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# *APC* mutations in FAP-associated desmoid tumours are non-random but not 'just right'

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**Analysis of *APC* mutations in colonic and duodenal tumours from familial adenomatous polyposis (FAP) patients has shown that the site of the first hit, the germline mutation, can predict the type and position of the somatic mutation or 'second hit'. The two *APC* mutations are selected on the basis of a 'just right' level of beta-catenin signalling in intestinal tumours achieved through retention of some of the seven 20-amino-acid beta-catenin degradation repeats. Desmoids are a life threatening extra-colonic manifestation in FAP patients. These aggressive tumours of mesenchymal origin are, at present, poorly characterized in terms of mutational *APC* spectra. We have investigated somatic mutations in the largest cohort of FAP-associated desmoids to date, and combined our results with previously published data. Somatic mutations were found to occur non-randomly and the position of the germline mutation shown to be a major determinant of the somatic mutation, a characteristic shared with intestinal tumours from FAP patients. In contrast to colonic polyps, loss of heterozygosity in desmoids involved deletion rather than mitotic recombination. While tumours from the colorectum and upper gastrointestinal tract usually retain one to two and three to four beta-catenin degradation repeats, respectively, most desmoids preferentially retain two repeats ($P < 0.001$, $\chi^2$ test). In addition, most desmoids with two *APC* hits (87%, 26/30) had one mutated allele with no 20-amino acid repeats ($P < 0.001$). This feature, unique among FAP tumours, indicates that a mutation deleting all repeats from one allele may be an important component in maintaining appropriate levels of beta-catenin signalling levels in desmoid tumour cells.**

## INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal, dominantly inherited, Mendelian cancer syndrome caused by germline mutations in the adenomatous polyposis coli (*APC*) gene, which encodes a large multifunctional protein. FAP is characterized by the development of hundreds or thousands of colorectal polyps, which almost inevitably progress to colorectal cancer unless prophylactic colectomy is undertaken. Following surgery, the mortality of patients with FAP remains in excess of the general population, due to extra-colonic manifestations such as upper gastrointestinal polyps and desmoid tumours (1).

Desmoid tumours are poorly understood, frequently aggressive tumours of mesenchymal origin, which arise in musculoaponeurotic structures. Intra-abdominal desmoids often appear to be surgically induced—typically by prophylactic colectomy—but can arise in the absence of surgery (2). The incidence of desmoids in FAP has been estimated to be around 850 times that of the general population and 10–25% of FAP patients will develop at least one desmoid within their lifetime (3–6). A number of independent predictors of increased desmoid risk have been identified, including female gender, a family history of desmoids and a germline mutation distal to codon 1399 (7,8).

*APC* is a tumour suppressor gene, and consistent with Knudson's two-hit hypothesis, both alleles are mutated in FAP-associated intestinal tumours: one in the germline and the other in the soma (9–12). Somatic *APC* mutations,

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principally protein truncating changes and loss of heterozygosity (LOH), have been reported in both colonic and duodenal polyps. These mutations occur non-randomly (13,14). In colonic polyps, truncating mutations tend to cluster in a segment of the *APC* gene known as the mutation cluster region (MCR), which lies between codons 1250 and 1450 (13). A similar MCR has been identified in the duodenum between codons 1400 and 1580 (14). LOH is the most likely second hit in colonic polyps from those patients with a germline mutation between the first and second 20-amino acid beta-catenin degradation repeats (20-AARs) of *APC*, close to 1300; LOH usually occurs by mitotic recombination, with no decrease in copy number. Other FAP patients generally have a truncating mutation as the second hit (13). It has been suggested that there needs to be a 'just right' level of beta-catenin signalling that results from the two *APC* mutations (15). In fact, the incipient colorectal tumour can tolerate some variation in its genotype, but most acquire mutant proteins that between them encode two, or less often one, 20-AARs.

Extra-colonic tumours in FAP show similar associations, but different regions of the *APC* gene are involved. Tumours taken from the upper gastrointestinal tract of FAP patients with germline *APC* mutations after codon 1400 tend to show allelic loss, whereas tumours from other patients have truncating mutations after codon 1400 as the somatic mutation (14). A 'first hit–second hit' association at *APC* has also been found in sporadic colorectal tumours (13). Overall, it appears that *APC* mutations are selected for their ability to produce an optimal level of beta-catenin activation in the intestinal tumour cell (16).

Combining the data from three studies (8,13,17) suggested that somatic truncating mutations distal to codon 1400 occurred in those desmoids with a germline mutation proximal to codon 1400 and, in contrast, allelic loss was found in lesions when the patient's germline mutation was distal to codon 1449. In order to extend these observations and so determine whether an association between first and second hits does exist in FAP-associated desmoids, we have undertaken the largest analysis to date of somatic *APC* mutations arising in these tumours.

## RESULTS

Somatic mutations were identified in 19/23 tumours (83%): truncating somatic mutations in 63% (12/19), allelic loss in 32% (6/19) and exonic deletion in 5% (1/19) of desmoids investigated. LOH was identified in six tumours using microsatellite markers; MLPA analysis was successful in four of these desmoids (only DNA from paraffin embedded tissue was available for the other two-data not shown), identifying the mechanism of loss as deletion rather than mitotic recombination.

Both germline and somatic mutations were identified in a total of 17 tumours (Ds1-17; Table 1). Most tumours (78%, 7/9) from patients with a germline mutation proximal to codon 1400, had somatic frameshift truncating mutation distal to 1400 and, in comparison, no desmoids from patients with germline mutations distal to 1400 displayed such truncating mutations ((0/8) $P = 0.002$, Fisher's exact test). The majority of desmoids from the latter group of patients displayed allelic loss as second hit (6/8) compared to none of the desmoids from patients with germline mutations proximal to 1400 (0/9; $P = 0.002$, Fisher's exact test).

The total number of 20-AARs remaining in desmoid cells after the first and second hit was established for the St Mark's cohort of 15 samples (Ds1–15) and for a combined set of 30 tumours which included a further 15 desmoids where the germline and somatic mutations have been reported previously in the literature (summarized in Table 2) (13). Tumours with LOH as the somatic mutation were included only if the mechanism of LOH—deletion or mitotic recombination—was known; allelic loss by deletion was considered to remove all beta-catenin degradation repeats. As with the St Mark's samples, interdependence between the first hit and second hit was found to be significant (St Mark's $P = 0.01$; Combined, $P < 0.001$; Fisher's exact test). The majority of desmoids with two *APC* hits (26/30) were found to have one mutated allele with zero 20-AARs ($P < 0.001$, Fisher's exact test) and most null alleles were contributed by the germline (17/26) rather than the soma (9/26), although this was of borderline significance ($P = 0.05$, Fisher's exact test).

We now examined whether the number of 20-AARs left intact after germline and somatic mutation arose by chance or were determined by factors intrinsic to cell survival and proliferation. For any given germline mutation, the number of 20-AARs left intact by the somatic mutation was not uniformly distributed ($P < 0.001$, Kolmogorov-Smirnov test). Hence, the position of the somatic mutation was not determined by chance. To investigate this further, the observed and expected number (calculated by assuming a uniform distribution of somatic mutations) of retained repeats were compared using the combined data set and found to be highly significant ($\chi^2$ with four degrees of freedom = 58.967; $P < 0.0001$, chi-squared goodness of fit test; Table 3). Ungrouped data shows that, overall, by far the biggest contribution was for a sum equal to two repeats ($(O-E)^2/E = 52.071$; Table 3); retention of two beta-catenin degradation repeats was observed much more frequently than would be expected by chance.

## DISCUSSION

The present study represents the largest analysis of mutation arising in a single series of FAP-associated desmoids to date. We have identified a relationship between the first (germline) and second (somatic) hit in desmoids, as has been previously demonstrated for both upper and lower gastrointestinal tract tumours in FAP. Desmoids from patients with a germline mutation proximal to codon 1400 are more likely to have a truncating somatic mutation distal to codon 1400, whereas desmoids carrying a germline mutation distal to 1400 are more likely to have LOH as their second hit. Importantly, desmoids from two patients (Ds5 and Ds15) had relatively 5′ truncating somatic mutations that gave the same outcome as LOH, namely that, in most cases, the two 20-AARs left intact were contributed by the germline mutation. These observations, and a subsequent analysis of

**Table 1.** Germline and somatic mutations in FAP-associated desmoid tumours

| Patient ID | Germline mutation | | Somatic mutation | | 20-aa repeats intact in germline/somatic (% of total intact out of 14 possible 20-aa repeats) |
|---|---|---|---|---|---|
| | AA change[a] | Nucleotide change[a] | AA change[a] | Nucleotide change[a] | |
| Ds1 | Q1242X | 3724C>T | 1516 FS | 4548 del1 bp | 0/2 (14) |
| Ds2 | T.gene.del | MLPA(T.gene.del) | 1454 FS | 4360del35 bp | 0/2 (14) |
| Ds3 | 1484 FS | 4450del2 bp | LOH | LOH | 2/0 (14) |
| Ds4 | 1484 FS | 4450del2 bp | LOH | LOH | 2/0 (14) |
| Ds5 | R1450X | 4348C>T | 1080 FS | 3240 del 5 bp | 2/0 (14) |
| Ds6 | R1450X | 4348C>T | LOH | LOH | 2/0 (14) |
| Ds7 | DelEx8-15a | MLPA(delEx8-15a) | 1542 FS | 4624del180 bp | 0/3 (21) |
| Ds8 | 451 FS | 1351del11 bp | 1282 FS | 3843del1 bp | 0/1 (7) |
| Ds9 | 451 FS | 1351del11 bp | 1357 FS | 4070 del1 bp | 0/1 (7) |
| Ds10 | Q1228X | 3682C>T | 1878 FS | 5632del31 bp | 0/4 (29) |
| Ds11 | Del Ex7-15 | MLPA(delEx7-15) | 1483 FS | 4447 del 4 bp | 0/2 (14) |
| Ds12 | Q237X | 706C>T | 1472 FS | 4416del1 bp | 0/2 (14) |
| Ds13 | I1557X | 4671 del2 bp | LOH | LOH | 3/0 (21) |
| Ds14 | 769 FS | 2307 del1 bp | 1490X | 4469 C>T | 0/2 (14) |
| Ds15 | 2310 FS | 6929 ins1 bp | 783FS | 2348 del1 bp | 7/0 (50) |
| Ds16 | 1940 FS | 5819 del5 bp | LOH | LOH | 5/NK (NK) |
| Ds17 | L1488X | 4463 ins1 bp | LOH | LOH | 2/NK (NK) |
| PLK126 | K848X | 2541A > T | 1458 FS | 4373del10 bp | 0/2 (14) |
| PLK59 | 1061 FS | 3244del4 bp | 1581 FS | 4742del8 bp | 0/3 (21) |
| P5 | 1061 FS | 3244del5 bp | 1472FS | 4415del14 bp | 0/2 (14) |
| GD13#61b | Y1075X | 3223T>A | 1461FS | 4415del4 bp | 0/2 (14) |
| P2 | D1084X | 3250G>A | 1542FS | 4625del1 bp | 0/3 (21) |
| PLK56 | 1105FS | 3313del1 bp | 1461FS | 4382del2 bp | 0/2 (14) |
| PLK111 | S1110X | 3329C>G | 1426FS | 4276ins82 bp | 0/2 (14) |
| P12 | Q1228X | 3682C>T | 1859FS | 5575del31 bp | 0/5 (36) |
| PLK124 | 1309FS | 3925del5 bp | V1452X | 5355del1 bp | 1/ 2 (21) |
| GD-11#63 | 1309FS | 3925del5 bp | 1551FS | 4652del4 bp | 1/3 (29) |
| P10 | E1397X | 4189G>T | 1543FS | 4628del1 bp | 1/3 (29) |
| P7 | 1461FS | 4382del2 bp | 1483FS | 4448del6bpins2 bp | 2/2 (29) |
| P8.1 | 1462FS | 4384del2 bp | LOH | LOH | 2/0 (14) |
| P8.2 | 1462FS | 4384del2 bp | LOH | LOH | 2/0 (14) |
| PLK42 | 1462FS | 4384del4 bp | LOH | LOH | 2/0 (14) |

[a]AA and nucleotide positions cal from transcription start site (ATG).
Mutations are recorded by site and type: del, deletion; FS, frameshift; LOH, allelic loss. NK, not known as mechanism of LOH not established. Ds numbers were given to St Mark's samples, other codes relate to desmoids referred to in literature Δ (8,13,16).

the number of 20-AARs retained by cells after both hits in a combined series of 30 desmoids, suggests that the most important aspect to the first hit–second hit relationship is likely to be maintenance of appropriate levels of beta-catenin protein. Curiously, the majority of desmoids (26/30) have one allele with zero 20-AARs ($P < 0.001$, Fisher's exact test), a feature that appears very different from intestinal polyps. Overall, our data demonstrated that mutations are selected to ensure that two to three repeats in total are left intact between the two mutant alleles, with retention of two repeats by far the most common outcome of genetic mutation.

The ability of the APC protein to target beta-catenin for degradation is dependent on the number of intact 20-AARs and, hence, the number of repeats is a crucial determinant of the selective advantage conferred by the mutant APC protein in tumours. Models describing the loss of beta-catenin degra-

**Table 2.** The number of β-catenin degradation repeats retained following germline and somatic *APC* mutation

| Germline mutant allele repeats | Somatic mutant allele repeats | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| 0 | 0 | 2 | 10 | 3 | 2 |
| 1 | 0 | 0 | 1 | 2 | 0 |
| 2 | 7 | 0 | 1 | 0 | 0 |
| 3 | 1 | 0 | 0 | 0 | 0 |
| 7 | 1 | 0 | 0 | 0 | 0 |

A total of 30 desmoids were analysed, 15 from St Mark's and 15 from literature (8,13,16).

**Table 3.** Total number of 20-AARs retained in FAP-associated desmoid tumours

| Sum of repeats retained | Observed | Expected | Observed | Expected | (O-E) (2)/E |
|---|---|---|---|---|---|
| 0 | 0 | 2.125 | 2 | 4.625 | 1.490 |
| 1 | 2 | 2.5 | | | |
| 2 | 17 | 3.5 | 17 | 3.5 | 52.071 |
| 3 | 5 | 3.625 | 11 | 21.875 | 5.406 |
| >3 | 6 | 18.25 | | | |

Values given are the number of desmoids with a given total of retained repeats, figures are observed and expected. $\chi^2$ on four degrees of freedom = 58.967 ($P < 0.001$).

dation repeats in tumours taken from the colorectum and upper gastrointestinal tract of FAP patients propose that, in general, one to two repeats and three to four repeats, respectively, are left intact (14,16). However, there are still a few exceptions indicating that variation in the efficiency of beta-catenin degradation is tolerable. In the case of the desmoids studied here, there are two desmoid tumours (Ds15 and P12) where after the first and second hits seven and five 20-AARs are left intact, respectively. This figure is much greater than two to three repeats seen in most other desmoids. It is possible that these tumours have a third *APC* hit as observed in some colonic polyps in FAP (18). The APC protein also contains SAMP (Ser-Ala-Met-Pro) motifs that are important in the regulation of beta-catenin. Conductin and axin both associate with beta-catenin and GSK3β and interact with APC polypeptides containing a SAMP motif, and most truncating somatic mutations are associated with the loss of all three SAMP motifs. Unusually, tumours Ds15 and Ds10 have retained SAMP motifs (2 and 3, respectively) again, raising the possibility of an, as yet, unidentified third hit.

In previous studies of colonic tumours in FAP, LOH generally occurred by mitotic recombination rather than deletion. The mechanism of allelic loss has not been studied in FAP associated desmoid tumours. A total of six desmoids were found to show LOH using microsatellite markers; four of these were successfully analysed by MLPA and a deletion event observed in each case. This might reflect an important difference between colonic and desmoid tumours or, more likely, that the mechanism is selected for based on conserving an optimal number of 20-AARs; LOH by deletion leaves no intact repeats whereas LOH by mitotic recombination leaves an equal number of repeats.

Overall, our data supports the current opinion that beta-catenin dysregulation is a fundamental component in the development of FAP associated tumours. Desmoids arise, therefore, following biallelic *APC* mutation, with one change usually occurring distal to the second beta-catenin binding/degradation repeat of the gene (3′ to codon 1399). The apparent differences between the number of repeats retained by colorectal and upper gastrointestinal polyps, and by desmoid cells, would suggest that tumour site and/or origin of tumour cell plays a role in selecting appropriate beta-catenin levels. We have suggested that because families with germline mutations in this region already have the requisite change, they are more likely to develop desmoids.

## MATERIALS AND METHODS

DNA was extracted from 23 samples of desmoid tumours (21 fresh frozen, 2 paraffin embedded) from 17 patients (males 47%, 8/17; females 53%, 9/17) with FAP at St Mark's Hospital. All patients had colonic polyposis and a family history of desmoid disease was know for 24% (4/17; Ds1, 5, 6, 14), 41% (7/17) did not have any history and 35% (6/17) were unknown. No patients matched the description provided where desmoids are the main clinical symptoms (19,20). Matched blood was available for 65% (11/17) of patients providing desmoid tissue samples. DNA extraction from fresh frozen material was performed using the Qiagen Tissue Extraction Kit (Hilden, Germany) while DNA extracted from blood and paraffin used standard laboratory protocols.

Polymorphic microsatellite markers (D5S346, D5S82, D5S1965 and D5S421) that map close to the *APC* gene were used to assess allelic loss, and loss was scored as given in Crabtree *et al*. (16). Detection of copy number changes was performed using Multiplication Ligation-Probe Amplification (MLPA; MRC-Holland, Amsterdam, The Netherlands) according to manufacturer's instructions. Products were analysed using the Applied Biosystems PRISM™ 3100 DNA sequencer and the Applied Biosystems Genotyper software (PE Applied Biosystems, Foster City, CA, USA).

*APC* coding sequence mutations were identified using denaturing high performance liquid chromatography (dHPLC) analysis. Heteroduplex analyses were carried out on an automated dHPLC instrument (WAVE, Transgenomic CA, USA). For samples not yielding sufficient product on PCR for dHPLC, fluorescence–single stranded conformational polypmorphisms analysis (SSCP) was performed using the ABI3100 system as described previously (14). Data were analysed using ABI Genescan and Genotyper software. Segments of exon 15 encompassing the MCR (codons 935–1773) and those samples displaying abnormal profiles by dHPLC or SSCP were bidirectionally sequenced using the BigDye Terminator sequencing kit (Applied Biosystems) according to the manufacturer's instructions on an ABI Prism 3100 instrument. Details of primers and PCR conditions used in dHPLC, SSCP and sequencing supplied on request.

Mutation data were analysed in Stata 8.2 (Stata statistical software release 8.0; Stata Corporation, College Station, TX, USA). Fisher's exact test was used to test for significance in $2\times2$ table; Kolmogorov–Smirnov test was used to examine whether distribution of somatic mutations differed significantly from a uniform distribution. Observed and expected distributions were compared using a $\chi^2$ goodness of fit test.

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