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Linkage to chromosome 2q32.2-q33.3 in familial serrated neoplasia (Jass syndrome)

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Abstract Causative genetic variants have to date been identified for only a small proportion of familial colorectal cancer (CRC). While conditions such as Familial Adenomatous Polyposis and Lynch syndrome have well defined genetic causes, the search for variants underlying the remainder of familial CRC is plagued by genetic heterogeneity. The recent identification of families with a heritable predisposition to malignancies arising through the serrated pathway (familial serrated neoplasia or Jass syndrome) provides an opportunity to study a subset of familial CRC in which heterogeneity may be greatly reduced. A genome-wide linkage screen was performed on a large family displaying a dominantly-inherited predisposition to serrated neoplasia genotyped using the Affymetrix GeneChip Human Mapping 10 K SNP Array. Parametric and nonparametric analyses were performed and resulting regions of interest, as well as previously reported CRC susceptibility loci at 3q22, 7q31 and 9q22,

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were followed up by finemapping in 10 serrated neoplasia families. Genome-wide linkage analysis revealed regions of interest at 2p25.2-p25.1, 2q24.3-q37.1 and 8p21.2-q12.1. Finemapping linkage and haplotype analyses identified 2q32.2-q33.3 as the region most likely to harbour linkage, with heterogeneity logarithm of the odds (HLOD) 2.09 and nonparametric linkage (NPL) score 2.36 ($P = 0.004$). Five primary candidate genes (*CFLAR, CASP10, CASP8, FZD7* and *BMPR2*) were sequenced and no segregating variants identified. There was no evidence of linkage to previously reported loci on chromosomes 3, 7 and 9.

Keywords Serrated neoplasia · Familial cancer · Linkage

Abbreviations

CRC	Colorectal cancer
HLOD	Heterogeneity logarithm of the odds
LOD	Logarithm of the odds
MMR	Mismatch repair
MSI	Microsatellite instability
MSI-V	Microsatellite instability-variable
NPL	Nonparametric linkage

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Introduction

The identification of a causative genetic variant in cancer-prone families such as those with Lynch syndrome is an important determinant of decreased mortality through more focussed cancer surveillance [1]. In addition, colorectal cancers (CRC) arising in Lynch syndrome show molecular and pathology features, such as immunohistochemical loss of DNA mismatch repair (MMR) gene expression and increased tumour infiltrating lymphocytes, which allow them to be distinguished from among unselected series. However, less than 3% of all CRC is accounted for by this well-characterised condition, in which the cancers develop from adenomatous polyps [2, 3].

Several studies examining linkage in non-syndromic CRC families have reported statistically significant associations at 3q22 [4–7] and 9q22 [8–11]. A more recent study found linkage to 7q31 in colorectal neoplasia families, and confirmed the region at 3q22 as a susceptibility locus for CRC [12]. To date no confirmed causative variants have been identified from these regions, though the reported associations remain robust.

The balance of families with CRC predisposition are likely to show genetic heterogeneity and variable penetrance, thereby limiting the power of genome-wide linkage approaches to define critical regions in the absence of stratification. The description of families with multiple cases of neoplasia in which serrated polyps are prominent has facilitated the exploration of a proportion of the remaining unexplained familial aggregation. Such families were initially reported from New Zealand by the late Professor Jass and colleagues in the mid-nineties [13, 14], and a further publication demonstrated the familial involvement of hyperplastic polyposis [15], associated with LOH at chromosome 1p. Families fulfilling the Amsterdam I criteria with mixed epithelial polyps and variable levels of microsatellite instability (MSI) in which Lynch syndrome could be excluded, and which showed some overlap with hyperplastic polyposis, were reported in 2005 [16]. Such families are characterised by a mixture of serrated and adenomatous polyps, and, in contrast to Lynch syndrome, somatic *BRAF* mutation is commonly observed in CRC. Only a minority of cancers demonstrate MMR deficiency, attributable to methylation of the *MLH1* gene promoter. We refer to this condition of serrated neoplasia and adenomas clustering in families as Jass syndrome, after the pathologist who first described it [13, 14].

Studies on unselected series of CRCs have shown that molecular features of the serrated pathway, such as widespread CpG island methylation and somatic *BRAF* mutation, as well as the presence of serrated lesions with atypical histology (sessile serrated adenomas), are associated with a family history of CRC [17–19], lending further support to

the idea that the observed familial aggregation of lesions arising through the serrated pathway is the result of an inherited predisposition. In addition, the presence of a sessile serrated adenoma is associated with polyp multiplicity [19, 20], and with conventional adenomas in patients who do not meet the criteria for hyperplastic polyposis [21]. In patients with hyperplastic polyposis, polyps with adenomatous elements increase the risk of CRC [22–24], and are the likely lesions of origin for at least some of the cancers occurring in this condition [25].

We have investigated genomic regions associated with Jass syndrome by performing a genome-wide linkage screen in a single large family, followed by finemapping in a further 10 families, and present evidence for linkage to chromosome 2q32.2-q33.3. Through further finemapping analysis, we also provide evidence that previously reported CRC susceptibility loci at 3q22, 7q31 and 9q22 are unlikely to contribute to Jass syndrome.

Materials and methods

Families

The 11 families in this study, five of which have been described previously [16], were enrolled from high-risk genetics clinics in Australia as part of the Colon Cancer Family Registry, an international collaborative registry for the study of genetics and epidemiology of colorectal cancer [26]. All participants gave written informed consent to take part in research, and the project was performed under QIMR Human Research Ethics Committee Approval P912 (Genetics of Serrated Neoplasia).

Polyps were reviewed by a specialist gastro-intestinal pathologist (JRJ). MSI status of tumours was determined using a panel of 10 microsatellite markers (BAT-25, BAT-26, BAT-34C4, BAT-40, D5S346, D10S197, D17S250, D18S55, ACTC and MYCL) and standard techniques [16, 27]. *BRAF* V600E mutation status of tumours was analysed as previously described [28]. Lynch syndrome was excluded in all families as determined by: (1) proficient expression of the MMR proteins in tumours; (2) absence of pathogenic mutations or variants of uncertain clinical significance in the MMR genes after sequencing of the coding and splice site regions and MLPA analysis for large deletions or duplications; and (3) methylation analysis of the *MLH1* gene promoter. No mutations were found in any patients. Three cancers showed loss of MLH1 protein expression, of which two had sufficient DNA available for methylation analysis and tested positive for methylation of the *MLH1* promoter in their tumour tissue [29].

Criteria for inclusion of families were: at least 2 individuals with CRC, with one aged under 60 years; AND at least 2 individuals with polyps, with one aged under 60 years; AND at least two of the following characteristics associated with serrated neoplasia:

1. a mixture of hyperplastic and adenomatous polyps;
2. variable levels of MSI in cancers and/or polyps
3. the presence of *BRAF* V600E somatic mutation in one or more cancers; and
4. at least one individual with multiple hyperplastic polyps under age 60.

Characteristics of each family are listed in Table 1.

Genome-wide linkage screen

The 10 K Xba 142 GeneChip Human Mapping Array (Affymetrix Inc., Santa Clara, CA, USA) was used to genotype seven affected individuals and one unaffected spouse from Family 1 (Fig. 1), a multi-case CRC family with multiple young-onset, *BRAF*-mutated, MSI-variable (MSI-V) CRCs, and multiple individuals with multiple serrated polyps or those with atypical histology including sessile serrated adenomas, serrated adenomas, and mixed polyps [16, 25]. Sample call rates were all greater than 98.5%, with a total of 990 no calls, and 1280 SNPs (1.3%) were uninformative in the pedigree. Sex was verified by observing heterozygosity rates of X-linked markers. PedCheck [30] was used to detect Mendelian errors, which were manually corrected via the removal of inconsistent genotypes.

Both parametric and nonparametric (NPL) linkage analyses were performed using GeneHunter version 2.1 [31] via a stepwise 900 SNP sliding window, implemented in the Alohomora GUI interface [32]. For parametric analysis, an autosomal dominant mode of inheritance was specified, with 60% penetrance, 10% phenocopy rate and a disease allele frequency of 0.001. Families segregating serrated neoplasia show clustering of CRC, including those with somatic *BRAF* mutation, and polyps of adenomatous, serrated and mixed histology. Individuals with CRC and/or large, multiple or atypical serrated polyps, or any serrated or adenomatous polyp diagnosed before age 60, were classed as affected. As the prevalence of adenomas in the population increases with age [33], a conservative approach was taken to the classification of affected individuals in the over 60 age group, requiring adenomas to be 5 mm or greater in size, or have villous components, and excluding individuals with diminutive rectal-only lesions. Haplotypes were constructed in GeneHunter and viewed graphically using HaploPainter version 029.5 [34].

Finemapping

Twelve microsatellite markers on chromosome 2p, 22 markers on 2q, 18 markers on chromosome 3, 10 markers

Table 1 Phenotypic characteristics of families

Family	Individuals with CRC	CRC ages	Polyp ages (and numbers)	MSI-V	BRAF CRC	Hyperplastic and adenomatous polyps	Individuals with multiple hyperplastic polyps under 60 (ages)	Evidence of linkage to Region 1
1	3	45, 53, 74	63 (1), 53 (3), 51 (2), 57 (2), 46 (1)	Yes	Yes	Yes	1 (57)	Yes
2a[a]	3	25[a], 31[a], 32	29 (1), 53 (1)	No	No	Yes	0	Yes
2b[a]	8	25[a], 31[a], 54, 72, 53, 65, 63, 52	57 (10), 53 (2), 63 (1)	No	Yes	Yes	1 (57)	No
3	4	52, 65, 67, 70	71(1), 66 (1), 65 (7), 55 (11), 60 (2), 49 (1)	No	No	Yes	1 (55)	No
4	4	37, 44, 58, 62	37 (2), 57 (11), 51 (4), 58 (5)	No	No	Yes	3 (51, 57, 58)	No
5	4	31, 47, 52, 59	49 (1), 38 (1), 45 (2), 50 (7), 28 (1)	Yes	No	Yes	1 (50)	Yes
6	3	43, 53, 74	53 (6), 43 (4), 52 (4)	Yes	Yes	Yes	2 (52, 53)	Yes
7	4	31, 39, 50, 61	49 (2), 31 (2)	No	No	Yes	1 (49)	No
8	3	27, 57, 60	52 (1)	Yes	Yes	Yes	0	Yes
9	3	54, 59, 76	54 (2), 75 (1), 50 (1)	Yes	No	Yes	0	No
10	2	55, 62	58 (2), 65 (6)	Yes	No	Yes	1 (58)	Yes
11	4	48, 53, 74, 85	85 (2), 48 (5), 50 (3), 51 (17), 53 (6)	Yes	Yes	Yes	2 (48, 51)	Yes

MSI-V variable levels of MSI between lesions

[a] Families 2a and 2b are branches of the same family which were analysed separately due to independent segregation of affected status. Two individuals with CRC were included as part of both families

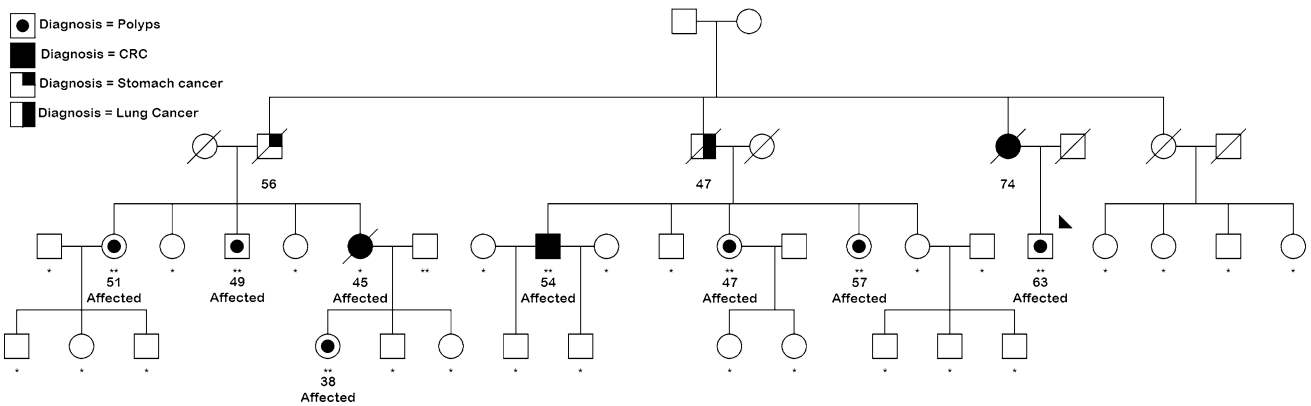

Fig. 1 Family 1. *Solid symbols*, individuals with colorectal cancer. *Dotted symbols*, individuals with large or atypical serrated polyps. Individuals marked with two asterisks were genotyped for the genome-wide linkage scan and for finemapping; those with a single asterisk were typed for finemapping only. Affected individuals are marked as such. A single individual in generation 2 with CRC was also considered as affected. *Numbers* indicate age at first onset of cancer or polyps

on chromosome 7, 12 markers on chromosome 8 and 13 markers on chromosome 9 were typed in the 11 families. Markers and primer sequences are listed in Supplementary Table 1. Markers were amplified using True Allele PCR Premix (Applied Biosystems, Foster City, CA, USA) or GoTaq Colorless Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. PCR products were labelled with FAM, HEX or NED fluorophores, either by labelling one primer in each pair or via an M13 tag and labelled M13 primer, and separated using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Data were analysed using GeneMarker version 1.70 or 1.75 (SoftGenetics, PA, USA). Mendelian error checking was performed using Progeny Lab 6 (Progeny Software, South Bend, IN, USA). Non-Mendelian error checking and parametric and nonparametric linkage analyses on full pedigrees were performed using SimWalk 2.91 [35], implemented under the easyLINKAGE Plus

interface version 5.05 [36]. Parametric linkage analysis was performed under a dominant model with 60% penetrance, 10% phenocopy rate and disease allele frequency of 0.001. Affected-only parametric analysis was performed using GeneHunter version 2.1, also under the easyLINKAGE interface, under a dominant model with 99% penetrance, 10% phenocopy rate and disease allele frequency of 0.001. For affected-only analysis, all individuals with unknown or unaffected status were classed as unknown. Marker map locations were based on the deCODE map [37]; positions for markers not on the deCODE map were interpolated from the nearest adjacent deCODE markers using the NCBI Map Viewer Build 36.3 (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606). Haplotypes were constructed by SimWalk and visualised using HaploPainter.

Sequencing of exons and exon–intron boundaries of candidate genes was performed using BigDye Terminator chemistry on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Where possible, variants were confirmed by sequencing in both forward and reverse directions.

Results

Genome-wide linkage screen

As there is currently little information on Jass syndrome in the literature, the parameters used for linkage analysis were estimated from the 11 families involved in the study. The pattern of affected individuals was consistent with a dominant mode of inheritance with 60% penetrance, and four affected spouses out of 37 resulted in an estimated phenocopy rate of 10%. Using these parameters, genome-wide linkage analysis on Family 1 revealed regions of interest at 2p25.2-p25.1 (logarithm of the odds (LOD) 1.36, nonparametric linkage (NPL) score 4.50 (nominal $P = 0.004$)), 2q24.3-q37.1 (LOD 1.38, NPL 4.62 (nominal $P = 0.003$)), and 8p21.2-q12.1 (LOD 1.36, NPL 4.28 (nominal $P = 0.007$)) (Fig. 2), although none of these attained genome-wide statistical significance.

Finemapping

Finemapping using microsatellite markers spanning each of the three regions of interest was carried out in Family 1 and a further 10 families (Supplementary Table 2). Family 2 showed independent segregation of affected status in two branches, each of which met the criteria for inclusion, and was therefore treated as two separate families for analysis, 2a and 2b. Five individuals overlapped between the two branches and were therefore included in the analysis as part of both Family 2a and Family 2b. In an effort to account for some of the uncertainties inherent in a newly described disease (such as penetrance, age at onset and the possibility of a proportion of affected individuals being asymptomatic), three separate analyses were performed: parametric analysis with full pedigrees, 10% phenocopy rate and 60% penetrance; nonparametric analysis; and affected-only

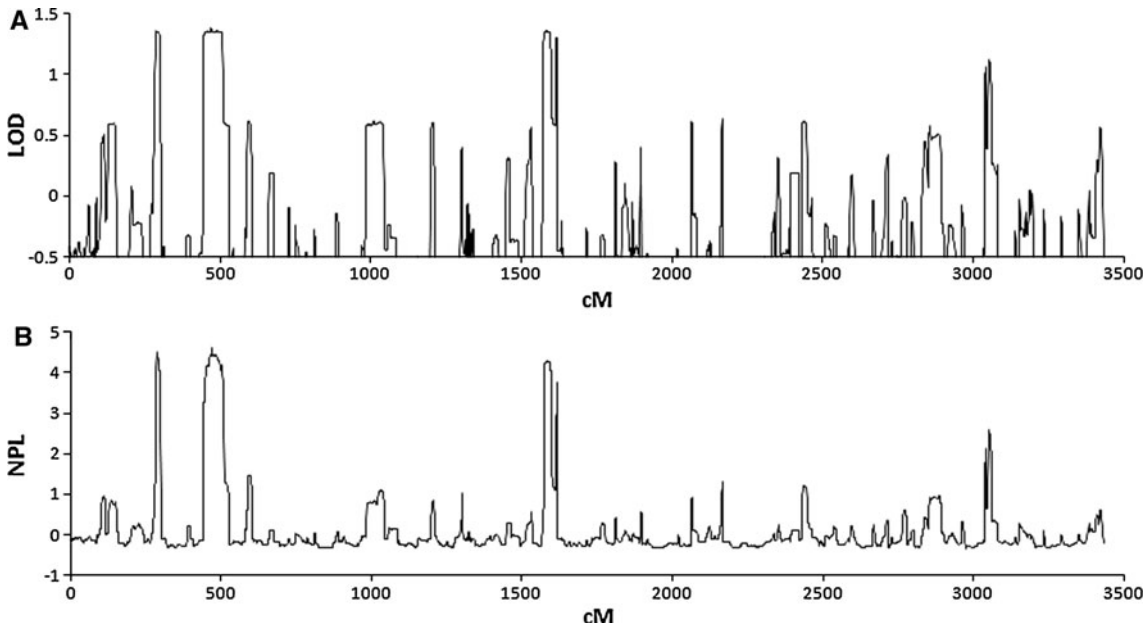

Fig. 2 Genome-wide linkage screen on Family 1. **a** Parametric and **b** Nonparametric analysis

Table 2 Finemapping results

Chr	cM	Parametric (full pedigrees)			Nonparametric		Affected only		
		LOD	HLOD	α	NPL	*P*	LOD	HLOD	α
Regions identified from genome-wide screen on Family 1									
2q (Region 1)	193–199	0.48	0.48	0.95	2.36	0.004	1.12	2.09	0.54
2q (Region 2)	224–231	0.79	0.96	0.60	1.75	0.018	0.82	1.73	0.54
2p	2–34	−1.06	0.04	0.15	1.36	0.04	−1.23	0.60	0.38
8	39–89	−1.56	0.00	0.00	0.86	0.14	−1.73	0.41	0.17

Chr	cM	Parametric (full pedigrees)		Nonparametric		Affected only	
		LOD	HLOD	NPL	*P*	LOD	HLOD
Previously published CRC susceptibility loci							
3	126–190	−1.22	0.18	0.57	0.27	−5.47	0.09
7	111–129	−2.76	0.00	0.83	0.15	−3.32	0.05
9	66–121	−1.86	0.00	0.37	0.42	−5.16	0.00

α proportion of linked families

parametric analysis with 10% phenocopy rate and 99% penetrance, in which unaffected individuals were retained only if they were parents of affected individuals, or were genotyped children of affected individuals for whom DNA was unavailable; these individuals were assigned unknown affected status.

LOD scores on chromosomes 2p and 8 were negative throughout. Evidence for linkage to these regions was limited, with no HLOD score greater than 0.6 and NPL reaching significance at the 5% level at a single location only (marker D2S262 at 30 cM on chromosome 2p, NPL 1.36 ($P = 0.044$)) (Table 2).

Parametric analysis on full pedigrees at 2q revealed LOD scores of 0.48 at 197 cM between markers D2S117 and D2S309, and 0.79 at 224 cM between markers D2S163 and D2S401, with corresponding HLOD scores of 0.48 and 0.96, respectively (Table 2). Nonparametric analysis revealed a single peak at 199 cM at D2S309 with NPL 2.36 ($P = 0.004$), while the strongest evidence for linkage in the affected-only analysis was at 193 cM between markers D2S118 and D2S115, with LOD 1.12 and HLOD 2.09, with a secondary peak at 225 cM between markers D2S163 and D2S133, with LOD 0.82 and HLOD 1.73 (Table 2; Fig. 3). Taking all three analyses into account, the strongest evidence for linkage was between markers D2S118 and D2S2309, with limited evidence for linkage between markers D2S163 and D2S133; these loci are defined respectively as Region 1 and Region 2. Affected-only linkage analysis accounting for heterogeneity gave estimates for the proportion of linked families (α) as 0.54 for both Region 1 and Region 2 (Table 2). Results for individual families are summarised in Supplementary Table 2.

Haplotype analysis at 2q and sequencing of candidate genes

Eight families revealed a haplotype segregating with affected status. Although there was no evidence of a common haplotype shared between families, a consensus region covering approximately 12 Mb, bordered at the centromeric end by D2S117 and at the telomeric end by D2S2358 and showing considerable overlap with Region 1, segregated with affected status in 48/53 affected individuals in seven families (Figs. 3, 4, Supplementary Table 2). Of these, six families demonstrated variable MSI in cancers and/or polyps (Table 1). In contrast, only one of the five families not showing linkage to this region had variable MSI. The consensus region contains 60 genes, from which five (*CFLAR*, *CASP10*, *CASP8*, *FZD7* and *BMPR2*) were selected based on previous evidence in the literature for a role in CRC or other cancers for sequencing of exons and exon–intron boundaries. At least two affected members of each family were screened, and where a variant showed possible segregation with affected status the rest of the family was also sequenced. Several variants were found, including two *CASP8* polymorphisms, namely a 6-nucleotide promoter insertion/deletion (rs3834129) and the D302H SNP (rs1045485), with previously reported associations with colorectal and other cancers [38–40]. Neither these nor any other identified variants segregated with affected status.

Exclusion of reported CRC susceptibility loci

The contribution of recently reported CRC susceptibility loci on chromosomes 3, 7 and 9 to Jass syndrome was

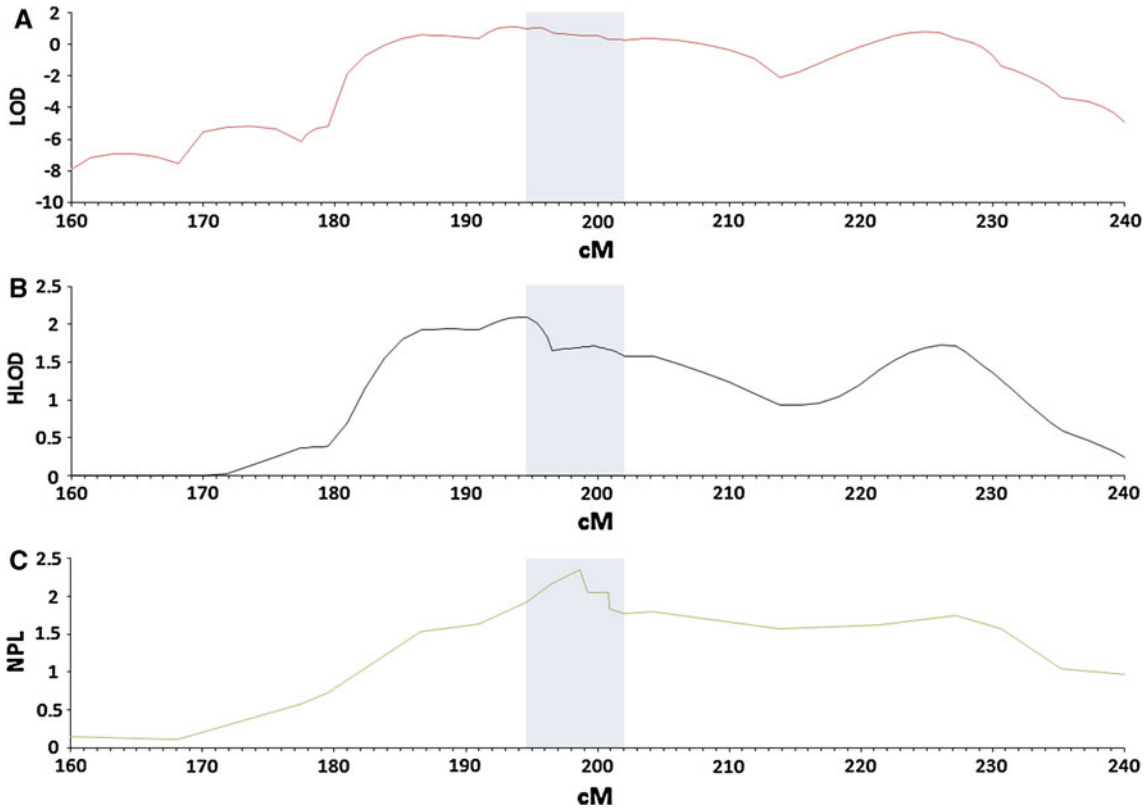

Fig. 3 Finemapping at chromosome 2q in 11 Jass syndrome families. Horizontal axis position along chromosome 2 in cM. **a** LOD score from affected-only analysis; **b** HLOD from affected-only analysis accounting for heterogeneity; **c** Nonparametric analysis. Shading indicates location of haplotype sharing at Region 1

tested using microsatellite markers selected to cover the reported regions, and parametric, nonparametric and affected-only analyses performed as above. No evidence was found to support linkage (Table 2).

Discussion

Since the discoveries of the genes responsible for Familial Adenomatous Polyposis, Lynch syndrome, and the hamartomatous polyposes, little progress has been made in the identification of genetic causes for the remaining unexplained majority of familial CRC. One of the reasons for this lack of success is likely to be extensive genetic heterogeneity, which has proved to be a difficult obstacle to overcome for investigators undertaking linkage studies on collections of CRC families. Other than exclusion of known syndromes, approaches aimed at limiting the heterogeneity within a sample set have included enrichment for families with young-onset cases [8, 41], exclusion of multiple polyposis phenotypes [8], exclusion or inclusion of adenomas and hyperplastic polyps [41, 42], and stratification by occurrence of extracolonic cancers [41]. While these approaches certainly limit phenotypic heterogeneity, the extent to which they reduce genetic heterogeneity is unknown.

The recent identification of a familial predisposition to colonic malignancies arising through the serrated pathway [13, 14, 16, 43] has presented an opportunity to study a subset of familial CRC in which genetic heterogeneity is more limited. Serrated neoplasia families show distinct features, such as variable levels of MSI and frequent somatic *BRAF* mutation in tumours and a mixture of serrated and adenomatous polyps, that allow them to be distinguished from the remainder of familial CRC. Such features characterised families with evidence of linkage to 2q. Incomplete penetrance, small family size, underreporting due to a proportion of individuals being asymptomatic, and practical difficulties in obtaining tumour and polyp samples for all patients, however, mean that all features will not be observed in all families. In an effort to account for this, a set of criteria was developed for this study that allowed for some flexibility while still requiring families to show a clustering of CRC and polyps in conjunction with characteristics that define Jass syndrome. In this attempt to include phenotypically homogeneous families, the possibility remains that a minority of these families may have a genetic predisposition different to that

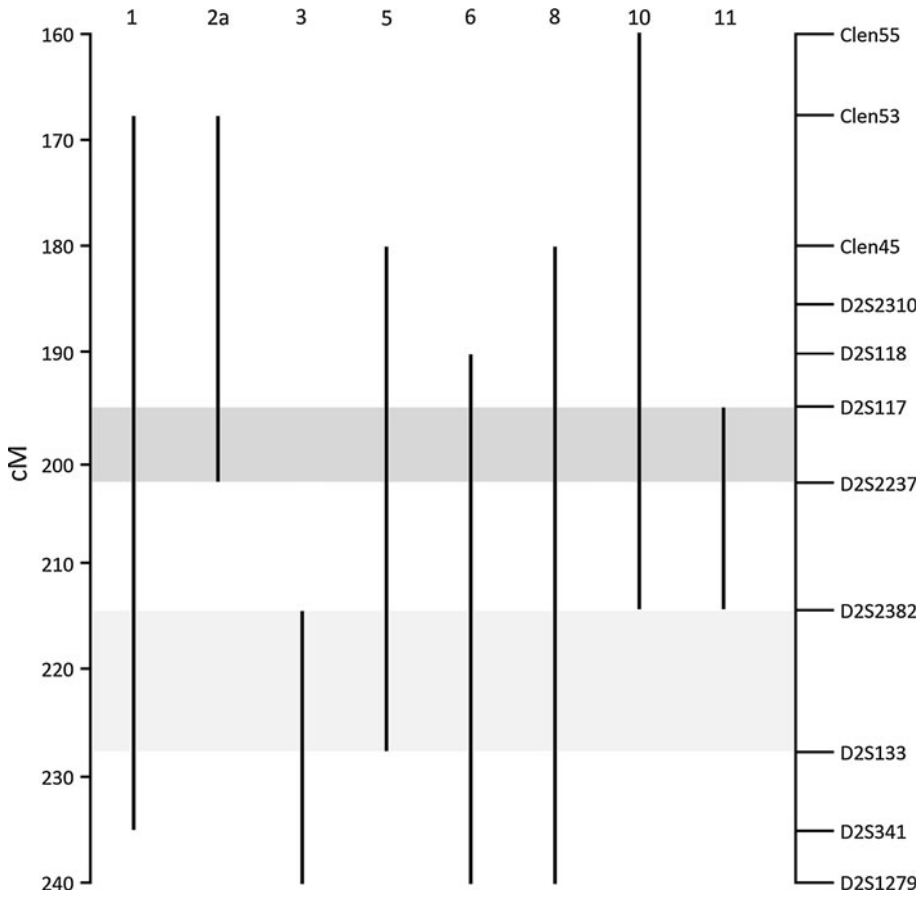

Fig. 4 Haplotype analysis at 2q. *Vertical lines* show chromosomal segments segregating with affected status within each family. *Dark* and *light shading* show areas of segregation at Region 1 and Region 2 respectively

responsible for the majority of Jass syndrome. While this leaves open the possibility of some families being incorrectly included, these are likely to be a minority.

A linkage strategy was selected that would maximise the possibility of identifying linkage, while minimising the effects of any heterogeneity that may remain within the selected families. To this end, the family considered the most likely to allow the identification of a strong linkage signal was subjected to a genome-wide linkage screen, followed by finemapping of regions of interest in the remaining 10 families. Loci at 2p25.2-p25.1, 2q24.3-q37.1 and 8p21.2-q12.1 were considered worthy of further investigation, with 2q24.3-q37.1 showing the strongest evidence of linkage. This region has also been identified in three previous genome-wide linkage studies [4, 6, 12] and one association study [40].

The consensus region at 2q32.2-q33.3 identified through finemapping linkage and haplotype analyses contains five genes that stand out as potential candidates and as a result were prioritised for sequencing. *CFLAR*, *CASP10* and *CASP8* are located in close proximity to each other at 2q33.1 and are all involved in the death receptor-induced apoptotic pathway [44]. *CASP8* somatic mutation has been reported in CRC [45], and a *CASP8* promoter polymorphism has been reported to be associated with susceptibility to multiple cancers including CRC [40], although the association has not been replicated in other populations [46, 47]. *CFLAR* has attracted attention as a therapeutic target as well as a potential oncogene due to its role in the TRAIL pathway [48]. *FZD7* is a member of the Frizzled family which plays an important role in Wnt signalling [49], central to the development and progression of CRC [50], and has recently been shown to activate the Wnt pathway in CRC cell lines [51]. *BMPR2* inactivation leads to epithelial cell proliferation and mixed polyp formation in the mouse colon [52] and reduced protein expression associated with somatic mutation is observed in human CRC and cell lines [53]. Although sequencing of these genes did not reveal any variants segregating with disease, only coding regions and exon–intron boundaries were sequenced, leaving noncoding regions, which may harbour regulatory elements and potential splicing variants, largely unexplored. With current re-sequencing technology and exome capture, the investigation of the remaining genes and regulatory regions becomes more feasible.

Several studies have reported evidence of linkage among a proportion of non-syndromic CRC families, with 3q22, 7q31 and 9q22 prominent [4–6, 8–10, 12]. As well as suggesting that they are unlikely to contribute to Jass syndrome, the failure to find evidence of linkage to these

loci in the present study suggests that further characterisation of the 3q22, 7q31 and 9q22 regions should aim to exclude families demonstrating molecular and/or histological evidence of serrated neoplasia.

Approximately half of the families studied showed evidence of linkage to 2q32.2-q33.3. The balance of the families did not demonstrate linkage, suggesting that other as yet unidentified loci may contribute to families with serrated neoplasia. Continuing efforts to identify and characterise serrated neoplasia families will serve the dual purpose of allowing a greater understanding of the phenotypic presentation of the syndrome, and facilitating further genetic studies which will allow confirmation and refinement of the linked region, paving the way for more comprehensive analysis of the locus, and potentially the identification of the underlying genetic defect.

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Conflict of interest Authors declare no conflict of interest.

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