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Oxidative DNA damage drives carcinogenesis in *MUTYH*-associated-polyposis by specific mutations of mitochondrial and MAPK genes

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MUTYH **is a DNA-base-excision-repair gene implicated in the activation of nuclear and mitochondrial cell-death pathways.** ***MUTYH*** **germline mutations cause an inherited polyposis,** ***MUTYH*****-associated-polyposis, characterized by multiple adenomas and increased susceptibility to colorectal cancer. Since this carcinogenesis remains partially unknown, we searched for nuclear and mitochondrial gene alterations that may drive the tumorigenic process. Ninety-six adenomas and 7 carcinomas from 12** ***MUTYH*****-associated-polyposis and 13 classical/attenuated adenomatous polyposis patients were investigated by sequencing and pyrosequencing for the presence of mutations in** ***KRAS***, ***BRAF***, **MT-CO1/MT-CO2 and MT-TD genes.** ***KRAS*** **mutations were identified in 24%** ***MUTYH*****-associated-polyposis** ***vs*** **15% classical/attenuated familial polyposis adenomas; mutated** ***MUTYH*****-associated-polyposis adenomas exhibited only c.34G>T transversions in codon 12, an alteration typically associated with oxidative DNA damage, or mutations in codon 13; neither of these mutations was found in classical/attenuated familial polyposis adenomas ($P<0.001$). Mutated** ***MUTYH*****-associated-polyposis carcinomas showed** ***KRAS*** **c.34G>T transversions, prevalently occurring with** ***BRAF*****V600E; none of the classical/attenuated familial polyposis carcinomas displayed these alterations. Comparing mitochondrial DNA from lymphocytes and adenomas of the same individuals, we detected variants in 82%** ***MUTYH*****-associated-polyposis** ***vs*** **38% classical/attenuated familial polyposis patients ($P=0.040$). MT-CO1/MT-CO2 missense mutations, which cause aminoacid changes, were only found in** ***MUTYH*****-associated-polyposis lesions and were significantly associated with** ***KRAS*** **mutations ($P=0.0085$). We provide evidence that** ***MUTYH*****-associated-polyposis carcinogenesis is characterized by the occurrence of specific mutations in both** ***KRAS*** **and phylogenetically conserved genes of mitochondrial DNA which are involved in controlling oxidative phosphorylation; this implies the existence of a colorectal tumorigenesis in which changes in mitochondrial functions cooperate with RAS-induced malignant transformation.**

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MUTYH encodes a DNA glycosylase which is expressed in nucleus as well as in mitochondria[1] where reactive oxygen species are produced as by-products of cellular respiration. Changes in mitochondrial functions, associated with reactive oxygen species production, have recently been associated with perturbation of cellular senescence process.[2] MUTYH takes part in this process since the accumulation of oxidative products in nuclear and mitochondrial DNAs results into the activation of two distinct cell-death pathways, both initiated by MUTYH-generated single-strand breaks.[3]

MUTYH-associated-polyposis is a recessive form of inherited polyposis associated with germline inactivating mutations of *MUTYH*; this gene encodes a base excision repair protein counteracting the DNA damage induced by the oxidative stress.[4,5] Although clinically variable, *MUTYH*-associated-polyposis phenotype resembles that of *APC*-linked attenuated familial polyposis with the onset in the fourth-fifth decade of life, a limited number of adenomas (generally 30–100) and an increased susceptibility to colorectal cancer. However, unlike attenuated familial adenomatous polyposis, hyperplastic and sessile serrated polyps can develop;[6,7] in addition, approximately 60% of

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patients with polyposis have colorectal cancer at presentation.[8]

Colorectal cancer cells of *MUTYH*-associated-polyposis patients contain an excess of c.34 G>T transversions in *KRAS* gene; this is due to failure to repair mismatches induced by 8-oxo-guanine (8-oxoG) variant base, a widely recognized hallmark of oxidative stress.[8–10] Although mutations of *BRAF* gene have been recognized as early genetic events in the initiation of the 'serrated neoplastic pathway',[11] limited *BRAF* analyzes on *MUTYH*-associated-polyposis colorectal cancers have been performed.[9] On the other hand, the pathogenetic role of *BRAF*V600E mutation in sebaceous gland hyperplasia, a well-known extracolonic manifestation of *MUTYH*-associated-polyposis syndrome, has been demonstrated in affected individuals from *MUTYH*-associated-polyposis pedigrees.[12]

On the whole, *MUTYH* driven carcinogenesis is only partially known. Overall, *MUTYH*-associated-polyposis cancers appear to follow a distinct pathway compared to other colorectal cancers: some features overlap with chromosomal instability colorectal cancer phenotype, including frequent *APC* and *KRAS* mutations,[9,13] some others with microsatellite instability phenotype, including loss of HLA class I protein expression.[14] Recently, Nieuwenhuis and collaborators[15] have suggested the occurrence of an accelerated disease progression in *MUTYH*-associated-polyposis, underlying the high risk for patients to develop colorectal cancer even under surveillance.

Several studies support the involvement of respiratory chain defects in the development of tumors, including colorectal cancer, indicating that progression of these lesions can be affected by a disturbed oxidative phosphorylation.[16] In addition to rRNA and tRNA genes, the phylogenetically conserved region of mitochondrial DNA includes sequences encoding for some of the polypeptides which have a role in the control of electron chain transport, such as cytochrome oxidase I and II subunits (MT-CO1/MT-CO2). Mutations affecting these genes have been identified in colorectal cancer cell lines as well as in tumor specimens from colorectal cancer patients.[17–19] In particular, by sequencing the whole mitochondrial DNA in gastrointestinal precancerous lesions, Sui and collaborators[20] found the highest mutation frequency in three of the conserved coding sequences (MT-CO1, MT-ND4 and MT-ND5 subunits of NADH dehydrogenase).

To date, no data concerning mitochondrial DNA alterations affecting *MUTYH*-associated-polyposis or classical/attenuated familial polyposis lesions have been reported. This study was therefore aimed at identifying molecular drivers of *MUTYH*-associated-polyposis with respect to classical/attenuated familial polyposis carcinogenesis. Due to the active role of *MUTYH* at both nuclear and mitochondrial level, we focused on the possible relationship between nuclear and mitochondrial alterations, searching for mutations in *KRAS* and *BRAF* genes as well as in phylogenetically conserved coding regions of mitochondrial DNA; in particular, we analyzed MT-CO1/MT-CO2 genes, involved in oxidative phosphorylation control, and the contiguously located tRNA aspartic acid (MT-TD) gene.

Patients and methods

Patients and Specimens

The study cohort consisted of 12 subjects carrying different biallelic *MUTYH* germline mutations (*MUTYH*-associated-polyposis patients) and 13 subjects with different *APC* germline alterations (11 classical-familial adenomatous polyposis and 2 attenuated-familial adenomatous polyposis). Among patients there were 2 pairs of *MUTYH*-associated-polyposis affected sisters (M1 and M2; M3 and M4, Table 1) and one pair of classical-familial adenomatous polyposis affected brothers (F1 and F2, Table 1). All patients were of Italian origin and had been previously undergone to *MUTYH/APC* genetic testing. Overall, we analyzed 55 colorectal adenomas and 5 colorectal carcinomas from *MUTYH*-associated-polyposis patients and 41 colorectal adenomas and 2 colorectal carcinomas from classical/attenuated-familial polyposis subjects. Adenomas were cross-matched for histotype, size (range: 2–10 mm), and grade of dysplasia. Grading and staging of colorectal adenocarcinomas were in accordance with the TNM system. In most cases, multiple adenomas (2–13) from the same patient were examined. For one *MUTYH*-associated-polyposis patient (M6) only adenocarcinoma was available (Table 1). All germline mutations as well as clinico-pathological data are reported in Table 1. This study was conducted according to the research code of our institutional medical ethical committee on human experimentation and appropriate informed consent was obtained from all individuals included in this study.

The molecular assessment was performed using 5–10 μm thick, microdissected histological sections. After deparaffinization, DNA was extracted from formalin–fixed, paraffin–embedded tissue sections according to manufacturer's instructions (QIAamp DNA FFPE Tissue Kit, Qiagen, Hilden, Germany). We used DNA from each patient's lymphocytes as germline control.

Mutational Analysis and Quantification of *KRAS* and *BRAF* Genes

For *KRAS* gene, exon 2 was amplified and sequenced by using internal primers (F1 5′-aaaaggtactggtggagtatttga-3′, R1 5′-ttgaaacccaaggtacatttca-3′, F2 5′-ttaaccttatgtgtgacatgttctaa-3′, R2 5′-tcatgaaaatggtcagagaaacc-3′). The same nested PCR approach was carried out for the amplification and sequencing of

Table 1 Clinicopathologic features and *MUTYH*/*APC* germline mutations of *MUTYH*-associated-polyposis and classical/attenuated adenomatous polyposis patients

MAP patient	*Gender*	*Age at diagnosis*	*MUTYH germline mutations*	*No. of adenomas*	*Hystotype of analyzed adenomas*	*Dysplasia grading of analysed adenomas*	*CRC*
M1	F	51	p.G396D c.1229insGG	<70	Tubular adenomas	LG	yes
M2	F	48	p.G396D c.1229insGG	<70	Tubular adenomas	LG	no
M3	F	47	p.Y179C p.Y179C	40–70	Tubular adenomas	LG+HG	yes
M4	F	44	p.Y179C p.Y179C	6	Tubular adenomas	LG+HG	no
M5	F	57	p.Y179C p.R182D	<100	Tubular adenoma	HG	yes
M6	M	47	p.Y179C p.G396D	50	NA	NA	yes
M7	M	35	p.Y104X p.W174X	20–30	Tubulovillous adenoma	LG	no
M8	F	50	p.Y179C p.G396D	70–100	Tubular adenomas	LG	no
M9	F	34	c.1145delC p.G396D	40–70	Tubular adenomas	LG	no
M10	F	65	c.241delGT c.1145delC	40–70	Tubulovillous adenomas	LG+HG	no
M11	F	39	p.G396D p.G396D	30–40	Mixed polyps[a]	LG	no
M12	M	51	p.Y179C p.W402L	150	Tubular adenomas	LG	yes
Tot 12							
FAP/AFAP patient	*Gender*	*Age at diagnosis*	*APC germline mutations*	*No. of adenomas*	*Hystotype of analyzed adenomas*	*Dysplasia grading of analysed adenomas*	*CRC*
F1	M	38	2523_2524insA	>100	Tubular adenoma	LG	no
F2	M	36	2523_2524insA	>100	Tubular adenoma	LG	no
F3	M	29	c.3577_3578del2	>100	Tubular adenoma	LG	no
F4	M	31	c.505del4	70–100	Tubular adenomas	LG	no
F5	M	61	c.677_684del8ins4	<100	Tubular adenomas	LG	no
F6	M	26	c.637C>T	>100	Tubular adenomas	LG	no
F7	M	40	exon 12 del.	<70	Tubular adenomas	LG	no
F8	F	36	c.3496_3497insA	>100	Tubular adenoma	LG	no
F9	F	30	c.4147delA	>1000	Tubular adenomas	LG	yes
F10	F	37	c.745A>T	>1000	Tubular adenomas	LG	no
F11	F	38	c.1289_1290insCT	<100	Tubular adenomas	LG	no
F12	F	48	whole gene del.	>1000	Tubular adenomas	LG	yes
F13	M	23	c.2734delT	>100	Tubular adenoma	LG	no
Tot 13							

MAP, *MUTYH*-associated-polyposis; FAP/AFAP, classical/attenuated adenomatous polyposis; CRC, colorectal cancer; LG, low grade; HG, high grade; NA, not available.
[a]mixed polyps: polyps with conventional adenomatous tissue intermingled with serrated glands.

BRAF exon 15 (F1 5′-ttgactctaagaggaaagatgaagt-3′, R1 5′-agaacactgatttttgtgaatactg-3′, F2 5′-tcataatgcttgctctgatagga-3′, R2 5′-ggccaaaaatttaatcagtgga-3′). For both exons, thermal cycling was carried out in a final volume of 50 μl containing 1X Buffer, 1.5 mM $MgCl_2$, 100 mM each dNTP, 10 pmole of primers, 150 ng of DNA extract and 5U of Taq polymerase (AccuPrime Taq DNA Polymerase, Invitrogen, Carlsbad, CA). Briefly, samples were denaturated at 94 °C for 5 min and amplified for 40 cycles as follows: 94 °C for 30 s, 56 °C for 30 s (*KRAS)* or 52 °C for 30 s (*BRAF*) and 72 °C for 30 s; final elongation at 72 °C for 7 min. Sequencing reactions were carried out according to manufacturer's instructions for the BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) with minor modifications. All samples were directly sequenced forward and reverse on a 3730 ABI DNA Analyzer (Applied Biosystems). The reference sequences for *KRAS* (NM_004985.3) and *BRAF* (NM_004333.4) were obtained from www.ncbi.nlm.nih.gov/gene.

The assessment of the percentage of *KRAS* and *BRAF* mutant alleles was performed by pyrosequencing analysis as we previously reported.[21] PCR reactions were carried out as before but using the following 5′-biotynylated primers: 5′-ggcctgctgaa aatgactg (forward) and 5′-biotin-gctgtatcgtcaaggcactct (reverse) for *KRAS* (codon 12 and 13); 5′-biotin-cttcataatgcttgctctgatagg-3′ (forward) and 5′-gcatct cagggccaaaaat-3′ (reverse) for *BRAF* (codon 600). Samples were denaturated at 94 °C for 5 min and amplified for 35 cycles consisting of 94 °C for 30 s, 57 °C for 30 s (*KRAS)* or 55 °C for 30 s (*BRAF*), 72 °C for 30 s and a final elongation at 72 °C for 7 min. PCR products were then analyzed according to manufacturer's instructions using PyroMark Vacuum Prep Workstation (Qiagen). The primed single-stranded DNA templates were subjected to real-time sequencing by using the following primer: *KRAS* 5′-cttgtggtagttggagct-3′, *BRAF* 5′-ccactccatcgagatt-3′. Pyrosequencing analysis was carried out using PyroMark Q24 Instrument (Qiagen). Quantification of mutant *vs* wild-type alleles was calculated by PyroMarkQ24 software (Qiagen).

Mutational Analysis of Mitochondrial DNA

This analysis was carried out by comparing mitochondrial DNA from lymphocytes and adenomas/

carcinomas for selected coding sequences of the following mitochondrial genes: MT-CO1, MT-CO2 and MT-TD. All these regions were contiguous and comprised in a 590 bp fragment (nt. 7333–7860), allowing to amplify them by a single pair of primers: 5′-cttcgaagcgaaaagtcctaata-3′ (forward) and 5′-tcgttgacctcgtctgttatgt-3′ (reverse); mitochondrial DNA was amplified with Accuprime Taq DNA Polymerase System (Invitrogen) in a final volume of 50 μl containing 1X Buffer, 1.5 mM $MgCl_2$, 100 mM each dNTP, 10 pmole of primers, 150 ng of DNA extract and 5U of Taq polymerase. Samples were denaturated at 94 °C for 4 min and amplified for 35 cycles as follows: 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min 30 s. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3730 ABI DNA Analyzer (Applied Biosystems).

Sequences were compared against human mitochondrial DNA sequence, NCBI accession # NC_012920.1, (www.ncbi.nlm.nih.gov) as well as a comprehensive mitochondrial databank, MITOMAP (www.mitomap.org). All mitochondrial DNA-positive samples were resequenced to confirm the results.

Statistical Analysis

The chi-square and Fisher's exact tests (when appropriate) were used to examine the significance of the association between *KRAS*, mitochondrial DNA mutations and the polyposis subgroups. Statistical significance was assumed with $P<0.05$. All tests were carried out with SPSS software, Statistics v. 17.0 (SPSS Inc., Chicago, IL).

Results

Frequency and Allelic Quantification of *KRAS* and *BRAF* Mutations in Adenomas from *MUTYH*-Associated-Polyposis and Adenomatous Familial Polyposis Patients

We analyzed 96 histologically comparable adenomas, 55 from 11 *MUTYH*-associated-polyposis and 41 from 13 classical/attenuated familial patients (2 up to 13 adenomas for each patient). Exon 2 activating mutations of *KRAS* gene were identified in 13/55 (24%) *MUTYH*-associated-polyposis and in 6/41 (15%) classical/attenuated familial polyposis adenomas, respectively (Table 2). Eight of the 13 mutated *MUTYH*-associated-polyposis adenomas (62%) exhibited codon 12 c.34G>T transversions, typically associated with oxidative DNA damage; the remaining 5 mutated adenomas showed mutations at codon 13, four transitions (3 c.38G>A and 1 c. 37G>A) and one transversion c.37G>C (Table 2). None of these mutations was found in classical/attenuated familial polyposis adenomas where we detected 6 substitutions at codon 12, namely 3 c.35G>A transitions and 3 c.35G>T transversions (Table 2) ($P<0.001$). Overall, taking into account only adenomas, *KRAS* activating mutations were found to affect predominantly *MUTYH*-associated polyposis patients (64 *vs* 31% classical/attenuated familial polyposis subjects; $P=0.115$), clustering in some individuals of both categories (patient M1, M3 and F1 of Table 2).

MUTYH-associated-polyposis and classical/attenuated familial polyposis adenomas were also evaluated for the percentage of *KRAS* mutated allele by pyrosequencing technology: *MUTYH*-associated-polyposis polyps exhibited a higher mean value compared to classical/attenuated familial polyposis lesions (23 *vs* 13%; $P=0.329$) (Table 3a).

We further tested the adenomas for the presence of *BRAF* exon 15 mutations. V600E mutation (c.1799A>T transversion) was found in 1/55 *MUTYH*-associated-polyposis and 1/41 classical/attenuated familial polyposis adenomas (Table 2). The two *BRAF* mutated adenomas were wild type for *KRAS* gene and showed comparable level of mutant allele (9 and 10%) (Table 3a). *KRAS* and *BRAF* mutations never coexisted in the same lesion, even if the two oncogenes were mutated in different polyps belonging to the same patient (see case M12 in Table 2).

Frequency and Allelic Quantification of *KRAS* and *BRAF* Mutations in Carcinomas from *MUTYH*-Associated-Polyposis and Familial Adenomatous Polyposis

We searched for *KRAS* exon 2 and *BRAF* exon 15 mutations in 7 adenocarcinomas, 5 derived from *MUTYH*-associated-polyposis and 2 from classical/attenuated familial polyposis patients. *KRAS* exon 2 activating mutations were present in 4/5 *MUTYH*-associated-polyposis colorectal cancers: all base substitutions were c.34G>T transversions. Three *KRAS* mutated colorectal cancers also contained *BRAF*V600E (Table 2). The following quantification of *KRAS* c.34G>T and *BRAF* c.1799A>T alleles showed that *KRAS* mutations were quantitatively more represented compared to *BRAF* in all three lesions (mean value: 38 *vs* 14%; $P=0.141$) (Table 3b and Figure 1a). On the other hand, colorectal cancers from the two classical/attenuated familial polyposis patients showed *KRAS* c.35G>A mutation but no *BRAF* genetic alterations (Tables 2 and 3b).

KRAS Mutations in Adenomas and Colorectal Cancers from *MUTYH*-Associated-Polyposis Affected Sisters

Our set included adenomas and carcinomas from two pairs of sisters with *MUTYH*-associated-polyposis: two sisters were homozygous carriers of p.Y179C *MUTYH* germline mutation (patients M3 and M4 of Table 2) while the two others were p.G396D/c.229insGG *MUTYH* compound heterozygotes (patients M1 and M2 of Table 2). In both cases, in addition to adenomas, one of the two sisters had also developed a colorectal cancer (patients M1 and M3 of Table 2). As far as p.Y179C homozygous sisters

Table 2 *KRAS* and *BRAF* gene mutations in *MUTYH*-associated-polyposis and classical/attenuated adenomatous polyposis adenomas and colorectal cancers (CRCs)

MAP patient	*MUTYH germline mutations*	*Adenomas*						*CRCs*		
		N. of analysed adenomas	*N. of KRAS mutated adenomas*	*Codon 12: c.34G>T*	*Codon 12: other mutations*	*Codon 13 mutations*	*N. of BRAF^{V600E} mutated adenomas*	*CRC*	*KRAS mutation*	*BRAF^{V600E}*
M1	p.G396D c.1229insGG	11	3	**1**	—	**2**	—	+	c.34G>T	+
M2	p.G396D c.1229insGG	6	1	—	—	**1**	—	—	—	—
M3	p.Y179C p.Y179C	6	5	**5**	—	—	—	+	c.34G>T	+
M4	p.Y179C p.Y179C	9	—	—	—	—	—	—	—	—
M5	p.Y179C p.R182D	1	1	**1**	—	—	—	+	c.34G>T	+
M6	p.Y179C p.G396D	NA	NA	NA	NA	NA	NA	+	c.34G>T	—
M7	p.Y104X p.W174X	1	1	**1**	—	—	—	—	—	—
M8	p.Y179C p.G396D	6	—	—	—	—	—	—	—	—
M9	c.1145delC p.G396D	5	1	—	—	**1**	—	—	—	—
M10	c.241delGT c.1145delC	3	—	—	—	—	—	—	—	—
M11	p.G396D p.G396D	3	—	—	—	—	—	—	—	—
M12	p.Y179C p.W402L	4	1	—	—	**1**	**1**	+	—	—
Tot 12		**55**	**13**	**8**	**0**	**5**	**1**	**5**	**4**	**3**

FAP/AFAP patient	*APC germline mutations*	*N. of analysed adenomas*	*N. of KRAS mutated adenomas*	*Codon 12: c.34G>T*	*Codon 12: other mutations*	*Codon 13 mutations*	*N. of BRAF^{V600E} mutated adenomas*	*CRC*	*KRAS mutation*	*BRAF^{V600E}*
F1	2523_2524insA	1	—	—	—	—	—	—	—	—
F2	2523_2524insA	1	—	—	—	—	—	—	—	—
F3	c.3577_3578del2	1	—	—	—	—	—	—	—	—
F4	c.505del4	8	—	—	—	—	**1**	—	—	—
F5	c.677_684del8ins4	13	3	—	**3**	—	—	—	—	—
F6	c.637C>T	2	—	—	—	—	—	—	—	—
F7	exon 12 del.	5	1	—	**1**	—	—	—	—	—
F8	c.3496_3497insA	1	—	—	—	—	—	—	—	—
F9	c.4147delA	2	—	—	—	—	—	+	c.35G>A	—
F10	c.745A>T	2	1	—	**1**	—	—	—	—	—
F11	c.1289_1290insCT	2	—	—	—	—	—	—	—	—
F12	whole gene del.	2	1	—	**1**	—	—	+	c.35G>A	—
F13	c.2734delT	1	—	—	—	—	—	—	—	—
Tot 13		**41**	**6**	**0**	**6**	**0**	**1**	**2**	**2**	**0**

MAP, *MUTYH*-associated-polyposis; FAP/AFAP, classical/attenuated adenomatous polyposis; CRC, colorectal cancer; NA, not available.

Table 3 Percentage of *KRAS* and *BRAF* mutated alleles in *MUTYH*-associated-polyposis and classical/attenuated adenomatous polyposis adenomas (a) and colorectal adenocarcinomas (b)

a. Adenomas

MAP patient	*Adenomas - Dysplasia grading*	*% of KRAS mutated alleles*	*% of BRAF mutated alleles*	*FAP/AFAP patient*	*Adenomas— Dysplasia grading*	*% of KRAS mutated alleles*	*% of BRAF mutated alleles*
M1	p1-LG	36	*wt*	F4	p1-LG	*wt*	10
	p2-LG	15	*wt*	F5	p1-LG	7	*wt*
	p3-LG	45	*wt*		p2 -LG	5	*wt*
M2	p1-LG	15	*wt*		p3-LG	10	*wt*
M3	p1-HG	36	*wt*	F7	p1-LG	10	*wt*
	p2-LG	20	*wt*	F10	p1-LG	19	*wt*
	p3-LG	30	*wt*	F12	p1-LG	25	*wt*
	p4-LG	20	*wt*				
M7	p1-LG	20	*wt*	**Average**		**13%**	
M9	p1-LG	5	*wt*				
M12	p1-LG	*wt*	9				
	p2-LG	12	*wt*				
Average		**23%**					

b. Colorectal adenocarcinomas

MAP patient	*% of KRAS mutated alleles*	*% of BRAF mutated alleles*	*FAP/AFAP patient*	*% of KRAS mutated alleles*	*% of BRAF mutated alleles*
M1	45	16	F9	39	*wt*
M3	30	15	F12	21	*wt*
M5	39	12			
M6	NA	*wt*	**Average**	**30%**	
Average	**38%**	**14%**			

MAP, *MUTYH*-associated-polyposis; FAP/AFAP, classical/attenuated adenomatous polyposis; wt, wild-type; LG, low grade; HG, high grade; NA, not available.

is concerned, we analyzed the colorectal cancer and 6 adenomas from patient M3, and 9 adenomas from patient M4; as far as p.G396D/c.1229insGG mutation carriers, we investigated the colorectal cancer and 11 adenomas from patient M1, and 6 adenomas from patient M2. *KRAS* c.34G>T transversions were found in 5/6 (83%) adenomas from patient M3 and in 1/11 (9%) adenomas from patient M1 (Table 2). This same transversion was also present in combination with *BRAF* V600E mutation in colorectal cancer cells of both M1 and M3 subjects. Interestingly, no c.34G>T *KRAS* mutation was present in the adenomas of M2 and M4 subjects who had not developed carcinomas (Table 2).

Mutational Analysis of Mitochondrial DNA Sequences in *MUTYH*-Associated-Polyposis and Familial Adenomatous Polyposis Lesions

By sequencing DNA extracted from lymphocytes and colorectal lesions of the same patients, we compared phylogenetically conserved MT-CO1, MT-CO2 and MT-TD. By only taking into account sequence changes absent in mitochondrial DNA from lymphocytes and present in mitochondrial DNA from the adenoma cells of the same patient, we found DNA variants in 18/47 (38%) *MUTYH*-associated-polyposis adenomas and in 6/29 (21%) classical/attenuated familial polyposis adenomas (Table 4 and Figure 1b); overall, variants were found in 82% (9/11) *MUTYH*-associated-polyposis *vs* 38% (5/13) classical/attenuated familial polyposis patients ($P=0.040$).

Taking into account both adenomas and colorectal cancers, 6/12 (50%) *MUTYH*-associated-polyposis subjects were carriers of both *KRAS* and mitochondrial DNA variants *vs* 3/13 (23%) classical/attenuated familial polyposis individuals ($P=0.163$) (Tables 2 and 4).

The majority of the identified mitochondrial DNA variants were G>A transitions, although other rare substitutions were also detected (transition C>T; transversions A>C and C>G), prevalently in *MUTYH*-associated-polyposis lesions (Table 4). Interestingly, variants affecting MT-CO1/MT-CO2 subunits were only found in *MUTYH*-associated-polyposis adenomas; with the exception of nt. 7768A>G recurrent base change, most variants caused aminoacid substitutions (Table 4). Variants causing an aminoacid change were also found in 2/5 of *MUTYH*-associated-polyposis carcinomas (nt. 7763G>A); these variants were detected in both adenomas and carcinomas of the same patients (M1 and M3) (Table 4). Similarly, the variants

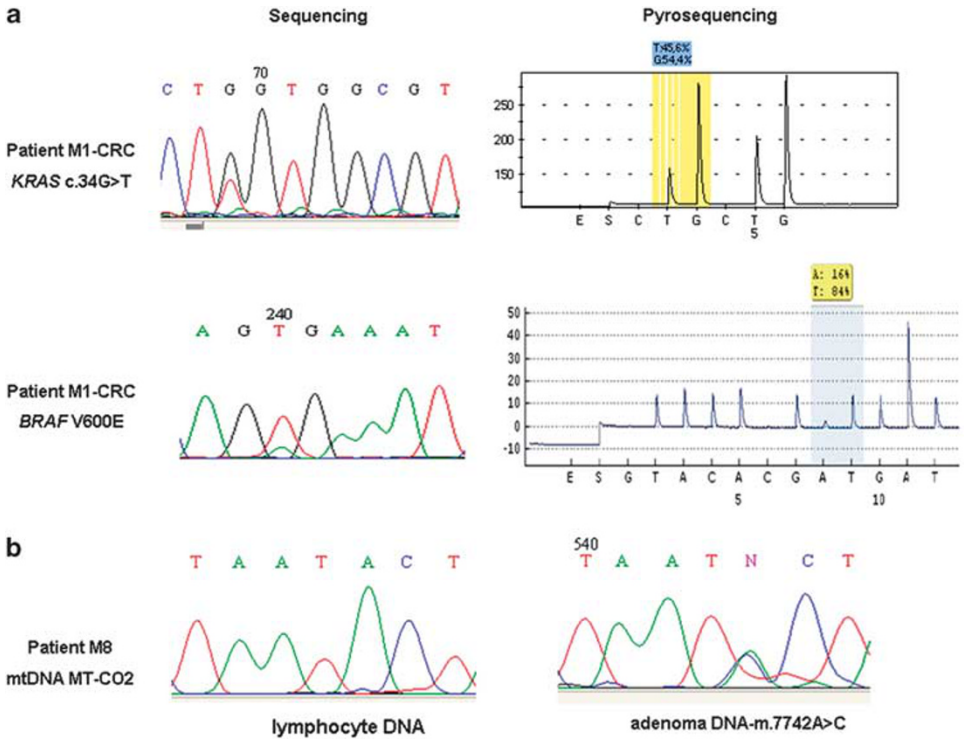

Figure 1 Mutational analysis of *KRAS*, *BRAF* and MT-CO2 in *MUTYH*-associated-polyposis colorectal cancers and adenomas (**a**) sequencing and pyrosequencing evaluation of *KRAS* c.34G>T transversion and *BRAF*V600E mutation in colorectal cancer of M1 patient; pyrosequencing detection for *KRAS* c.34G>T and *BRAF*V600E mutated alleles is 45 and 16%, respectively. (**b**) comparison of MT-CO2 sequencing in lymphocyte and adenoma mitochondrial DNAs derived from M8 patient; mitochondrial DNA from adenoma shows non-synonymous coding mutation m. 7742A>C.

involving MT-TD were more frequent in *MUTYH*-associated-polyposis than in familial polyposis adenomas (10 *vs* 6). Most of the identified changes (15/16) caused G>A transition of nt.7521 (Table 4). *MUTYH*-associated-polyposis patients with MT-TD variants did not show any MT-CO1/MT-CO2 mutations. Moreover, in *MUTYH*-associated-polyposis lesions, only MT-CO1/MT-CO2 coding mutations were significantly associated with *KRAS* mutations ($P=0.0085$; Table 5).

Discussion

The preponderance of c.34G>T transversion in *KRAS* gene is a well-assessed molecular feature of *MUTYH* associated carcinogenesis. However, a detailed analysis of the role of this oncogenic mutation throughout the *MUTYH*-associated-polyposis progression has never been investigated.

By comparing adenomas from *MUTYH*-associated-polyposis and familial adenomatous polyposis patients, carrying different types of constitutional mutations in *MUTYH* and *APC* genes, we showed that *KRAS* activating mutations differ in frequency and type. Not only mutations affect more frequently adenomas from *MUTYH*-associated-polyposis than from familial adenomatous polyposis subjects (64 *vs* 31%), but *KRAS* mutated alleles are also quantitatively more represented in *MUTYH*-associated-polyposis adenomas compared to classical/attenuated familial polyposis lesions (23 *vs* 13%). A limited influence of *KRAS* mutations in familial adenomatous polyposis tumorigenesis was suggested by Obrador-Hevia and coworkers[22] who reported that these mutations affect only 10% of FAP adenomas without achieving the WNT pathway activation. Therefore, while *KRAS* exerts a marginal role in classical/attenuated familial polyposis progression, it appears to have a key role in *MUTYH*-driven colorectal tumorigenesis.

As far as the type of *KRAS* mutation is concerned, we definitely showed that c.34G>T transversions are the most common alterations in *MUTYH*-associated-polyposis adenomas (62% of the mutated lesions). In addition to this hallmark of DNA oxidation, we also detected *KRAS* activating mutations at codon 13. Both types of mutations were found in carriers of different *MUTYH* germline alterations, but neither was detected in classical/attenuated familial polyposis adenomas ($P<0.001$). Van Puijenbroek and coworkers[23] pointed out that, among patients with family history of polyposis and less than 10 adenomas, *KRAS* c.34G>T prescreening of formalin-fixed, paraffin-embedded carcinomas may represent a valuable tool to identify *MUTYH*-associated-polyposis cases. Our results indicate that this approach could be

Table 4 MT-CO1/MT-CO2 and MT-TD gene mutations in *MUTYH*-associated-polyposis and classical/attenuated adenomatous polyposis adenomas and colorectal cancers (CRCs)

MAP patient	*MUTYH germline mutations*	*Adenomas*									*CRCs*	
		MT-CO1		*MT-CO2*					*MT-TD*			
		N. of analysed adenomas	*N. of mtDNA mutated adenomas*	*7441C>T Ser>Phe*	*7644T>C Leu>Pro*	*7742A>C Thr>Pro*	*7763G>A Glu>Lys*	*7768A>G*	*7521G>A*	*7533C>G*	*CRC*	*mutations*
M1	p.G396D c.1229insGG	9	2	—	—	—	**2**	—	—	—	+	7763G>A
M2	p.G396D c.1229insGG	5	1	**1**	—	—	—	—	—	—	—	—
M3	p.Y179C p.Y179C	6	2	—	**1**	—	**1**	—	—	—	+	7763G>A
M4	p.Y179C p.Y179C	9	5	—	—	—	—	—	**5**	—	—	—
M5	p.Y179C p.R182D	1	1	—	—	—	—	—	**1**	—	+	7768A>G
M6	p.Y179C p.G396D	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	—
M7	p.Y104X p.W174X	1	—	—	—	—	—	—	—	—	—	—
M8	p.Y179C p.G396D	4	1	—	—	**1**	—	—	—	—	—	—
M9	c.1145delC p.G396D	3	2	—	—	—	—	—	**2**	—	—	—
M10	c.241delGT c.1145delC	4	1	—	—	—	**1**	**1**	—	—	—	—
M11	p.G396D p.G396D	3	—	—	—	—	—	—	—	—	—	—
M12	p.Y179C p.W402L	2	2	—	—	—	—	—	**2**	—	+	—
Tot 12		**47**	**17**	**1**	**1**	**1**	**4**	**1**	**10**	**0**	**5**	**3**
FAP patient	*APC germline mutations*	*N. of analysed adenomas*	*N. of mtDNA mutated adenomas*	*7441C>T Ser>Phe*	*7644T>C Leu>Pro*	*7742A>C Thr>Pro*	*7763G>A Glu>Lys*	*7768A>G*	*7521G>A*	*7533C>G*	*CRC*	*mutations*
F1	2523_2524insA	1	—	—	—	—	—	—	—	—	—	—
F2	2523_2524insA	1	1	—	—	—	—	—	—	**1**	—	—
F3	c.3577_3578del2	1	—	—	—	—	—	—	—	—	—	—
F4	c.505_508del4	5	—	—	—	—	—	—	—	—	—	—
F5	c.677_684del8ins4	6	2	—	—	—	—	—	**2**	—	—	—
F6	c.637C>T	3	1	—	—	—	—	—	**1**	—	—	—
F7	exon 12 del	4	1	—	—	—	—	—	**1**	—	—	—
F8	c.3496_3497insA	1	—	—	—	—	—	—	—	—	—	—
F9	c.4147delA	2	—	—	—	—	—	—	—	—	+	—
F10	c.745A>T	1	—	—	—	—	—	—	—	—	—	—
F11	c.1289_1290insCT	1	—	—	—	—	—	—	—	—	—	—
F12	whole gene del.	2	1	—	—	—	—	—	**1**	—	+	—
F13	c.2734delT	1	—	—	—	—	—	—	—	—	—	—
Tot 13		**29**	**6**	**0**	**0**	**0**	**0**	**0**	**5**	**1**	**2**	**0**

MAP, *MUTYH*-associated-polyposis; FAP/AFAP, classical/attenuated adenomatous polyposis; CRC, colorectal cancer; NA, not available.

Table 5 Association between *KRAS* and mitochondrial DNA mutations in *MUTYH*-associated-polyposis adenomas and colorectal adenocarcinomas

mtDNA status	*KRAS status*		P *value*
	mutated KRAS	*wild-type KRAS*	
mutated MT-CO1/MT-CO2	7	3	**0.0085**[*]
wild-type MT-CO1/MT-CO2	9	30	
mutated MT-CO1/MT-CO2	7	3	0.0698[**]
wild-type MT-TD	2	8	
mutated MT-CO1/MT-CO2 and MT-TD	8	11	0.2179[***]
wild-type MT-CO1/MT-CO2 and MT-TD	7	22	

[*]*P* value for the presence of both *KRAS* and MT-CO1/MT-CO2 mutations.
[**]*P* value for the presence of both *KRAS* and MT-TD mutations.
[***]*P* value for the presence of *KRAS*, MT-CO1/MT-CO2, and MT-TD mutations.

adopted at the level of pre-malignant lesions in order to specifically address germline mutation analysis towards either *MUTYH* or *APC* gene.

Presently, the relation between *KRAS* mutations at codon 13 and *MUTYH* impairment is unknown. At any rate, the mutated *MUTYH*-associated-polyposis adenocarcinomas of our cohort showed only c.34G>T transversions (p.G12C); moreover, in adenomas, the frequency of c.34G>T mutation was two times higher than that of mutations at codon 13. Overall, this suggests that *KRAS* activation through either codon 12 or codon 13 mutations might have a different impact on *MUTYH*-associated-polyposis carcinogenesis. A variable carcinogenic potential of *KRAS* mutations was reported by Al-Mulla and co-workers[24] who demonstrated a preferential association of p.G12V (c.35G>T) with high-grade tumors and bad prognosis compared to p.G12D (c.35G>A) mutation. More recently, De Roock and coworkers[25] showed that colorectal cancer patients with p.G13D-mutated tumors treated with cetuximab had longer overall and progression-free survival.

In our set, *MUTYH*-associated-polyposis carcinomas differed from familial adenomatous polyposis colorectal cancers not only for the type of *KRAS* mutations, but also for the concomitant presence of both *KRAS* and *BRAF* mutated alleles (in 3/5 of the examined *MUTYH*-associated-polyposis neoplasias). By also taking into account intratumoural heterogeneity,[26] *KRAS* and *BRAF* mutations are well demonstrated mutually exclusive events in colorectal cancer.[27,28] Therefore, their coexistence, although very likely in different cellular subclones, might represent a peculiar feature of *MUTYH*-associated-polyposis carcinogenesis. Boparai and coworkers[6] reported that about 50% of *MUTYH*-associated-polyposis patients, besides adenomas with *APC* somatic mutation, show hyperplastic polyps and sessile serrated adenomas harbouring *KRAS* c.34G>T mutations. On the other hand, *BRAF* mutations are known to identify the subset of colorectal cancers following the serrated neoplastic pathway.[29] On this basis, our results support the hypothesis of a *MUTYH*-associated-polyposis progression with a distinct genetic pattern at the interface between the Vogelstein's classical model and the serrated carcinogenesis.

We analyzed cytochrome c oxidase subunit I (MT-CO1) cytochrome c oxidase II (MT-CO2), and tRNA aspartic acid (MT-TD) genetic variant by comparing mitochondrial DNA sequences from lymphocytes and colorectal lesions of the same patients; taking into account only adenomas, we found mutations in 82% of *MUTYH*-associated-polyposis with respect to 38% of classical/attenuated familial polyposis patients ($P=0.040$). Mostly, non-synonymous substitutions causing aminoacid change in MT-CO1 and MT-CO2 were only found in *MUTYH*-associated-polyposis lesions, both adenomas and carcinomas. Mutations targeting these genes and leading to aminoacid changes have previously been found in colorectal cancer,[30] while data concerning mitochondrial DNA mutations in colorectal adenomas are extremely scanty. Sui and coworkers,[20] performing a whole genome analysis of mitochondrial DNA from gastrointestinal precancerous lesions, including sporadic colorectal adenomas, found MT-CO1 among the most frequently mutated sequences.

Cytochrome c oxidase genes encode the enzyme acting as the final acceptor in the electron transport chain and catalyses the reduction of oxygen to water. Besides regulation of respiratory chain, the enzyme is also known to activate a cell death pathway associated with mitochondrial dysfunction. Indeed, apoptosis can occur by changes in mitochondrial integrity initiated by effectors like Ca^{2+} and reactive oxygen species, leading to the release of cytochrome c. *In vitro* analysis by Oka and coworkers[3] demonstrated that the accumulation of 8-oxoG, due to oxidative stress, causes mitochondrial dysfunction and Ca^{2+} release, thereby activating calpain; at this context, cell death is triggered by single-strand-breaks that accumulate in the DNA, and is suppressed by knockdown of *MUTYH* thus indicating that excision of adenine opposite 8-oxoG leads to the accumulation of single-strand-breaks that cause mitochondrial depletion. In this scenario, the functional impairment of MUTYH and the presence of MT-CO1 and MT-CO2 mutations are expected to deeply compromise the control of mitochondrial-mediated apoptosis.

In our cohort, coding mutations targeting MT-CO2 were also found in 2/5 *MUTYH*-associated-polyposis colorectal cancers, thereby suggesting a contribution of this mitochondrial gene in *MUTYH*-associated-polyposis progression. If so, MT-CO2 alterations might be regarded as 'driver mutations' in *MUTYH*-associated-polyposis syndrome. Interestingly, MT-CO1/

MT-CO2 mutations in *MUTYH*-associated-polyposis were inversely correlated to the presence of MT-TD alterations, thus suggesting a possible 'mutational balance' of mitochondrial DNA mutations in tumor progression.

In classical/attenuated familial polyposis patients, mitochondrial DNA mutations were limited to MT-TD with almost the same mutation frequency compared to *MUTYH*-associated-polyposis. Mitochondrial tRNA genes have previously been found to be mutation hot spots in mitochondrial DNA and their defects are expected to affect both replication and transcription;[31] an altered regulation of these processes appears to be common in both *MUTYH*-associated-polyposis and classical/attenuated familial polyposis lesions.

In this study *MUTYH*-associated-polyposis subjects mutated in both *KRAS* and mitochondrial DNA genes were two times more that of classical/attenuated familial polyposis individuals. Moreover, only MT-CO1/MT-CO2 coding mutations were significantly associated with the presence of *KRAS* mutations in *MUTYH*-associated-polyposis lesions ($P = 0.0085$). Some evidences indicate a role of *KRAS* in activating tumor cell growth by inducing mitochondrial dysfunction and increasing the production of ROS.[32,33] In *MUTYH*-associated-polyposis adenomas the early occurrence and the high frequency of *KRAS* mutations can result into the increase of mitochondrial functional mutations; indeed our results show a relationship between *KRAS* activation and mitochondrial DNA mutations affecting a gene specifically involved in controlling oxidative phosphorylation.

Our results clearly demonstrate, for the first time, that *MUTYH*-associated-polyposis carcinogenesis can be driven by the early occurrence of specific and statistically associated mutations in both *KRAS* and mitochondrial genes, MT-CO1/MT-CO2. These findings cast new insights into the existence of a colorectal carcinogenesis in which changes in mitochondrial functions, which are important for the control of oxidative phosphorylation, cooperate with the process of *RAS*-induced malignant transformation.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Ohtsubo T, Nishioka K, Imaiso Y, *et al.* Identification of human MutY homolog (hMYH) as a repair enzyme for 2-hydroxyadenine in DNA and detection of multiple forms of hMYH located in nuclei and mitochondria. Nucleic Acids Res 2000;28:1355–1364.
2 Passos JF, Simillion C, Hallinan J, *et al.* Cellular senescence: unravelling complexity. Age (Dordr) 2009; 31:353–363.
3 Oka S, Ohno M, Tsuchimoto D, *et al.* Two distinct pathways of cell death triggered by oxidative damage to nuclear and mitochondrial DNAs. EMBO J 2008; 27:421–432.
4 Al-Tassan N, Chmiel NH, Maynard J, *et al.* Inherited variants of *MYH* associated with somatic G:C->T:A mutations in colorectal tumours. Nat Genet 2002; 30:227–232.
5 Sieber OM, Lipton L, Crabtree M, *et al.* Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in *MYH*. N Engl J Med 2003;348:791–799.
6 Boparai KS, Dekker E, Van Eeden S, *et al.* Hyperplastic polyps and sessile serrated adenomas as a phenotypic expression of *MYH*-associated polyposis. Gastroenterology 2008;135:2014–2018.
7 Zorcolo L, Fantola G, Balestrino L, *et al. MUTYH*-associated colon disease: adenomatous polyposis is only one of the possible phenotypes. A family report and literature review. Tumori 2011;97:676–680.
8 Nielsen M, Joerink-van de Beld MC, Jones N, *et al.* Analysis of *MUTYH* genotypes and colorectal phenotypes in patients with MUTYH-associated-polyposis. Gastroenterology 2009;136:471–476.
9 Lipton L, Halford SE, Johnson V, *et al.* Carcinogenesis in *MYH*-associated polyposis follows a distinct genetic pathway. Cancer Res 2003;63:7595–7599.
10 Jones S, Lambert S, Williams GT, *et al.* Increased frequency of the k-ras G12C mutation in MYH polyposis colorectal adenomas. Br J Cancer 2004; 90:1591–1593.
11 Kambara T, Simms LA, Whitehall VL, *et al. BRAF* mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut 2004;53:1137–1144.
12 Ponti G, Venesio T, Losi L, *et al. BRAF* mutations in multiple sebaceous hyperplasias of patients belonging to MYH-associated polyposis pedigrees. J Invest Dermatol 2007;127:1387–1391.
13 Johnson V, Lipton LR, Cummings C, *et al.* Analysis of somatic molecular changes, clinicopathological features, family history, and germline mutations in colorectal cancer families: evidence for efficient diagnosis of HNPCC and for the existence of distinct groups of non-HNPCC families. J Med Genet 2005; 42:756–762.
14 de Miranda NF, Nielsen M, Pereira D, *et al. MUTYH-associated-polyposis* carcinomas frequently lose HLA class I expression - a common event amongst DNA-repair-deficient colorectal cancers. J Pathol 2009; 219:69–76.
15 Nieuwenhuis MH, Vogt S, Jones N, *et al.* Evidence for accelerated colorectal adenoma—carcinoma progression in *MUTYH-associated-polyposis*? Gut 2012;61: 734–738.
16 Sánchez-Aragó M, Chamorro M, Cuezva JM. Selection of cancer cells with repressed mitochondria triggers colon cancer progression. Carcinogenesis 2010;31: 567–576.

17 Polyak K, Li Y, Zhu H, *et al.* Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet 1998;20:291–293.
18 Lièvre A, Chapusot C, Bouvier AM, *et al.* Clinical value of mitochondrial mutations in colorectal cancer. J Clin Oncol 2005;23:3517–3525.
19 Wang CY, Li H, Hao XD, *et al.* Uncovering the profile of somatic mitochondrial DNA mutations in Chinese colorectal cancer patients. PLoS One 2011;6:e21613.
20 Sui G, Zhou S, Wang J, *et al.* Mitochondrial DNA mutations in preneoplastic lesions of the gastrointestinal tract: a biomarker for the early detection of cancer. Mol Cancer 2006;5:73.
21 Venesio T, Chiorino G, Balsamo A, *et al.* In melanocytic lesions the fraction of *BRAF* V600E alleles is associated with sun exposure but unrelated to ERK phosphorylation. Mod Pathol 2008;21:716–726.
22 Obrador-Hevia A, Chin SF, González S, *et al.* Oncogenic *KRAS* is not necessary for Wnt signalling activation in APC-associated FAP adenomas. J Pathol 2010;221:57–67.
23 van Puijenbroek M, Nielsen M, Tops CM, *et al.* Identification of patients with (atypical) *MUTYH-associated-polyposis* by KRAS2 c.34G > T prescreening followed by *MUTYH* hotspot analysis in formalin-fixed paraffin-embedded tissue. Clin Cancer Res 2008;14:139–142.
24 Al-Mulla F, Going JJ, Sowden ET, *et al.* Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. J Pathol 1998; 185:130–138.
25 De Roock W, Jonker DJ, Di Nicolantonio F, *et al.* Association of *KRAS* p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. JAMA 2010;304:1812–1820.
26 Richman SD, Chambers P, Seymour MT, *et al.* Intratumoural heterogeneity of *KRAS* and *BRAF* mutation status in patients with advanced colorectal cancer (acolorectal cancer) and cost- effectiveness of multiple sample testing. Anal Cell Pathol (Amst) 2011; 34:61–66.
27 Heideman DA, Lurkin I, Doeleman M, *et al. KRAS* and *BRAF* mutation analysis in routine molecular diagnostics: comparison of three testing methods on formalin-fixed, paraffin-embedded tumour-derived DNA. J Mol Diagn 2012;14:247–255.
28 Krol LC, 't Hart NA, Methorst N, *et al.* Concordance in *KRAS* and *BRAF* mutations in endoscopic biopsy samples and resection specimens of colorectal adenocarcinoma. Eur J Cancer 2012;48:1108–1115.
29 Burke CA, Snover DC. Editorial: sessile serrated adenomas and their pit patterns: we must first see the forest through the trees. Am J Gastroenterol 2012; 107:470–472.
30 Greaves LC, Preston SL, Tadrous PJ, *et al.* Mitochondrial DNA mutations are established in human colonic stem cells, and mutated clones expand by crypt fission. Proc Natl Acad Sci USA 2006;103:714–719.
31 Wei YH, Lee HC. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. Exp Biol Med 2002;227:671–682.
32 Moiseeva O, Bourdeau V, Roux A, *et al.* Mitochondrial dysfunction contributes to oncogene-induced senescence. Mol Cell Biol 2009;29:4495–4507.
33 Yun J, Rago C, Cheong I, *et al.* Glucose deprivation contributes to the development of *KRAS* pathway mutations in tumour cells. Science 2009;325: 1555–1559.