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Aberrant intestinal stem cell lineage dynamics in Peutz–Jeghers syndrome and familial adenomatous polyposis consistent with protracted clonal evolution in the crypt

Danielle Langeveld^{1,2}, Marnix Jansen², D V de Boer¹, Mariska van Sprundel¹, Lodewijk A A Brosens¹, Folkert H Morsink¹, Francis M Giardiello³, G Johan A Offerhaus^{1,2}, and Wendy W J de Leng¹

¹Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands

²Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands ³Division of Gastroenterology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Abstract

Objective—Genetic predisposition to cancer in Peutz–Jeghers syndrome (PJS) and the role of germline serine–threonine kinase (*LKB1*) mutations are poorly understood. The authors studied the effect of germline *LKB1* mutations on intestinal stem cell dynamics in unaffected flat PJS mucosa. Recent research has documented that the intestinal crypt houses multiple equipotent stem cell lineages. Lineages continuously compete through random drifts, while somatically inherited methylation patterns record clonal diversity.

Design—To study the effect of germline *LKB1* mutations on clonal expansion, the authors performed quantitative analyses of cardiac-specific homeobox methylation pattern diversity in crypts isolated from unaffected colonic mucosa obtained from archival PJS patient material. The authors compared methylation density and methylation pattern diversity in patients with PJS to those in patients with familial adenomatous polyposis and age-matched controls.

Results—The percentage of total methylation is comparable between groups, but the number of unique methylation patterns is significantly increased for patients with familial adenomatous polyposis and patients with PJS compared to control subjects.

Conclusions—Monoallelic *LKB1* loss is not silent and provokes a protracted clonal evolution in the crypt. The increased methylation pattern diversity observed in unaffected PJS mucosa predicts that premalignant lesions will arise at an accelerated pace compared to the general population.

Correspondence to: Wendy W J de Leng, Department of Pathology, University Medical Center, Utrecht, H 04.312, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands; w.w.j.deleng@umcutrecht.nl.

Competing interests None.

Patient consent Study materials are archival tissues of anonymised patients from different institutions that were used with patient consent and according to the medical ethical guidelines of the institutions.

Ethics approval The ethics committee of the University Medical Center Utrecht and Academic Medical Center Amsterdam granted approval to the study.

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INTRODUCTION

Evolutionary change is dependent on the presence of heritable variation within a population. Malignant tumour progression is characterised by the successive emergence of competing neoplastic clones. Cancer can therefore be viewed as a disease of Darwinian somatic evolution.¹ The selection of clonal populations varying in fitness is accelerated by increased genetic diversity. Indeed, in Barrett's oesophagus, the degree of clonal genetic diversity can predict progression to malignancy.² The adenoma–carcinoma sequence in the colorectum describes the stepwise progression from low-grade dysplasia to invasive carcinoma through the accumulation of genetic lesions. The study of tumour evolution at each of these stages rests on measuring changes in the frequency of (epi)genetic variants in a tumour population. To measure the number of variants present, one ideally compares clonal markers, such as point mutations in tumour suppressor genes, to document the dynamics of various clones competing over time.

Polymorphic markers can be used to visualise the spread of mutations during the earliest stages of tumour formation.³ In fact, several previous studies have shown that non-dysplastic epithelia may harbour pathogenic DNA mutations.^{4,5} The phase of tumour progression whereby normal epithelia collect mutations in the absence of phenotypic change is termed 'pre-tumour progression'.⁶ We can compare the rates of pre-tumour progression by retrospective analyses of genomic differences between populations of progenitor cells in epithelia. In the colorectum, every crypt is a single clonal unit, and each crypt harbours multiple equipotent stem cells that together give rise to all other postmitotic differentiated cells populating the crypt epithelium.⁷ All stem cells generate daughter progeny through continuous self-renewal; every individual stem cell and its clonal daughter progeny represent one lineage in the crypt. Stem cell lineages carrying polymorphic marker mutations may be randomly lost from the crypt or attain dominance through drifts, showing that individual lineages are in neutral competition.⁸ New potential markers, such as genomic methylation changes or somatic mutations, arise continuously. However, as we have no practical method of prospectively identifying and tracing these markers in the gut over time, the process of lineage competition through genetic drift is obscure in routine histology.

Elegant studies by Darryl Shibata and colleagues on a patient with familial adenomatous polyposis (FAP) material,^{9,10} formulated a statistical approach to drifts in the allele frequencies of polymorphic marker mutations in the crypt. These dynamic modelling studies are based on a retrospective analysis of genomic differences within a population of intestinal crypt progenitor cells through the concept of a molecular clock. In paleontology, molecular clocks record time between a last common ancestor and species divergence based on the random accumulation of genetic changes according to a predetermined neutral mutation rate between related genomes.¹¹ Analogously, phylogenies of progenitor lineages within individual crypts can be revealed based on the genetic changes accumulated over time since progenitor lineages underwent a clonal homogenisation event (known as 'crypt niche succession').¹² A marker under neutral selection shows an increased measure of heterogeneity as lineages diverge over time. Following this reasoning, Kim *et al* analysed the diversity in the methylation patterns of the CpG island of the cardiac-specific homeobox (*CSX*) gene (which is not expressed in the colorectum) between individual crypts in fresh colectomy specimens. Heterogeneity of methylation patterns was significantly greater in histologically normal FAP crypts than in normal control crypts.⁹

Whether intestinal stem cell dynamics are also affected in other gastrointestinal polyposis syndromes carrying an increased cancer risk is unknown. Peutz–Jeghers syndrome (PJS) is an autosomal-dominant cancer susceptibility syndrome characterised by the formation of characteristic non-dysplastic polyps throughout the gastrointestinal tract¹³ and an 18-fold

increased risk of developing intestinal and extraintestinal cancers.¹⁴ PJS is caused by a germline mutation in serine–threonine kinase 11 (*LKB1*).^{15,16} Whether *CSX* methylation pattern analysis is also suitable for formalin-fixed paraffin-embedded archival material has, thus far, not been studied. The aim of this study was to explore clonal expansion and pretumour progression in patients with PJS. We find that flat (ie, unaffected) PJS mucosa demonstrates a significantly increased diversity in methylation patterns, corresponding to a protracted clonal evolution scenario in the *LKB1* hemizygous crypt.

MATERIALS AND METHODS

Patients

Paraffin-embedded archival material of normal non-dysplastic colon mucosa was studied. Five well-documented classic FAP cases (polyp number >100) and five well-documented patients with PJS with a previously identified *LKB1* germline mutation were age-matched to five control subjects (table 1). The research was carried out in accordance with the ethical guidelines of the research review committee of our institution.

DNA isolation from single crypts

Paraffin-embedded tissue was cut into 5 µm sections and mounted onto PALM slides (Microlaser Technologies, Bernried, Germany). The slides were deparaffinised and briefly counter-stained with haematoxylin. Single crypts were recovered with microdissection using the PALM Laser Microbeam System, followed by DNA extraction in 20 µl volumes of PicoPure extraction solution (PicoPure DNA extraction Kit; MDS Analytical Technologies, Nieuwerkerk ad IJssel, The Netherlands) according to the manufacturer's instructions.

Sodium bisulphite conversion and *CSX* methylation analysis

DNA was treated with sodium bisulphite (EpiTect Bisulphite Kit; Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The *CSX* gene was amplified using nested PCRs. The first PCR was performed in a final volume of 20 µl with 0.375 mM dNTPs, 3.41 mM MgCl₂, 0.85 units of Platinum Taq DNA Polymerase (Invitrogen, Breda, the Netherlands), 1 µM of each primer, Methylation Specific PCR (MSP) buffer (10× MSP buffer, 166 mM (NH₄)₂ SO₄, 670 mM Tris (pH 8.8), 67 mM MgCl₂ and 100 mM β-mercaptoethanol) and 5 µl of sodium-bisulfate-treated DNA. The following primers were used: 5'-GGGGAGAAGGGGTTTTTAATAT-3' and Rev 5'-AAAAACACTCCTAAAAAACTAA-3'. The first PCR started with 5 min denaturation at 95°C, followed by 45 cycles of 30 s denaturation at 95°C, 1.30 min annealing at 61°C and 1 min elongation step at 72°C. The program ended with 5 min elongation at 72°C. The second PCR was performed in a final volume of 20 µl with 0.375 mM dNTPs, 2.5 mM MgCl₂, 1.25 units of Platinum Taq DNA Polymerase (Invitrogen), 1 µM of each primer and 1 µl of the first PCR product diluted 1:100. The following primers were used: 5'-GGAGATTTAGGAATTTTTTTTGT-3' and Rev 5'-ACACCAAACACTACAAAATCACTCATTA-3'. The second PCR started with 5 min denaturation at 95°C, followed by 25 cycles of 30 s denaturation at 95°C, 1.30 min annealing at 58°C and 1 min elongation at 72°C. The program ended with 5 min elongation at 72°C. An overhang was created with the Illustra GFX PCR DNA and Gel Band purification kit (GE Healthcare, Eindhoven, The Netherlands) according to the manufacturer's instructions. Next, purified PCR products were cloned with the pGEM-T Vector System I (Promega, Leiden, The Netherlands). Cloned inserts were then sequenced using ABI Big Dye Terminator mix (Applied Biosystems, Nieuwerkerk ad IJssel, The Netherlands) and run on an ABI 3100 Genetic Analyzer. Sequences were determined with the use of Codon Code Aligner (CodonCode Corporation, Dedham, MA, USA). Sequences with incomplete bisulphite conversion were omitted from analysis.

Data analysis

SPSS version 15.0 software package was used for statistical analysis. Independent t test was applied for analyses, and statistical significance was defined as $p < 0.05$.

RESULTS

The diversity between stem cell lineages present in the colonic crypt can be investigated by analysing polymorphic sequences in the CpG island of the *CSX* gene. This gene is not expressed in the colorectum, and changes in the methylation status of individual CpG sites are therefore expected to be effectively neutral. Previous studies show that, as a consequence of methylation errors during cell division, CpG islands in mitotically active tissues randomly accumulate methylation errors with normal ageing.¹⁷ With ageing, methylation patterns drift within and between individual crypts. Since methylation errors are somatically inherited, a stretch of CpG sites can be read by bisulphite sequencing as a clonal tag marking an individual stem cell lineage in the crypt.¹⁰

Following the method outlined in figure 1, we analysed crypt patterns from the archival material of five patients with FAP and five patients with PJS and compared these to five control subjects. For every individual, 10 crypts were microdissected; for every crypt, 10 sequences were analysed. Thus, for every group, a total of 500 sequences were compared. Validation experiments showed that increasing the number of tags analysed per crypt to greater than 10 did not further increase diversity measures (data not shown). Pertinent to the *CSX* CpG island data used as readout in our study are data recently reported by Graham *et al.*,¹⁸ who investigated clonal relationships between adjacent micro-dissected crypts in a similar set-up. This investigation revealed that, among the polymorphic markers studied, the *CSX* CpG island showed the most dynamic behaviour. For this reason, the *CSX* CpG island is an attractive marker for recording drifts in allele frequencies in stem cell niches. Preferentially though, multiple loci are investigated to further increase the level of statistical confidence.

Unique methylation patterns

The number of unique methylation patterns is defined as the total number of different methylation patterns found within a single crypt. It indicates the amount of diversity in a crypt and reflects the time since the population last went through a clonal homogenisation event. As such, recently related lineages will have comparable methylation patterns, whereas more distantly related lineages will show greater divergence. The average number of unique methylation patterns was calculated for patients with FAP, patients with PJS and control subjects, and the results are presented in figures 2 and 3A,B. Multiple unique methylation patterns were observed in most crypts of normal PJS and FAP mucosa, consistent with genetic drift in allele frequencies through neutral competition. The average number of unique methylation patterns in patients with FAP (2.78) and patients with PJS (2.90), compared to controls (2.16), was significantly increased ($p = 0.030$ and $p = 0.008$, respectively; table 2). The maximum number of unique methylation patterns was 8, 7 and 5 for patients with FAP, patients with PJS and control subjects, respectively. These results reveal a significant increase in clonal diversity within crypt stem cell niches in these two polyposis syndromes when compared to control subjects.

Percentage of methylation

A given cell lineage has a chronological age (time since birth) and a mitotic age (total number of divisions since zygote stage). Ideally, three cohorts of an identical mitotic age are compared. However, since adequate patient material is scarce, we carefully selected and age-matched patients and control subjects to include a broad range of mitotic ages in our

study groups. In the mitotically active colonic epithelium, mitotic age increases with chronological age and is reflected by the percentage of methylation.¹⁰¹⁹ Per cent methylation is defined as the total number of methylated CpG sites compared to the total possible number of methylated sites. For example, the *CSX* sequence tag 00100100 shows 25% methylation because two out of eight sites are methylated. Since a greater mitotic age would spuriously provoke a greater number of random methylation errors (and possibly a greater number of unique methylation patterns), it is of essence to compare the average level of methylation between groups as a control for mitotic age. The percentage of methylated CpG sites was calculated for patients with FAP, patients with PJS and control subjects. Our data show that in spite of a minimal decrease in the average percentage of methylated sites in both patient groups, the overall percentage methylation of the analysed *CSX* tags is not significantly different between groups (figures 2 and 3C,D). Crypts in control subjects, patients with FAP and patients with PJS harbour stem cell lineages of comparable mitotic age.

Intracrypt distance

Intracrypt distance is the average distance between methylation tags within a single crypt. It is a second measure of diversity and reflects ancestry in the same way branch points along a genealogical diagram connote descent. For instance, when comparing two *CSX* sequences (00010100 and 00100110), the intracrypt distance is 3 since, in theory, it would require at least three methylation events to retrace the original methylation pattern. The maximum difference between two tags in the *CSX* CpG island gene sequence is 8; the average intracrypt distance is the mean of all intracrypt distances within a crypt. Methylation patterns are expected to closely resemble each other in case of recent niche succession, and an increase in intracrypt distance therefore reflects a longer time since the last clonal homogenisation event. Intracrypt distance was calculated for patients with FAP, patients with PJS and control subjects, and is presented in figures 2 and 3E,F. Controls, patients with FAP and patients with PJS had an intracrypt distance of 0.78, 0.93 and 1.10, respectively. Although we observed a trend for patients with PJS and patients with FAP towards an elevation in intra-crypt distance compared to control subjects, this does not reach statistical significance (table 2). Nonetheless, this trend is consistent with the increase in the number of unique methylation patterns found in patients with FAP and patients with PJS.

DISCUSSION

PJS and FAP are widely studied as familial cancer-prone disorders. In FAP, a detailed characterisation of the consequences of germline *adenomatous polyposis coli* (*APC*) mutation has translated into a clinicopathological understanding of tumour formation in mutation carriers. The situation remains much less clear for PJS. With the addition of techniques such as multiplex ligation dependent probe amplification (MLPA), virtually all patients with PJS can be linked to pathogenic *LKB1* germline mutations, but excluding the locus heterogeneity for this disorder. Patients with PJS develop unconventional gastrointestinal polyps characterised by an arborising core of smooth muscle covered with non-dysplastic epithelium. Whether these polyps carry premalignant potential remains unsettled, although we have previously postulated that these lesions are a clinical signpost to the cancer-prone condition, not the obligatory precursor of invasive cancer.²⁰ Regardless of whether the polyps are premalignant, elucidation of their formation will reveal an important insight into the coexistent cancer-prone condition in these patients. A firm understanding of tumour progression in PJS directs our studies into the tumour suppressor function of the *LKB1* tumour suppressor gene.

Many investigations have examined the effects of acute biallelic *LKB1* loss in mouse models. For example, recent studies show that *LKB1* plays a critical role in maintaining the

quiescence and metabolic homeostasis of haematopoietic stem cells.^{21–23} However, earlier work shows that *LKB1* may not necessarily conform to a classic ‘two-hit’ tumour suppressor scenario. The *LKB1*^{+/-} mouse accurately phenocopies patients with PJS who similarly develop large non-dysplastic polyps in the small intestine. However, multiple groups have independently demonstrated that these polyps retain the wild-type *LKB1* allele, thereby showing that *LKB1* is haploinsufficient with regard to the suppression of the formation of these characteristic non-dysplastic PJS polyps.^{24,25} Moreover, studies on *LKB1* loss in mouse lung cancer models reveal that while monoallelic *LKB1* loss greatly accelerated tumour formation, the second allele need not necessarily be lost for tumour formation.²⁶

We studied flat (ie, unaffected) mucosa in patients with PJS. If *LKB1* indeed exerts a dose-dependent effect, then the consequences of a germline mutation should be detectable in normal mucosa. Kim *et al* previously showed that in classic cases of FAP that carry an *APC* mutation, normal-appearing colon crypts display an increase in the diversity of methylation patterns. However, these analyses were performed on fresh frozen tissue. Since adequate PJS material is scarce, we investigated crypt methylation pattern diversity in paraffin-embedded archival tissue with the use of FAP material as reference. Reassuringly, FAP crypts show a significant increase in the number of unique methylation patterns, validating the application of our assay on archival material (figure 2). We find that the absolute difference between controls and patients with FAP is smaller than that described by Kim *et al*⁹ in fresh frozen tissue. This is likely explained by a slight decrease in the sensitivity of the assay on formalin-fixed paraffin-embedded tissue. Nonetheless, the range of unique *CSX* CpG island methylation patterns and the percentage of methylation conform to other published crypt analyses.¹⁸ We therefore confirm and extend previous findings on clonal expansion in FAP.

Next, we studied clonal expansion in the archival material of normal colon mucosa obtained from patients with PJS. In PJS, the mean number of unique methylation patterns is significantly increased compared to the mean number of unique patterns in the control group (figure 3). Importantly, methylation percentage, a reflection of mitotic age, is not statistically different between patients with PJS and our control group, validating our comparative analyses.

Our data show that crypts in flat PJS mucosa display an increased methylation pattern diversity (figure 4). Since stem cell populations and their pool of accumulated mutations continuously change, the diversity in methylation patterns is a reflection of the time elapsed since progenitor lineages last went through a clonal homogenisation event (a molecular clock hypothesis). Epithelia may silently carry many potentially oncogenic mutations (such as *p53* mutations) that only become clinically relevant given the right context of conspiring genomic hits. However, most passenger mutations in stem cell lineages will be lost because only one current stem cell lineage attains future dominance.¹² These mutations need not evoke a selective advantage and may be initially neutral. Individual mutations without selective advantage may thus initially ‘hitchhike’ along with the inherent clonal evolution of stem cell niches. In fact, the analysis of crypt heterogeneity patterns through neutral markers such as *CSX* methylation tags is based on this hitchhiking pattern. Eventually, combinations of mutations (eg, a biallelic gatekeeper mutation) confer a visible tumour such as a tubular adenoma. Differences in the time required for a crypt to undergo clonal homogenisation may allow mutations to accumulate at different frequencies even though the mutations themselves arise at a normal background mutation rate. This is because the rate at which mutations are shed from the crypt depends on the rate of clonal evolution.⁶

Mutation and selection are the twin driving forces of evolution.²⁷ Cells in premalignant and malignant neoplasias evolve by natural Darwinian selection. Broadening the mutational repertoire present in the crypt increases the odds of fortuitously incurring an accidental combination of mutations conferring a tumour phenotype. Our data on *CSX* crypt methylation patterns in unaffected PJS mucosa are consistent with a protracted clonal evolution scenario in PJS. In this scenario, patients with PJS are expected to retain a greater number of mutations incurred at a normal background mutation rate. An increased level of genetic clonal diversity accelerates somatic evolution.²⁸ Indeed, modelling studies demonstrate that time to gatekeeper mutation is decreased in a stem cell niche that retains potentially pathogenic mutations over longer periods of time.⁹ The increased methylation pattern diversity observed in unaffected PJS mucosa therefore predicts that premalignant lesions will arise at an accelerated pace in comparison to the general population. Thus, both PJS and FAP are characterised by a prolonged crypt niche succession scenario, wherein neoplastic transformation is driven by increased Darwinian selection accelerating somatic evolution. In contrast, neoplastic transformation in Lynch syndrome (formerly known as hereditary non-polyposis colorectal cancer) is driven by increased mutation. Curiously, our analyses reveal a nearly similar degree of methylation pattern diversity in PJS and FAP crypts. This is striking because patients with FAP develop a far greater number of preneoplastic lesions. It may well be that the situation in FAP is exacerbated by the fact that the germline mutation provokes loss of the somatic allele, which in itself drives the next phenotypic stage (ie, adenoma formation).²⁷ We stress that even though the observed differences in lineage diversity between groups appear small, this lottery-like process of accumulating pathogenic hits in stem cell lineages is played out in each of the more or less 15 million crypts that constitute the human colon,⁹ greatly increasing the oncogenic impact of minor differences in clonal evolution rates.

Definitively testing the concept of the impact of *LKB1* hemizygosity on lineage succession in the intestinal stem cell compartment will require lineage tracing experiments wherein multiple individual lineages can be traced over time in individual crypts. This is now open to scrutiny, with markers such as *LGR5* being available to unambiguously identify and genetically target progenitor cell populations.²⁹ Two recent studies have established that lineage competition through neutral drift is the driving force behind clonal evolution in the crypt.¹⁸³⁰ Disruption of *LKB1* homologues in model organisms such as fly and nematode shows that *LKB1* plays a domineering role in stem cell turnover in these systems. We envisage that future studies analysing lineage turnover in these elegant mouse models¹⁸³⁰ on an *LKB1*^{+/-} background will validate our findings obtained from patients with PJS.

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Significance of this study

What is already known on this subject?

- Polymorphic methylation patterns record genetic clonal evolution.
- In fresh frozen tissue, a protracted clonal evolution is found in patients with familial adenomatous polyposis.
- Clonal diversity is increased in stem cell niches undergoing a protracted clonal evolution scenario.

What are the new findings?

- Clonal evolution can be evaluated in patients with familial polyposis using formalin-fixed paraffin-embedded archival tissue.
- LKB1 germline mutations affect stem cell dynamics in histologically normal flat PJS mucosa.
- A protracted clonal evolution scenario predicts that premalignant lesions in patients with PJS will arise at an accelerated rate in comparison to the general population.

How might it impact on clinical practice in the foreseeable future?

- In the future, determination of methylation pattern diversity in individual patients may be used for personalised risk assessment in a diagnostic setting. The percentage of total methylation is comparable between groups, but the number of unique methylation patterns is significantly increased for FAP (p-value 0.030) and PJS patients (p-value 0.008) compared with control subjects.

Figure 1.

Schematic overview of the *CSX* tag diversity assay. The assay is completed on archival paraffin-embedded material from unaffected tissue. Single crypts are laser microdissected, and DNA is isolated. Next, the extracted genomic DNA is treated with sodium bisulphite to convert unmethylated cytosines into uracil; methylated cytosines are protected from this turnover. A nested PCR is performed to target the *CSX* sequence. The PCR products are cloned into bacterial vectors to distinguish between the different methylation patterns in individual stem cell lineages. The various methylation patterns per crypt can then be enumerated after a sequence analysis of cloned inserts. *CSX*, cardiac-specific homeobox.

Figure 2.

(A, B) Unique methylation patterns presented in scatter plots for controls and patients with FAP as a group (A) and as box plots for individual patients (B), respectively (50% of the data are within the box; the median is represented by a horizontal line, the mean is represented by the plus sign, outliers are represented with dots and whiskers reveal the maximum and minimum values). p Values were determined using independent t test. Outliers were not excluded from statistical analyses. (C, D) Per cent methylation presented as detailed in (A). (E, F) Intracrypt distance presented as detailed in (A). FAP, familial adenomatous polyposis.

Figure 3.

(A, B) Unique methylation patterns presented in scatter plots for controls and patients with PJS as a group (A) and as box plots for individual patients (B), respectively. p Values were determined using independent t test. (C, D) Per cent methylation presented as detailed in (A). (E, F) Intracrypt distance presented as detailed in (A). PJS, Peutz-Jeghers syndrome.

Figure 4.

(A) Unique methylation patterns are a reflection of genetic clonal diversity. The number of unique methylation patterns is increased in patients with PJS. (B) Per cent methylation is a reflection of the mitotic age of a given crypt progenitor cell population. Note that per cent methylation is not significantly different between groups. (C) Intracrypt distance is a reflection of diversity whereby more distantly related cells harbour more diverse patterns. The intracrypt distance in patients with PJS is moderately increased. (D) Increased selection drives tumorigenesis in PJS. Even though clonal marker mutations such as genomic methylation changes arise all the time, at a phenotypic level, the normal crypt shown on the left and the PJS crypt shown on the right are indistinguishable. However, methylation pattern diversity analysis reveals that, on average, the PJS crypt shown on the right accumulates and retains a greater number of (potentially oncogenic) variants. This increased level of heritable diversity in a population of stem cells accelerates somatic evolution. PJS, Peutz–Jeghers syndrome.

Table 1

Data for controls, patients with FAP and patients with PJS used to investigate stem cell lineage dynamics

Age (years)	Group	Average number of unique methylation patterns per crypt	Unique methylation patterns range	Per cent methylation	Intracrypt distance
15	Control	2.5	1-5	13.7	0.9
31	Control	2.2	1-3	17.3	0.9
35	Control	2.2	1-4	17.4	0.9
43	Control	1.9	1-3	30.7	0.7
68	Control	2.0	1-3	11.1	0.5
12	FAP	2.7	1-8	9.2	0.9
15	FAP	2.8	1-5	20.1	1.0
28	FAP	3.1	1-7	13.1	1.1
41	FAP	2.8	1-8	15.5	0.8
52	FAP	2.5	1-4	23.7	0.7
21	PJS	3.0	1-6	23.9	1.5
32	PJS	3.3	1-6	16.8	1.4
35	PJS	3.5	1-6	6.4	0.5
44	PJS	2.0	1-4	11.4	1.1
65	PJS	2.8	1-7	7.5	0.9

For every individual, 10 crypts were microdissected. For every crypt, 10 sequences were analysed.

FAP, familial adenomatous polyposis; PJS, Peutz-Jeghers syndrome.

Table 2

Statistical analysis of the differences in the number of unique methylation patterns, per cent methylation and intracrypt distance for patients with FAP and patients with PJS compared to controls

	Unique methylation patterns			Per cent methylation			Intracrypt distance		
	Mean unique methylation patterns	SD	p Value (compared to control)	Mean per cent methylation	SD	p Value (compared to control)	Mean intracrypt distance	SD	p Value (compared to control)
Control	2.16	0.98		18.0	13.5		0.78	0.81	
FAP	2.78	1.81	0.030	16.4	17.9	0.60	0.93	0.86	0.36
PJS	2.90	1.66	0.008	13.2	10.2	0.10	1.10	0.87	0.06

FAP, familial adenomatous polyposis; PJS, Peutz–Jeghers syndrome.