of Mas-Moya *et al.*,[35] colorectal cancer precursor lesion was observed in 7 out of 16 cases and all lesions were adenomatous polyps. Molecularly, *APC* and *KRAS* mutations are an integral part of adenoma-carcinoma sequence irrespective of MSI and also common in serrated adenomas associated with *MLH1* promoter methylation, although *KRAS* mutations are less frequent than *BRAF* mutations in the latter group. Determined from our panel sequencing data, 6 of 14 LLS tumors (43%) had truncating *APC* mutations. Three LLS tumors (21%) showed (non-truncating) mutations in *KRAS*, with the known pathogenic hotspots[41,42] involved in two cases, G12D in one tumor and A146T in another one. Among 18 LS colorectal carcinomas studied for comparison, 11 (61%) and 4 (22%) were mutant for *APC* and *KRAS*, respectively (all *KRAS* mutations affected the hotspot sites, G12 V and G13D in one tumor each and A146T in two tumors). Thus, colorectal tumorigenesis in LLS is associated with frequent *APC* and *KRAS* mutations, comparable to LS. It has been hypothesized that mutant *KRAS*, like mutant *BRAF*, may contribute to CIMP as they belong to the same signaling pathway.[34] This mechanism may explain CIMP in *BRAF*-V600E negative tumors (LS and LLS). Taken together, while the question about histology of LLS precursor lesions requires additional investigations, our results define a molecular pathway where *APC* and *KRAS* mutations combined with general hypermutability (MSI) and tumor suppressor gene inactivation by promoter methylation (CIMP) are important players.

Several potential clinical implications can be foreseen. The frequent occurrence of CIMP in LLS tumors may have prognostic relevance since CIMP has been shown to be associated with worse prognosis of colorectal cancer irrespective of MSI status.[43] The predictive value of CIMP remains unsettled.[43] *PIK3CA* mutations may predict resistance to anti-EGFR therapy.[44] Kloth *et al.*[36] combined LLS and LS colorectal cancers to a single *BRAF*-wild type MSI-H group and found that 15% of the tumors showed activating mutations in *ERBB2* that might indicate responsiveness of pan-HER inhibitors. High-frequency mutations in *ERBB2* were present in 7% (1/14) of our LLS tumors. Among the different members of the ErbB gene family, *ERBB3* revealed the most common involvement, being one of the top mutant genes in LLS tumors (29%, 4/14; (Fig. 2*a*). *ERBB3* mutations occur in up to one-fifth of various cancers overall and sensitivity to inhibitors of HER family has been documented.[45] Finally, hypermutability and neoantigen-induced immunoreactions make MSI-H tumors good candidates for PD-1 blockade-based immunotherapy.[46] While no data are available of LLS tumors specifically, Le *et al.*[47] reported that non-Lynch patients with MMR-deficient tumors responded significantly better than LS patients. The mechanisms behind the difference remain unknown. Our observation of fewer somatic mutations in LLS *vs.* LS colorectal carcinomas is by no means in conflict with the above; LLS (like LS) tumors are MMR-deficient and hypermutable, and the presence of certain particularly immunogenic neoantigens, rather than the total mutational burden, may ultimately determine the response to PD-1 blockade.[48]

In conclusion, LLS comprises a significant share of MMR-deficient colorectal carcinomas, ranging from 13% (our study) to 32%.[5] The reported clinicopathological features of LLS relative to the other two main groups of MMR-deficient colorectal carcinomas vary, in part likely to reflect the rather limited sizes of study series and/or possible geographical variation. LLS cannot be reliably distinguished from LS on clinical (Refs. 5,35 and our study) or histological (Refs. 5,35,49 and our study) grounds. The novel epigenetic and genetic features we

describe for LLS colorectal carcinomas can aid in the recognition of LLS as a separate entity. Our findings shed light to the pathogenesis of LLS carcinomas beyond MMR defects and may facilitate targeted approaches for treatment.

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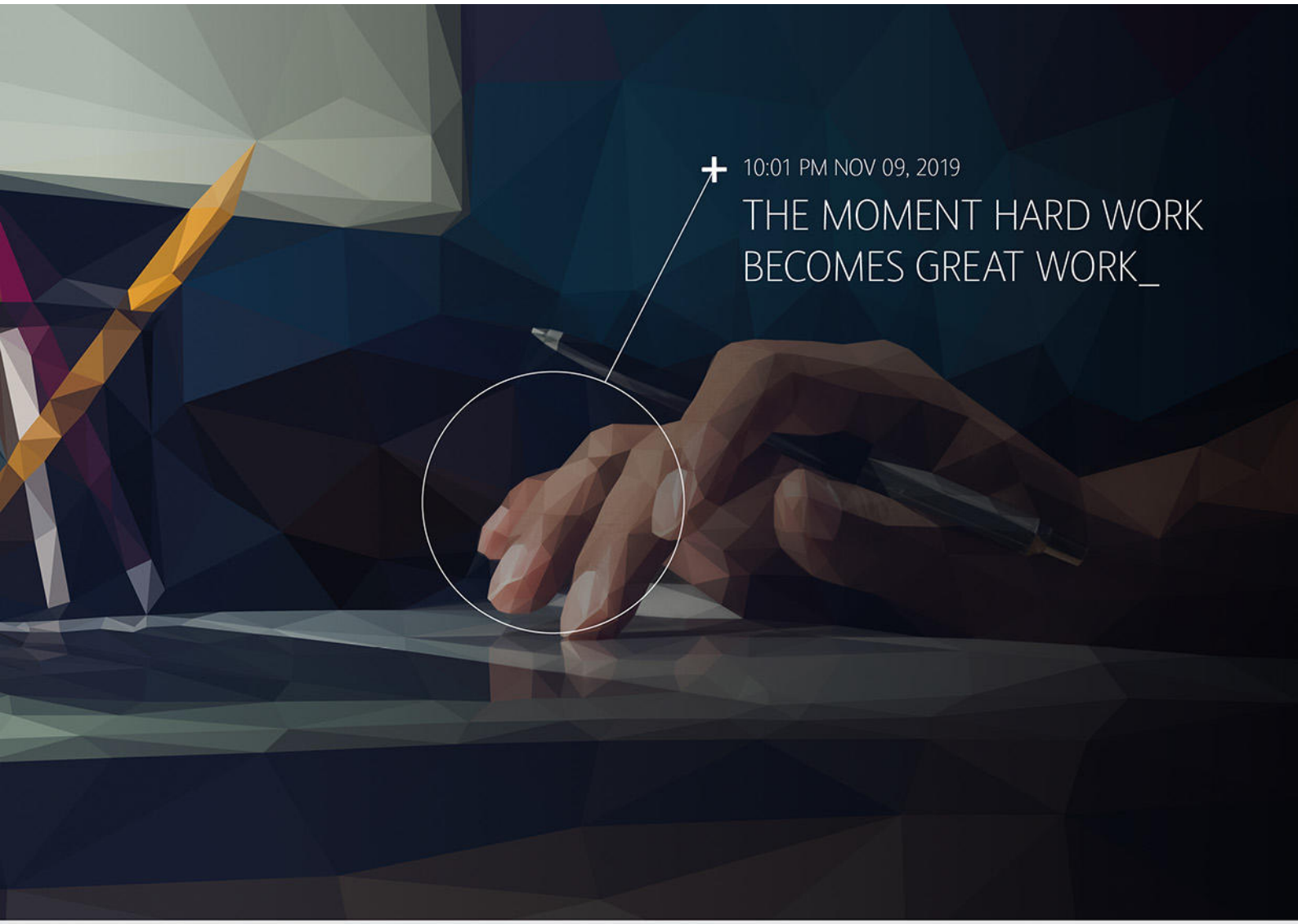

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