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ALLELE-SPECIFIC EXPRESSION OF *APC* IN ADENOMATOUS POLYPOSIS FAMILIES

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Abstract

Backgound & Aims—Germline mutations of the *APC* gene are the pathogenic cause of most cases of familial adenomatous polyposis (FAP) and a lesser proportion of attenuated FAP (AFAP). Systematic analysis of *APC* at the RNA level may provide insight into the pathogenicity of identified mutations and uncover the molecular basis of FAP/AFAP in families without identifiable mutations. Here, we analyzed the prevalence of imbalances in the allelic expression of *APC* in polyposis families with germline mutations in the gene and without detectable mutations in *APC* or and *MUTYH*.

Methods—Allele-specific expression (ASE) was determined by single nucleotide primer extension using an exon 11 polymorphism as an allele-specific marker. In total, 52 *APC*-mutation-positive (36 families) and 24 *APC*/*MUTYH*-mutation-negative (23 families) informative patients were analyzed. Seventy-six controls were also included.

Results—Of the *APC*-mutation-positive families, most of those in which the mutation was located before the last exon of the gene (12 of 14) showed ASE imbalance, which is consistent with a mechanism of nonsense-mediated decay (NMD). Of the *APC*/*MUTYH* mutation-negative families, two (9%) showed ASE imbalance as a hallmark of the putative pathogenic cause of the disease. Normal allele expression was restored after treatment of short-term cultured lymphocytes with puromycin, supporting the NMD hypothesis.

Conclusions—ASE analysis may be an indicator of pathogenicity for some cases of FAP and AFAP in which *APC* mutations are not found. ASE might also be useful for prioritizing the order in which different areas of *APC* should be tested. Our results underline the importance of RNA-level studies in molecular diagnosis of FAP.

Keywords

Familial adenomatous polyposis; Allele-specific expression; APC; Nonsense-mediated decay

BACKGROUND & AIMS

Familial adenomatous polyposis (FAP), an autosomal dominant disease predisposing to colorectal cancer, is mainly caused by truncating germline mutations in the *APC* gene (5q21–22)[1]. Widespread use of sequencing techniques has led to the identification of an increased number of missense mutations[2], variants with a putative impact on mRNA splicing[3], and cases of somatic and germline mosaicisms[4]. Exon dose analysis has detected gross rearrangements in a minority of cases[5]. Although in a lesser proportion of cases, attenuated FAP (AFAP) is also associated with *APC* mutations and with biallelic germline mutations in the *MUTYH* gene, showing an autosomal recessive pattern[6].

In other cancer predisposition genes such as *MSH2*, *MLH1*, *BRCA1*, *BRCA2* and *NF1*, studies at the RNA level have shown that mutations causing a premature termination codon (PTC) usually trigger nonsense-mediated decay (NMD) of the mRNA[7-11]. This mRNA surveillance mechanism reduces the abundance of premature stop-codon-harboring mRNA and of the corresponding truncated proteins. NMD of the affected transcript occurs if the PTC is located approximately 55 bp upstream of the last intron-exon boundary[12]. Although some RNA studies of the *APC* gene have been published, focused mainly on the presence of aberrant splicing due to missense, silent or unclassified variants[3,13], to date there are no published studies offering a systematic analysis of the impact of pathogenic *APC* mutations at the RNA level.

The proportion of familial adenomatous polyposis (FAP) families with identifiable *APC* mutations has shown a slight increase with the incorporation of new techniques into diagnostic algorithms. However, a large subset has undetectable pathogenic changes [designated here as *APC*(−)/*MUTYH*(−)]. Small decreases in *APC* mRNA have been detected in *APC*(−)[14,15] and *APC*(−)/*MUTYH*(−) families[16-18]. In contrast, high germline levels of an *APC* mRNA isoform resulting from an exon 10-15 connection have been observed in a case of *APC*(−)/*MUTYH*(−) AFAP[19]. Germline imbalances in allele-specific expression (ASE) of the *APC* gene have been detected in FAP and AFAP families[14, 15, 18, 20], but their potential contribution to diagnostic yield is unknown.

The prevalence of imbalances in *APC* allelic expression was analyzed in two sets of polyposis families: *APC* mutation-positive [designated here as *APC*(+)] and *APC*(−)/*MUTYH*(−). Here we show that mutations outside exon 15 are associated with a clear allelic imbalance at the RNA level that is secondary to NMD, and that these imbalances are also detected in a low proportion of *APC*(−)/*MUTYH*(−) families.

PATIENTS AND METHODS

Patients

One hundred and twenty-seven FAP and AFAP families from the Catalan Institute of Oncology (ICO) and the University of Michigan (UM) were initially tested for germline *APC* and *MUTYH* mutations by comprehensive diagnostic methods (sequencing or SSCP analysis of all exons and exon-intron boundaries, gross deletion analysis by QMPSF (Quantitative Multiplex PCR of Short Fluorescent Fragments) and/or MLPA (Multiplex Ligation-dependent Probe Amplification) and/or Southern blot for the *APC* gene, presence of germline epimutations in *APC* promoter in conjunction with sequencing of the two most prevalent variants of the *MUTYH* gene – Y165C and G382D). Mutations were found in 76 families, while the molecular pathogenic cause of the disease remained unknown in 51 families. The mutation detection rates were 83% for FAP and 25% for AFAP.

Individuals from 59 of the families tested heterozygous for the rs2229992 *APC* coding SNP in exon 11 of the *APC* gene and were the informative population of the present study. These families were divided according to mutational status: (i) 36 *APC*(+) families (24 classical FAP, 34 carrier individuals; 12 AFAP, 18 carrier individuals); and (ii) 23 *APC*(−)/*MUTYH*(−) families (5 FAP, 6 individuals; 18 AFAP, 18 individuals).

A total of 76 heterozygous controls were included: 29 non-carriers from *APC* mutation families, 6 carriers and 24 non-carriers from *MSH2* and *MLH1* mutation families, and 17 Ashkenazi Jewish individuals diagnosed with sporadic microsatellite-unstable colorectal cancer that form part of the Molecular Epidemiology of Colorectal Cancer (MECC) study. This is a population-based case-control study of incident colorectal cancer (CRC), including histopathologically confirmed cases from all incident colorectal cancer cases diagnosed in northern Israel from 31 March 1998 onwards. Informed consent was obtained from all of the subjects who participated in the study.

Genotyping: SNaPshot analysis of genomic DNA

Peripheral blood lymphocyte DNA was obtained using the FlexiGene DNA kit (Qiagen). Twenty-five ng of genomic DNA were amplified in a final volume of 25 μL containing 0.13 μmol/L primers, 0.2 mmol/L dNTPs, 2.5 mmol/L $MgCl_2$ and 1.25 U *Taq* polymerase (Thermoprime Plus DNA Polymerase; ABgene). After an initial denaturing step, samples underwent 5 initial cycles (1 min at 94°C, 30 s at 65°C, 30 s at 72°C), 20 cycles (1 min at 94°C, 30 s at 65°C decreasing 0.5°C/cycle, 30 s at 72°C), and 10 final cycles (1 min at 94°C, 30 s at 55°C, 30 s at 72°C). Primers in exon 11 (Forward: 5′-

GGGACTACAGGCCATTGCA-3′ and reverse: 5′-CAAGTTTGTCAAAGCCATTCCAGC-3′) were used to amplify the rs2229992 SNP. To remove unincorporated primers and dNTPs, PCR fragments were purified using illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). For the single nucleotide primer extension reaction, primer extension was carried out with the SNaPshot Multiplex Kit (Applied Biosystems) with 5′-ATTGCAAGTGGACTGTGAAATGTA-3′ according to manufacturer's instructions. Briefly, reactions were performed in a total volume of 10 μL containing 1.5 μL treated PCR product, 4.5 μL SNaPshot Ready Reaction Mix and 0.2 μmol/L extension primer. Primer extension thermocycling conditions consisted of 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. SNaPshot reaction products were treated with 1 U shrimp alkaline phosphatase (usb) for 60 min at 37°C and then 15 min at 75°C. Products were run in an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper v4.0 (Applied Biosystems). Heterozygous samples showed a profile with two peaks (black and red peaks represent C and T alleles, respectively), while only one peak was observed for homozygous samples.

Measuring ASE: SNaPshot analysis of cDNA

Total RNA was isolated from frozen lymphocytes using Trizol Reagent (Invitrogen) according to manufacturer's instructions. Two hundred and fifty ng of RNA were reverse-transcribed into cDNA using pdN_6 primers and MMLV reverse transcriptase (Invitrogen). To specifically amplify rs2229992 SNP in cDNA, we used the same exon 11 forward primer as for DNA amplification and a reverse primer targeting the exon 11-12 junction (5′-ATAGAGCATAGCGTAGCCTTGTTG -3′). PCR reactions were performed in a final volume of 25 μL containing 2 μL of cDNA, 0.2 μmol/L primers, 0.2 mmol/L dNTPs, 2.5 mmol/L $MgCl_2$ and 1.25 U *Taq* polymerase (Thermoprime Plus DNA Polymerase; ABgene). After a denaturing step, 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C were performed. The remaining steps were the same as described for genotyping, including purification, SNaPshot reaction, phosphatase treatment and capillary electrophoresis.

ASE was measured using peak intensities in heterozygous samples. Allelic frequencies were calculated as freq. C = C/(C+*k*T) or freq. T = T/(T+*k'*C), where *k* and *k'* are constants given by the mean of C/T (*k*) and T/C (*k'*) proportions in control samples. These constants were used to correct for unequal representation of alleles in known control heterozygotes, caused by differential PCR amplification of alleles, differential efficiencies of ddNTP incorporation in the extension reaction, unequal emission energies of fluorescent dyes[21], or putative differences in physiological RNA levels due to alternative splicing affecting exons 1, 7, 9, 10A and 14 of the *APC* gene[22-27]. ASE values are expressed as the proportion of frequencies of the two alleles (freq. C:freq. T). Three independent replicates of all experiments were obtained, and a set of controls was included in every experiment. A Mann-Whitney test was used to evaluate ASE differences among groups.

To validate the SNaPshot analysis as a quantitative technique, two homozygous samples representing the two alleles were selected. After reverse transcription, cDNAs were mixed in different proportions (0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0) and analyzed in triplicate. As seen in Figure 1, SNaPshot assay of rs2229992 SNP provides a quantitative measurement of ASE of the *APC* gene with an analytical sensitivity of at least 10% (Figure 1). To set the cut-off points for equal and unequal expression we used 14 controls from the ICO subset of samples and obtained a mean value of 1.002±0.055 (Figure 2, A). From these results we established a conservative range for normal ASE values of 0.836-1.168 with a confidence of 99% (mean values ± 3·SD). These values were independently validated in an additional set of 14 controls with a range for normal values of 0.732-1.198 (mean values ± 3·SD), which has been consistently replicated in independent experiments using different control sets. In the presence of modest inter-experiment variability, a set of control samples

was always run in the same experiment. Pyrosequencing was used to confirm the range of normal ASE values in an independent set of 18 controls. Briefly, germline cDNA and germline DNA were PCR-amplified using the forward primer (GAATTATTGCAAGTGGACTGTGAA) and the reverse biotinylated primer (GCCATTCCAGCATATCGTCTTA) to yield an 83 base-pair amplicon containing rs2229992. The PCR reaction mixture (25μL) contained 5ng of genomic DNA or 1μL of cDNA, 2.5μl of 10X PCR buffer (Applied Biosystems), 2μL of 25mM MgCl2 (Applied Biosystems), 1.25μL of 2.5mM dNTP (New England Biolabs), 0.5μL of each 10μM primer, and 0.75 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). Cycling conditions were as follows: initial denaturation at 95°C for 3 min, 50 cycles of 95°C for 34 sec, 60°C for 45 sec, 72°C for 45 sec, and a final extension at 72°C for 10 min. 5μL of the resulting PCR product was used for pyrosequencing with the sequencing primer (GCAAGTGGACTGTGAAAT) according to the standard Qiagen protocol. Normal ASE values were similar using this technique, ranging from 0.732 to 1.198 (mean values ± 3·SD).

Puromycin analysis

Frozen lymphocytes from a subset of patients and controls were short-cultured in RPMI 1640 + GlutaMAX medium (10% FBS, 1% penicillin-streptomycin) (Gibco) and phytohemagglutinin (Sigma). After 6-9 days, two subcultures were derived: one was treated with 250 μg/mL puromycin (Sigma) for 5 hours, while the other was left untreated. RNA was extracted and ASE calculated as described above.

APC cDNA molecular analysis and promoter analyses

APC cDNA molecular analysis was performed in all *APC*(−)/*MUTYH*(−) families showing ASE imbalance. Six overlapping primer pairs covering all exon-exon boundaries of the *APC* gene were designed to test for abnormal cDNA products in these families. Differential running patterns between test samples and controls were assessed visually using agarose gel electrophoresis and observed abnormal bands were sequenced. Sequencing of the 1A and 1B promoter regions of the *APC* gene was also performed. Finally, *APC* gene expression levels were analyzed in all ICO samples by qRT-PCR using a primer pair targeting exons 2 and 3. The primer sequences and experimental conditions for these analyses are detailed in Supplementary Table 1.

RESULTS

ASE analysis in *APC*(+) polyposis families

ASE was assessed in 52 individuals (34 FAP and 18 AFAP) *APC*(+) from 36 families (24 FAP and 12 AFAP) that tested heterozygous at rs2229992, in parallel with non-carrier individuals and controls. To categorize ASE, a cut-off range was calculated as described in Patients and Methods. ASE values <0.836 designated C-allele underexpression and values >1.168 designated C-allele overexpression, in both cases depicting allelic imbalance. The average and standard deviation (SD) ASE values for non-carriers (n=24) were 0.964±0.038 (Figure 2, A), with none outside the normal range. Twenty-two families (24 individuals) showed normal ASE values (range=0.876-1.155). The remaining 14 (39%) showed ASE imbalances: five families (8 individuals) showed C-allele overexpression (range=1.214-8.706), eight families (18 individuals) showed C-allele underexpression (range=0.677-0.830), and one family showed the borderline value of 0.838. Another family, harboring a deletion from the promoter to exon 4, showed complete loss of T-allele expression (F-UM-8) and was used as an internal control (Figure 2, A; Table 1).

In accordance with the canonical model of NMD, a strong correlation was observed between the location of the mutation outside exon 15 (leading to a premature termination codon

before the last exon of the gene) and the presence of ASE imbalance. Twelve of 14 (86%) families with mutations outside exon 15 showed abnormal ASE, whereas the proportion dropped to 2 of 22 (9%) for families with exon 15 mutations (p-value=7.3e-13, obtained from ASE values) (Table 1; Figure 2, B). Interestingly, the 4 cases that did not fit with the model showed borderline ASE values. Thus, the ASE values for the two families with mutations outside exon 15 and with no imbalance (F-UM-2 and F-UM-9) were 1.155 and 1.133 (upper cut-off value 1.168). The two cases with exon 15 mutations and classified as harboring putative imbalances (F-8 and F-9) showed ASE values of 0.830 and 0.838 (lower cut-off value 0.836). F-8 harbors a mutation at intron 14 that is predicted to alter intron-exon processing, leading to a truncated protein at exon 15. F-9 has a recurrent mutation, p.Gln1062X, that was not associated with ASE in other cases in our series sharing the same alteration (F-13 and F-22). However, F-9 was classified as putatively abnormal because the imbalance essentially coincided with the diagnostic threshold value and was confirmed in cultured lymphocytes (Table 1).

Variations in ASE imbalance depending on the location of the mutation associate with different *APC* expression levels. Values of *APC* germ-line expression in 43 controls were 3.793±2.933. Expression levels in polyposis with exon 15 mutations were similar (3.647±0.911), while expression levels in polyposis with mutations outside exon 15 were lower (2.887±0.867; p=0.014).

More than one member (range=2-5) was analyzed in 8 FAP and AFAP families, and all carriers showed concordant ASE values in 7 of these families (Table 1). In one family (F-2), two of the three carriers analyzed showed abnormal ASE values (0.823 and 0.717) and the third carrier showed a normal borderline value (0.853), which probably reflects a degree of variability between individuals. ASE values were similar among families with the same mutation. For example, in families F-4 (FAP) and F-23 (AFAP), carriers of the same splicing mutation (c.834+1G>A), ASE values were similar, although they did not account for phenotype differences. Allelic imbalance cosegregated with the disease in all cases analyzed (data not shown).

In nine of the allelic imbalance cases, short-term lymphocyte cultures showed clearer ASE imbalance than observed in cDNA from peripheral blood lymphocytes. Puromycin treatment completely reverted imbalance in 8 of the 9 cases. In the remaining case, only partial reversion was achieved (Table 1). Cultured control lymphocytes showed normal ASE values that remained unchanged after puromycin treatment (data not shown).

ASE analysis in *APC*(−)/*MUTYH*(−) polyposis families

Twenty-three (5 FAP and 18 AFAP) *APC*(−)/*MUTYH*(−) families (6 and 18 individuals, respectively) were heterozygous for the SNP at exon 11 and were subsequently analyzed. Mean ASE values in controls were 1.001±0.047. Initially, three of the 23 families showed abnormal *APC* ASE values (F-29, F-39 and UM-11) (Figure 3 and Table 2). Puromycin treatment of short-term cultured lymphocytes was performed in the two families from whom lymphocytes were available (F-29 and F-39) (Table 2). In family F-29, the observed imbalance was more intense in cultured cells than in PBL and was completely reverted by puromycin treatment, as expected. In F-39, with an ASE value of 0.819, the imbalance was no longer observed in cultured lymphocytes. Thus, two of the 23 (9%) *APC*(−)/*MUTYH*(−) families in our series (F-29 and UM-11) harbored ASE imbalance. In F-29, two point mutations in the promoter region were identified: g.[112043282C>G(+) 112072889C>T]. These changes were not detected in a small set of 9 controls. However, they were not located in a conserved sequence or a sequence consensus region for transcription factors, and were not associated with clear changes in *APC* expression levels as assessed by qRT-PCR. At the RNA level, the wt transcript coexisted with a second transcript containing a 60

bp insertion r.[=, 1408_1409ins1408+1315_1408+1369] in UM-11 RNA. These transcripts were also present in three of five additional samples consisting of either controls or FAP patients with no ASE imbalance. Interestingly, UM-11 family did not express a transcript skipping exon 11 that was present in other samples (data not shown). Finally, germline *APC* expression levels were lower in *APC*(−)/*MUTYH*(−) polyposis families (2.963±1.21) than in controls (p=0.04) or and *APC*(+) polyposis families with exon 15 mutations (p=0.01).

DISCUSSION

In this study we adapted the SNuPE (single nucleotide primer extension) methodology to perform a detailed allele-specific expression analysis of the *APC* gene in 59 FAP and AFAP families divided in two groups: those harboring a pathogenic mutation and those with no identified *APC* or *MUTYH* mutation.

In the presence of a detectable deleterious *APC* gene mutation, ASE imbalance was strongly associated with mutations located outside exon 15. Conversely, balanced ASE was common in cases with exon 15 mutations. Both observations fit, albeit imperfectly, with the nuclear scanning model of NMD in mammals[10,12]. Our observations are in disagreement with the findings of Renkonen and colleagues, who reported no ASE imbalance in a small series of 4 families harboring mutations in exons 6-9 of the *APC* gene, possibly due to differences in technique or sample processing[20]. To assess the contribution of NMD to the observed imbalance, lymphocytes were short-term cultured and treated with puromycin, a known inhibitor of NMD[28]. Similarly to previous observations, enhanced allelic expression imbalance was observed *in vitro* before puromycin treatment, pointing to higher degradation of nonsense transcripts associated with culture conditions[29]. Puromycin treatment completely restored normal ASE in most of the cases analyzed. However, only partial reversion was achieved in the case showing the greatest imbalance prior to treatment (F-24, I-106), and it is unclear whether this can be attributed to the intensity of allelic imbalance, the limitations of the *in vitro* assay or other unknown causes.

As has been reported for *MSH2*, *MLH1*, *BRCA1-2* and *NF1*[7-11], the nuclear scanning NMD model loosely fits with our observations for the *APC* gene. One of the exceptions is a mutation at intron 14 that creates a skipping of exon 14 and a PTC at the very beginning of exon 15 (data not shown) that associates with imbalanced ASE. This may be due to the special position of the PTC and its sequence context, both of which are factors known to influence the extent of NMD.

NMD can be considered a modifier of the phenotypic consequences of PTC and has contributed to our understanding of genotype-phenotype correlations in various genetic disorders[30]. In mutations outside exon 15, NMD may promote partial loss-of-function or haploinsufficiency. Of note, family 24, harboring a frameshift mutation at exon 12, showed the highest ASE imbalance and an attenuated phenotype. It can be speculated that, in this case, the mutant allele is degraded leading to a bigger proportion of wt APC homodimers and a milder phenotype. In mutations causing PTCs in exon 15, absence of NMD activity may enhance the dominant-negative effect. As such, it is notable that our preliminary observations indicate lower germline *APC* expression levels in polyposis with mutations outside exon 15. An exon-15-mutated allele is not amenable for degradation by NMD, so this mechanism cannot act as an additional regulatory mechanism. However, no clear phenotypic correlation between clinical features and location of the mutation was observed in our series.

Finally, imbalanced ASE was observed in the absence of a detectable mutation in a subset of *APC*(−)/*MUTYH*(−) families assessed by conventional methods. A more detailed analysis of

cDNA and promoter regions revealed the presence of alterations in *APC* promoter that may be functionally relevant in one family, while in the other family a complex pattern of *APC* transcripts in the exon 11 region was of unknown significance. We cannot rule out the possibility that a genetic change deep within an intron may account for the observed ASE imbalance. In addition, *cis*-acting regulatory SNPs with an allele-specific effect on *APC* might also be responsible for the imbalance[31]. However, the failure to detect imbalances in most of the AFAP cases reflects a putative mechanism for their attenuated nature, which may be more closely related to subtle changes at the RNA level that could lead to delayed phenotypic expression of symptoms. In fact, germline expression of the APC allele is slightly lower in these *APC*(−)/*MUTYH*(−) and shows no apparent correlation with ASE imbalance.

Methodological issues should be taken into account in the interpretation of results and have both theoretical and practical implications. According to our own and others' results, the SNaPshot approach is a suitable technique with excellent analytical sensitivity[32-35]. Importantly, pyrosequencing (a robust technique) yielded similar normal range values. In any case, the definition of cut-off values was improved by the inclusion of control set in every experimental run. The putative clinical relevance of borderline values emphasizes the importance of the controls. Sample processing is likely to influence the results. Aged blood has been shown to decrease NMD, thus precluding the detection of loss of allelic imbalance in the *NF1* gene[29]. The balanced allelic expression of the two samples with mutations outside exon 15 may be due to differences in the isolation process of peripheral blood lymphocytes. Finally, the complex pattern of transcripts at exon 11 should be considered, as it might influence ASE results. Notably, we have identified the expression of transcripts with skipping of exon 11 in controls (data not shown).

Our results may have clinical implications. Allelic imbalances detected in a small proportion of *APC*(−)/*MUTYH*(−) families point to the presence of a pathogenic event in the *APC* gene. Although further studies are needed to elucidate the correlation between allelic imbalance and the disease in families with no detectable mutation, it could be used as a diagnostic marker of elusive mutations in the *APC* gene that might be otherwise only detected by the more costly conversion approach[33] or by next-generation sequencing when implemented in the routine clinical setting. Based on our results, we propose a new molecular diagnostic algorithm for polyposis families that undergo *APC* mutation screening beginning with analysis of gross rearrangements (Figure 4). In samples with ASE imbalance, sequencing of exons 1-14 at the cDNA or gDNA level would be followed by analysis of the promoter region and, possibly, the 3′UTR region. This strategy would have made it unnecessary to sequence the largest exon of the gene (exon 15, 6574 bp) in approximately 21% of the families included in the present study (12/58). The pathogenic cause of the disease would remain unknown if sequencing of the whole coding region of *APC* and the promoter and 3′UTR regions, and cDNA transcript analyses targeting aberrant skipping or cryptic exons, revealed no functionally relevant changes. However, a processing protocol for preserving lymphocytes and RNA would be needed to incorporate this RNA-based assay into routine screening. Also, the addition of more polymorphisms as allele-specific markers would improve informativeness and dependability. We do not know whether missense or unclassified variants from introns 1-14 are associated with ASE imbalance. However, transcript-level analysis has shown that a significant proportion of these mutations can be classified as pathogenic and has revealed the presence of PTCs in some cases[3].

Taken together, our results reinforce the importance of RNA-level studies not only for a better understanding of the disease but also for optimizing the molecular diagnostic algorithm for FAP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AFAP	attenuated familial adenomatous polyposis
ASE	allele-specific expression
FAP	familial adenomatous polyposis
FISH	fluorescent in-situ hybridization
MLPA	multiplex ligation-dependent probe amplification
NMD	nonsense-mediated decay
PTC	premature termination codon
QMPSF	quantitative multiplex PCR of short fluorescent fragments
SNuPE	single nucleotide primer extension

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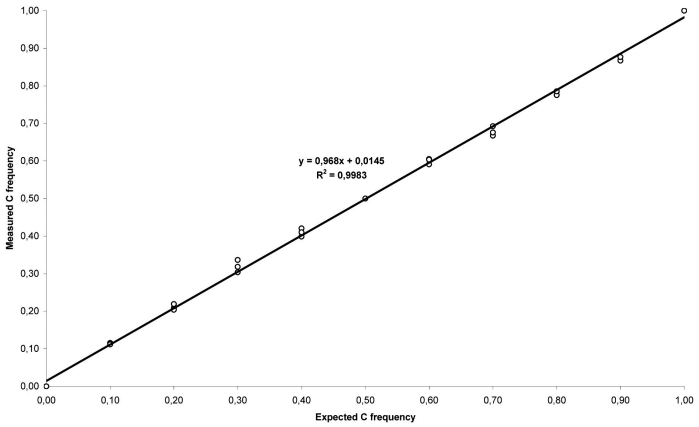

Figure 1. SNaPshot analysis of rs2229992 SNP as a quantitative test

Regression analysis of the amplification products of two homozygous control cDNAs mixed in different proportions and analyzed in triplicate by SNaPshot. Allele frequencies were calculated by the peak heights: freq. C = C/(C+*k*T), where the correction factor *k* is determined from the mix simulating an actual allele frequency of 0.5 (5:5). Measured allele frequencies were plotted against the expected values. A near-linear relationship was observed across all data points (R2 = 0.9983).

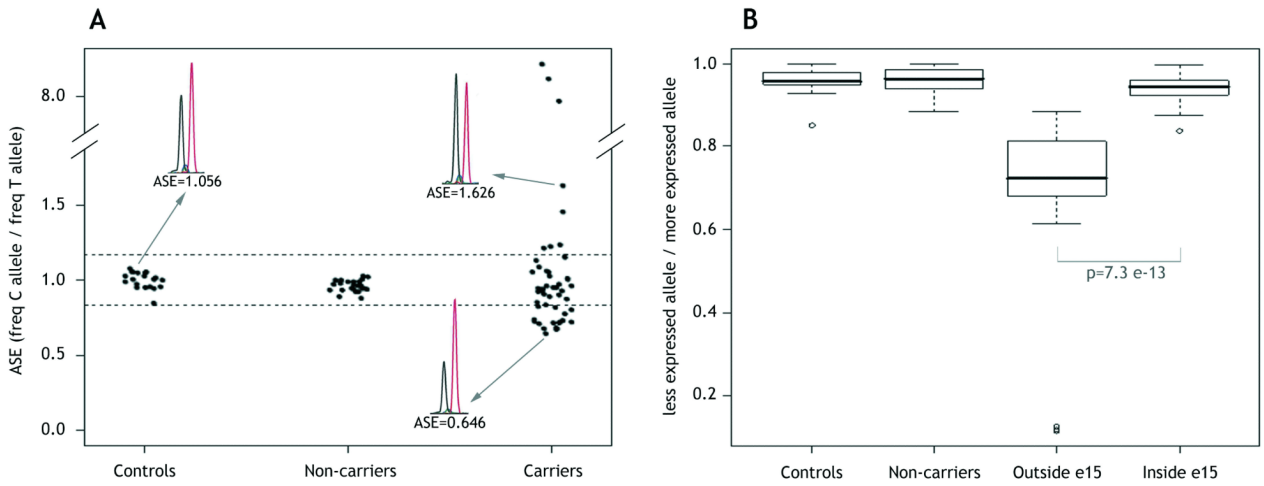

Figure 2. Quantification of ASE in *APC*(+) polyposis patients

A. Distribution of *APC* ASE in controls, non-carriers and carriers of a pathogenic *APC* mutation from the ICO and UM subsets. ASE values are the proportion between the C and T allele frequencies of the *APC* coding SNP rs2229992. Each point represents the mean of the triplicate measurements, and each replicate was performed for all samples in each subset. Allelic ratios ranged from 0.646 to 8.706. The normal ASE cut-off range is indicated by dashed horizontal lines (at 1.168 and 0.836) and determined by control ASE values, as indicated in Patients and Methods. Three sample electropherograms obtained from the SNaPshot reaction, and their corresponding ASE values, are also shown. **B.** Box plots of ASE values for the location of the mutation in the *APC* gene. Patients were stratified into two groups by mutation site ("Outside e15", mutation located from exons 1-14; and "Inside e15", mutation located in exon 15). To give an overview of our results, ASE values are represented as the proportion of allelic frequencies between the underexpressed and the overexpressed allele in each sample, independently if whether it is C or T. The interquartile range includes 50% of the samples and is shown by white boxes. The interdecile range includes 90% of the patients and is shown as whiskers. Outliers are shown by empty dots. Mutated groups were compared using the non-parametric Mann-Whitney test.

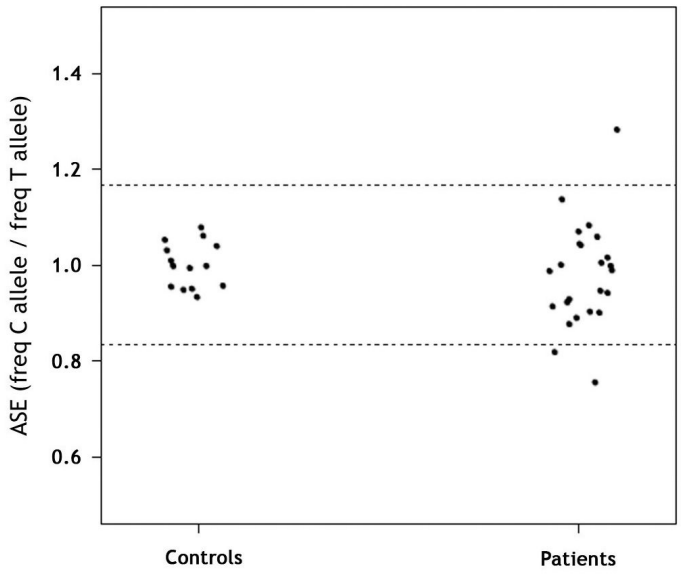

Figure 3. Quantification of ASE in *APC*(−)/*MUTYH*(−) polyposis patients

Distribution of *APC* ASE in controls and polyposis patients with no previously identified mutation. ASE values are the proportion between the C and T allele frequencies of the *APC* coding SNP rs2229992. Each point represents the mean of the triplicate measurements, and each replicate was performed for all samples in each subset. Allelic ratios ranged from 0.756 to 1.284. The normal ASE cut-off range is indicated by dashed horizontal lines (at 1.168 and 0.836).

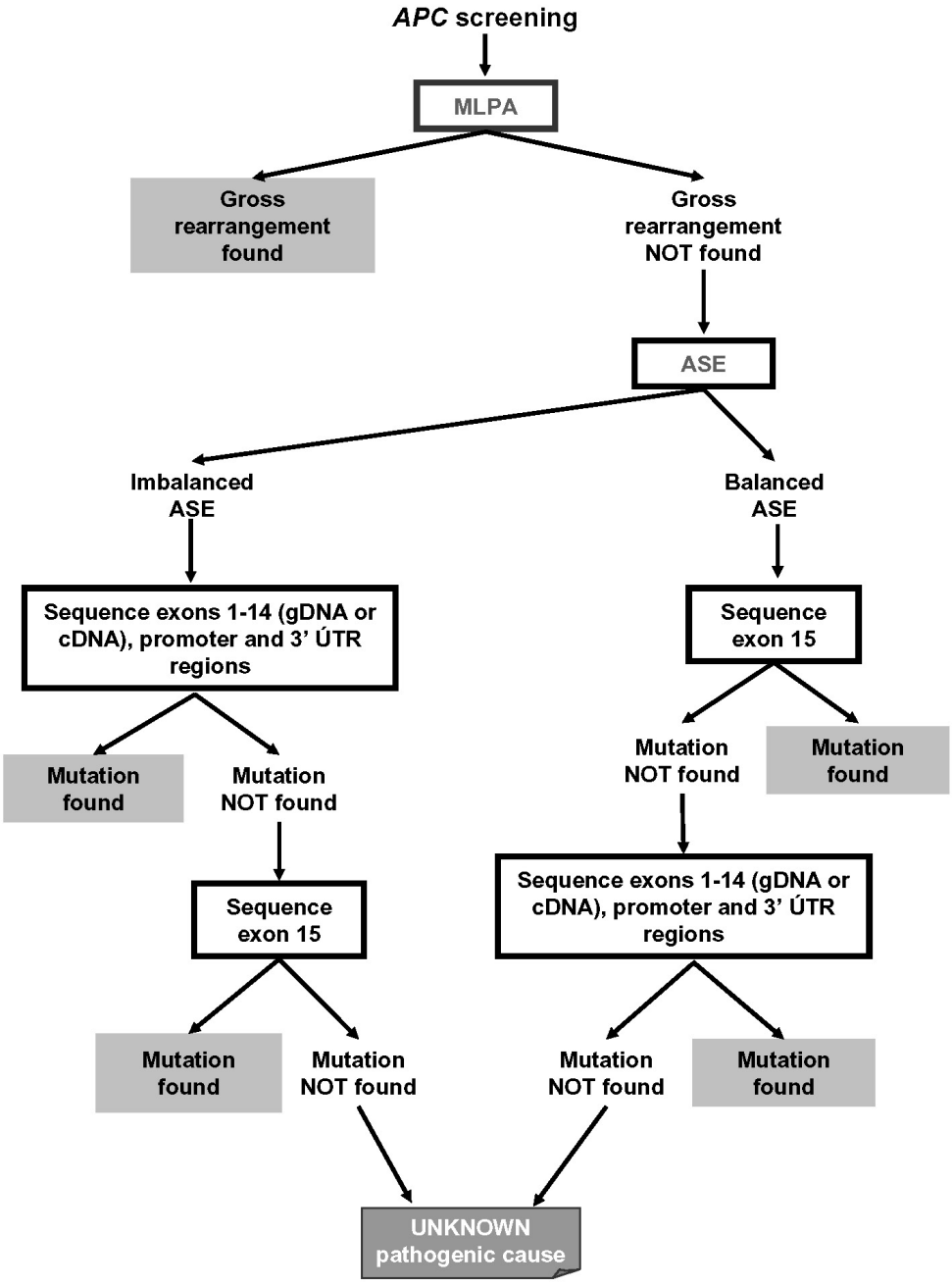

Figure 4.
Proposed diagnostic algorithm for *APC* mutation screening including ASE measurement in polyposis families.

Table 1

Characteristics of the *APC*(+) polyposis families analyzed for ASE

Individual	Family	Phenotype	Mutation*	Exon or intron	Type of mutation	Reference	ASE±SD (freq C/freq T)	ASE (−)Puromycin (freqC/freqT)	ASE (+)Puromycin (freqC/freqT)
41	8	FAP	c.1958+3A>G	i14^	Splicing	2,3	**0.830±0.036**		
UM-6	UM-6	AFAP	c.2004del; p.Leu669X	e15	Frameshift	This study	0.911±0.250		
78	19	FAP	c.2116del; p.Met706CysfsX12	e15	Frameshift	This study	0.964±0.010		
61	15	FAP	c.2397T>A; p.Tyr799X	e15	Nonsense	3	0.876±0.018		
38	14	FAP	c.2701C>T; pGln901X	e15	Nonsense	This study	1.062±0.025		
39	14	FAP	c.2701C>T; pGln901X	e15	Nonsense	This study	0.949±0.010		
25	11	FAP	c.2727del; p.Thr910LeufsX6	e15	Frameshift	This study	0.964±0.032		
127	27	AFAP	c.3077A>G, p.Asn1026Ser	e15	Missense	[36]	0.974±0.049		
97	9	FAP	c.3183_3187del; p.Gln1062X	e15	Frameshift	1,2,3	**0.838±0.030**	0.773	1.090
37	13	FAP	c.3183_3187del; p.Gln1062X	e15	Frameshift	1,2,3	1.055±0.093		
100	22	FAP	c.3183_3187del; p.Gln1062X	e15	Frameshift	1,2,3	0.907±0.042		
33	12	FAP	c.3562del; p.Pro1188LeufsX77	e15	Frameshift	This study	0.924±0.073		
124	26	AFAP	c.3631A>G; p.Met1211Val	e15	Missense	This study	0.892±0.050		
UM-4	UM-4	FAP	c.3688C>T; p.Gln1230X	e15	Nonsense	1,2,3	1.015±0.121		
66	16	FAP	c.3838_3839del; p.Leu1280ValfsX4	e15	Frameshift	This study	0.944±0.039		
77	18	FAP	c.3927_3931del; p.Glu1309AspfsX4	e15	Frameshift	1,2,3	0.950±0.023		
76	17	FAP	c.4189G>T; p.Glu1397X	e15	Nonsense	1,3	0.943±0.013		
81	20	FAP	c.4393_4394del; p.Ser1465TrpfsX3	e15	Frameshift	1,2,3	0.932±0.045		
96	21	FAP	c.4393_4394del; p.Ser1465TrpfsX3	e15	Frameshift	1,2,3	0.951±0.039		
23	10	FAP	c.4608_4614del; p.Glu1536AspfsX27	e15	Frameshift	This study	1.028±0.016		
24	10	FAP	c.4608_4614del; p.Glu1536AspfsX27	e15	Frameshift	This study	1.009±0.049		
UM-7	UM-7	AFAP	c.4782_4785del; p.Ala1595ArgfsX54	e15	Frameshift	This study	0.929±0.029		
UM-1	UM-1	AFAP	c.5936_5939del; p.Asn1979ThrfsX64	e15	Frameshift	1	1.054±0.039		
UM-10	UM-10	AFAP	c.5936_5939del; p.Asn1979ThrfsX64	e15	Frameshift	1	1.086±0.099		
UM-8	UM-8	AFAP	Deletion promoter-e4	?-e4	Gross deletion	This study	**loss of T**		

Individual	Family	Phenotype	Mutation*	Exon or intron	Type of mutation	Reference	ASE±SD (freq C/freq T)	ASE (−)Puromycin (freqC/freqT)	ASE (+)Puromycin (freqC/freqT)
54	1	FAP	c.423-2A>G	i3	Splicing	This study	**0.738±0.031**	0.621	0.997
161	2	FAP	c.423-2A>G	i3	Splicing	This study	**0.823±0.069**		
163	2	FAP	c.423-2A>G	i3	Splicing	This study	0.853±0.028		
165	2	FAP	c.423-2A>G	i3	Splicing	This study	**0.717±0.019**	0.497	0.952
UM-2	UM-2	AFAP	c.426_427del; p.Leu143AlafsX4	e4	Frameshift	2	1.155±0.061		
UM-3	UM-3	AFAP	c.426_427del; p.Leu143AlafsX4	e4	Frameshift	2	**1.214±0.020**		
UM-9	UM-9	AFAP	c.464_465del; p.Lys155ArgfsX12	e4	Frameshift	This study	1.133±0.043		
46	3	FAP	c.607C>T; p.Gln203X	e5	Nonsense	1	**1.626±0.008**		
47	3	FAP	c.607C>T; p.Gln203X	e5	Nonsense	1	**1.451±0.049**	2.164	1.083
116	23	AFAP	c.834+1G>A	i7	Splicing	2,3	**0.680±0.027**		
117	23	AFAP	c.834+1G>A	i7	Splicing	2,3	**0.677±0.006**		
118	23	AFAP	c.834+1G>A	i7	Splicing	2,3	**0.728±0.011**		
119	23	AFAP	c.834+1G>A	i7	Splicing	2,3	**0.713±0.006**		
122	23	AFAP	c.834+1G>A	i7	Splicing	2,3	**0.734±0.021**		
10	4	FAP	c.834+1G>A	i7	Splicing	2,3	**0.682±0.029**		
11	4	FAP	c.834+1G>A	i7	Splicing	2,3	**0.722±0.029**		
12	4	FAP	c.834+1G>A	i7	Splicing	2,3	**0.646±0.024**		
13	4	FAP	c.834+1G>A	i7	Splicing	2,3	**0.687±0.042**		
14	4	FAP	c.834+1G>A	i7	Splicing	2,3	**0.725±0.014**		
18	5	FAP	c.1262_1263delinsAA; p.Trp421X	e9	Nonsense	This study	**0.779±0.022**		
21	5	FAP	c.1262_1263delinsAA; p.Trp421X	e9	Nonsense	This study	**0.721±0.024**	0.525	1.069
106	24	AFAP	c.1557_1561del; p.Cys520TyrfsX15	e12	Frameshift	This study	**7.911±0.337**	13.800	6.353
107	24	AFAP	c.1557_1561del; p.Cys520TyrfsX15	e12	Frameshift	This study	**8.706±0.468**		
108	24	AFAP	c.1557_1561del; p.Cys520TyrfsX15	e12	Frameshift	This study	**8.387±0.882**		
105	25	AFAP	c.1699G>T; p.Gly567X	e13	Nonsense	This study	**0.804±0.022**	0.692	1.090
63	6	FAP	c.1660C>T; p.Arg554X	e13	Nonsense	1,2,3	**1.238±0.047**	2.152	1.069
32	7	FAP	c.1660C>T; p.Arg554X	e13	Nonsense	1,2,3	**1.223±0.039**	2.255	1.153

SD, standard deviation. *e*, exon. *i*, intron.

*Reference accession number: NM_000038. Imbalanced ASE values are depicted in bold letters.

^Considered as exon 15-mutation because leads to a PTC in exon 15. For further details about the mutations reported we refer to three *APC* gene mutation databases: (1) the Universal Mutation Database http://www.umd.be/APC/, (2) http://www.LOVD.nl/APC and .(3) http://www.hgmd.cf.ac.uk/ac/index.php.

Table 2

Characteristics of the *APC*(−)/*MUTYH*(−) polyposis families analyzed for ASE

Individual	Family	Phenotype	ASE±SD (freq C / freq T)	ASE (−)Puromycin (freqC/freqT)	ASE (+)Puromycin (freqC/freqT)
158	29	FAP	**1.284±0.060**	2.099	0.963
166	30	FAP	1.083±0.047		
170	31	FAP	1.059±0.136		
171	32	FAP	1.045±0.055		
172	32	FAP	1.042±0.011		
175	33	FAP	0.890±0.030		
129	34	AFAP	0.947±0.034		
132	35	AFAP	0.903±0.041		
134	36	AFAP	1.016±0.066		
136	37	AFAP	0.922±0.027		
138	38	AFAP	0.988±0.019		
140	39	AFAP	**0.819±0.024**	0.989	0.998
145	40	AFAP	0.991±0.025		
147	41	AFAP	1.005±0.033		
152	42	AFAP	0.901±0.047		
153	43	AFAP	0.929±0.015		
UM-11	UM-11	AFAP	**0.756±0.122**		
UM-12	UM-12	AFAP	0.943±0.024		
UM-14	UM-14	AFAP	0.914±0.034		
UM-15	UM-15	AFAP	1.002±0.074		
UM-16	UM-16	AFAP	1.138±0.026		
UM-18	UM-18	AFAP	1.070±0.061		
UM-19	UM-19	AFAP	0.877±0.029		
UM-20	UM-20		0.999±0.073		

SD, standard deviation. Imbalanced ASE values are depicted in bold letters.