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REVIEW ARTICLE

Familial Colorectal Cancer Type X

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Abstract: The genetic background is unknown for the 50-60% of the HNPCC families, who fulfill the Amsterdam criteria, but do not have a mutation in an MMR gene, and is referred to as FCCTX. This study reviews the clinical, morphological and molecular characteristics of FCCTX, and discusses the molecular genetic methods used to localize new FCCTX genes, along with an overview of the genes and chromosomal areas that possibly relate to FCCTX. FCCTX is a heterogeneous group, mainly comprising cases caused by single high-penetrance genes, or by multiple low-penetrance genes acting together, and sporadic CRC cases. FCCTX differs in clinical, morphological and molecular genetic characteristics compared to LS, including a later age of onset, distal location of tumours in the colon, lower risk of developing extracolonic tumours and a higher adenoma/carcinoma ratio, which indicates a slower progression to CRC. Certain characteristics are shared with sporadic CRC, *e.g.* similarities in gene expression and a high degree of CIN^+, with significanly increased 20q gain in FCCTX. Other molecular characteristics of FCCTX include longer telomere length and hypomethylation of LINE-1, both being a possible explanation for CIN+. Some genes in FCCTX families (*RPS20, BMPR1A, SEMA4A)* have been identified by using a combination of linkage analysis and sequencing. Sequencing strategies and subsequent bioinformatics are improving fast. Exome sequencing and whole genome sequencing are currently the most promising tools. Finally, the involvement of CNV's and regulatory sequences are widely unexplored and would be interesting for further investigation in FCCTX.

Keywords: Amsterdam positive, Colorectal cancer, Familial cancer, FCCTX, HNPCC, Microsatellite stable, MMR negative.

1. INTRODUCTION

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) accounts for about 5-10% of cases of colorectal cancers (CRC) [1] and the diagnosis is given to families who fulfil the Amsterdam Criteria (AC (-I/-II)) (Table **1**) [1, 2]. Lynch Syndrome (LS) accounts for 50 - 60% of families fulfilling the AC [3, 4] and is mainly caused by a hereditary defect in one of the mismatch repair (MMR) genes: *MLH1, MSH2, MSH6, PMS1* or PMS2 [5, 6]; mainly in *MLH1 and MSH2* (90%). Mutations in MMR genes lead to microsatellite instability (MSI), because microsatellites (stretches of DNA with repetitive sequences of nucleotides) are particularly susceptible to DNA errors when MMR genes are damaged [6]. Deletions of the 3' end of *EPCAM*, which leads to methylation of the promoter region of *MSH2* [7], and mutations in *MSH3* (a MMR gene) are other causes behind LS, though the clinical significance of *MSH3* in LS is still not fully clarified [8-10]. An alternative to the AC is the revised Bethesda Guidelines used to identify CRC patients with elevated risk of LS [11] (Table **2**).

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The terms HNPCC and LS are often used as synonyms [12]. In this review, LS is defined as cancer caused by a defect in one or more of the MMR genes, while HNPCC is defined as fulfilment of the AC. Many families with LS will, however, fulfil the AC. Families, who fulfil the AC or the revised Bethesda Guidelines, comprise a risk group and should be tested further for MMR deficiency. Sequencing of the MMR genes is necessary in order to identify the exact disease-causing, germline mutations (point mutations, small insertions, splice site alterations and deletions), but sequencing cannot identify mutations in which the entire gene or whole exons are deleted. These deletions are identified by multiplex ligation-dependent probe amplification (MLPA) [6]. In addition, targeted sequencing platforms, using Next-Generation Sequencing (NGS) technology, have been developed for use in clinical settings. These platforms comprise several genes of importance in CRC, making comprehensive genetic information available for individual patients [13]. Other strategies, applied prior to sequencing, include testing for MSI or absence of MMR proteins by immunohistochemistry (IHC). MSI-status is assessed using a panel of microsatellite markers and tumours are classified as MSI-high (MSI-H), MSI-low (MSI-L) or microsatellite stable (MSS). MSI-H or absence of MMR proteins are indicators of LS, but the diagnosis cannot be made solely on this basis, because 10-15% of sporadic CRC's also exhibit MSI or absence of

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Table 1. Amsterdam criteria II and I.

Amsterdam Criteria I (AC-I) [1]	Amsterdam Criteria II (AC-II) [2]
At least 3 family members with CRC and presence of all the following criteria:	Inclusion of HNPCC-associated cancer (CRC, cancer of the endometrium, small bowel, ureter and renal pelvis), which can substitute CRC in AC-1 in all sub points
1) One affected family member should be a first-degree relative of the other two	
2) Family members from at least two generations should be affected	
3) At least one family member should have the diagnosis CRC before age 50	
4) Familial Adenomatous Polyposis (FAP) should be excluded	
5) Tumours should be verified by pathological examination	

Table 2. The revised bethesda guidelines [11].

1) CRC diagnosed in a patient younger than 50 years
2) Presence of synchronous, metachronous colorectal or other HNPCC-associated tumours[1] regardless of age
3) CRC with MSI-H[2] histology diagnosed in a patient younger than 60 years
4) CRC diagnosed in at least one first-degree relative with a HNPCC-related cancer with one of the cancers being diagnosed before the age of 50 years.
5) CRC in at least two first- or second-degree relatives with HNPCC-related tumours regardless of age

[1] Tumours in colon, rectum, endometrium, ventricle, ovaries, pancreas, ureter, renal pelvis, biliary tract, brain, jejunum and duodenum and sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome. [2] MSI-H: microsatellite instability high grade.

MLH1, due to promoter hypermethylation. Therefore, supplements must be made, for instance molecular testing of tumour tissue for somatic methylation of *MLH1* and somatic mutations in *BRAF* (p.V600E) (because *BRAF* mutations are rare in LS) [6]. The genetic background is unknown for the remaining 40-50% of the HNPCC families, which do not have a MMR defect. This group, referred to as Familial Colorectal Cancer Type X (FCCTX), is characterised by being microsatellite stable (MSI-L/MSS) and by the absence of mutations in MMR genes [6, 12, 14-16]. Knowing the cause of disease and the risk associated with the causal genes allows for the development of optimal counselling, screening programs and treatment, which is important in detecting and treating tumours before they become malignant, or at least before they become metastatic. In addition, testing for the family´s disease-causing mutation in relatives, gives the opportunity to initiate early control and prevent cancer development in mutation carriers, and release the non-carriers from anxiety and further examinations [17]. Still, the research results in this area are sparse for FCCTX. Investigation of the genetic background for FCCTX is therefore of great importance when aiming for targeted treatment and setting up prophylactic procedures for the relevant family members. This study reviews the clinical, morphological and molecular characteristics of FCCTX and discusses the molecular genetic methods used to localize new FCCTX genes, along with an overview of genes and chromosomal areas that are possibly related to FCCTX.

2. METHOD

The literature was retrieved from PubMed [18] using the search term: *"Familial Colorectal Cancer Type X"*, primarily, and supplemented with search term: *"Hereditary Non-polyposis Colorectal Cancer Microsatellite Stable"*. Relevant articles were selected based on abstracts. The table of susceptibility genes and loci associated with CRC (Table **9**) was based on the search term: *"Colorectal cancer, susceptibility to"* in OMIN.org [19] after exclusion of genes/loci present in tumours, only. The search results were sorted by *"Relevance",* which is one of the sorting options in OMIN.org. The first 30 results are included, after exclusion of results that deal with already known hereditary, monogenic diseases and mutations, results that deal with sporadic CRC, results that deal with other primary phenotypes such as hereditary mixed polyposis syndrome, prostate and lung cancer, and finally, results based on animal studies. Search result from #31 and higher were assessed not to be relevant and therefore excluded. Houlston *et al.* conducted a meta-analysis of two genome-wide association studies (GWAS) in 2008 [20], which were expanded into a meta-analysis of three GWAS in 2010 [21]. The analysis from 2010 did not appear in the search result at OMIN.org, but is referred to in other studies and assessed as relevant, and therefore included in (Table **9**).

3. CLINICAL, MORPHOLOGICAL AND MOLECULAR GENETIC CHARACTERISTICS OF FCCTX

3.1. Clinical and Morphological Differences Between FCCTX and LS

FCCTX shows significant clinical differences compared to LS, as demonstrated in a study of 161 families, including 3422 individuals, fulfilling the AC-I. Tumours were analysed for MSI and grouped into MSI-H, reflecting MMR

deficiency (LS), and MSS/MSI-L, reflecting no MMR deficiency (FCCTX). All MSI-H and MSI-L were further tested by IHC for loss of expression of MLH1, MSH2 and MSH6. It was demonstrated that FCCTX cases develop cancer at a higher age, do not develop extracolonic cancer and have a lower risk of developing CRC compared to LS [14]. These results have been supported by several other studies [4, 22-31], and have given rise to further studies of clinical and morphological differences between FCCTX and LS [14, 15, 23-33], as summarised in (Table **3**). FCCTX is characterised by a predominant distal location of tumours in the colon/rectum, a lower rate of lymphocyte infiltration, peritumorous lymphocytes and synchronous and metachronous tumours, a higher adenoma/carcinoma ratio, and a higher differentiation of tumour cells compared to LS, which indicate a slower progression of cancer in FCCTX. Also, FCCTX tumours show a more heterogeneous architecture with a high frequency of tubular architecture, dirty necrosis (glands filled with necrotic debris), tumour budding, a low frequency of mucin production and the absence of Crohn-like reactions. FCCTX tumours, furthermore, show an infiltrative growth pattern, while LS tumours show an expansive growth pattern (Table **3**).

3.2. Molecular Genetic Differences and Similarities Between FCCTX, LS and MMR Proficient Sporadic CRC

Gene expression also clearly differs between FCCTX and LS. In 37 FCCTX tumours and 39 LS tumours, 2188 genes were differentially expressed between the two groups. Genes relating to the G-protein coupled signalling pathway were up regulated in FCCTX, while genes relating to cell cycle, mitosis and oxidative phosphorylation were up regulated in LS, indicating that different molecular pathways are involved in tumour genesis in FCCTX and LS. However, gene expression in FCCTX tumours were closely related to sporadic MMR proficient tumours (n=21), with only 4 differentially expressed genes. Specific cancer-related genes with increased expression in FCCTX included *MYC* and *AXIN2*, while *NDUFA9* expression was decreased [30]. Similar results have been observed in a study of 9 FCCTX and 9 MSS sporadic tumours and adjacent normal tissue, which were analysed for the gene expression of nine potentially cancer-specific genes. Three genes were cancer-specific and their gene expression did not differ significantly in FCCTX and sporadic MSS cases [34].

3.3. Chromosomal Changes in FCCTX

3.3.1. Gains and Losses

LS tumours are generally chromosomal stable (CIN-), while FCCTX tumours act like sporadic CRC, with up to 74% of FCCTX tumours being chromosomal unstable (CIN+) [15, 33]. Several duplications and deletions have been identified, some of which were common in sporadic CRC and presented with similar frequency. The most frequent aberrations included gains on 1q, 6p, 7p, 7q 8q, 13q, 10p, 17, 19, 20p and 20q, and losses on 1q, 4q, 5q, 8p, 12, 15q, 17p, 18p, 18q and 20p [15, 33, 35-37]. In FCCTX tumours, 20q gains were significantly increased (54-77% of FCCTX tumours showed a gain of 20q), indicating that 20q harbour an important area for FCCTX tumour genesis, most likely around 20q12 – 20q13.33 [15, 33, 35, 36]. In addition, FCCTX tumours showed high levels of copy neutral loss of heterozygosity (cnLOH) (32-40% of tumours showed cnLOH) compared to sporadic CRC (14% of tumours showed cnLOH) [35, 36]. A candidate gene in 20q was *GNAS* (20q13.32), which is involved in the G-protein coupled pathway found to be upregulated in FCCTX. The gene is thought to promote tumour genesis through the Wnt signalling pathway. Other candidate genes located in 20q include *CDH26*, *SRC* and *ASIP* [30].

3.3.2. Telomere Length

Telomere length is another chromosomal change of importance in FCCTX cancer risk. Telomeres are regions of repetitive nucleotide sequences (TTTAGG) at each end of the chromatids, that shorten during cell division, and which eventually lead to senescence and apoptosis signalling. In the case of the cell bypassing senescence, critically short telomeres may develop, which leads to genomic instability and tumour development. In cancer cells, the enzyme telomerase is expressed, adding new repeats to the telomeres, and thereby preventing apoptosis/senescence, which increases the risk of genomic abnormalities accumulating. By measuring the length of telomeres in chromosomes from peripheral blood in 57 cancer-affected and 57 cancer-free family members from 34 FCCTX families and from 234 unrelated controls, it was demonstrated that cancer-affected family members had significantly longer telomeres than the unaffected family members from the same families ($p = 0.009$) and from the unrelated cancer-free controls ($p = 0.013$) [38]. This indicates that longer telomeres in chromosomes from peripheral blood are a risk factor in FCCTX, but it has yet to be demonstrated whether this phenomenon can be used to distinguish between those unaffected family members who will develop CRC in the future and those who could stop attending surveillance programmes.

3.4. Methylation

When comparing tumour DNA to normal DNA, an increase and decrease in the methylation of specific DNA sequences are often observed. DNA hypomethylation might explain the increased gains and losses observed in FCCTX, because global hypomethylation can result in Long Interspersed Element-1 (LINE-1) activation, leading to CIN+. A study including 22 FCCTX, 21 LS, 92 sporadic MSS and 46 sporadic MSI tumours showed a significantly lower degree of LINE-1 methylation in FCCTX cases, compared to the other three groups ($p = 0.001$-0.015) [39]. In another analysis of 168 FCCTX, LS and sporadic colorectal tumours, LINE-1 methylation was decreased in tumour DNA relative to normal DNA in all groups, but FCCTX tumours showed lower levels of LINE-1 methylation compared to LS and sporadic CRC in tumours and also in the patients' normal mucosa. Levels of LINE-1 methylation in FCCTX tumours were significantly lower compared to sporadic MSI tumours, and levels of LINE-1 methylation in FCCTX normal mucosa were significantly lower compared to LS, sporadic MSS and sporadic MSI normal mucosa. These results indicate that germline hypomethylation of LINE-1 is a distinguishing feature of FCCTX, that may predispose normal tissues for cancer development (Table **4**). The basic mechanisms of LINE-1 hypomethylation are still unknown and need to be further investigated [40].

Table 3. Clinical and morphological comparison of LS and FCCTX [14, 15, 23-33].

—	FCCTX	LS	P-value, OR[1] and RR[2]	References
Location	Predominantly distal location of tumour in sigmoideum and/or rectum (left colon and/or rectum)	Predominantly location of tumour proximal for the splenic flexure (right colon)	<0.0001 0.15 0.010 0.001 Not given 0.00001 0.001 Not given	Klarskov *et al.* [27] Llor *et al.* [26] Mueller Koch *et al.* [28] Valle *et al.* [29] Francisco *et al.* [32] Dominguez-Valentin *et al.* [30] Shiovitz *et al.* [31] Benatti *et al.* [25]
Mean age-of-onset of CRC	60.7 years 51 years 54 years 63.4 years 60.2 years 60 years 55 years 53 years 58 years 53.3 years	48.7 years 46/45 years[3] 44 years 47.6/49 years[4] 53.8 years 54 years 41 years 41 years 53 years 50.5 years	Not given 0.001 Not given <0.05 0.0.036 0.01 <0.001 <0.001 Not given OR[1]= 1.02 CI[5]: 1.00-1.03	Lindor *et al.* [14] Russo *et al.* [23] Dove-Edwin *et al.* [24] Benatti *et al.* [25] Llor *et al.* [26] Klarskov *et al.* [27] Mueller Koch *et al.* [28] Valle *et al.* [29] Dominguez-Valentin *et al.* [30] Shiovitz *et al.* [31]
CRC risk	2.3 (SIR[6]) Lower than LS	6.1 (SIR[6]) Higher than FCCTX	< 0.001 RR[2] = 2.08, CI[5]: 1.16-3.73	Lindor *et al.* [14] Benatti *et al.* [25]
Risk for developing extra-colonic cancers	Lower than LS	Higher than FCCTX	Not given 0.025 <0.001 RR[2] = 7.59, CI[5]: 1.71-33.7	Lindor *et al.* [14][7] Mueller Koch *et al.* [28] Valle *et al.* [29][8] Benatti *et al.* [25][9]
Incidence of extracolonic HNPCC-related cancer	6.4% 3.3%	25%/18.2%[4] 5.1%	0.03 Not given	Benatti *et al.* [25][9] Llor *et al.* [26][10]
Differentiation	High differentiation	Poor differentiation	<0.0001 0.091[11] Not given 0.00001 OR[1] = 0.33, CI[5]: 0.14-0.78[12]	Klarskov *et al.* [27] Llor *et al.* [26] Francisco *et al.* [32] Dominguez-Valentin *et al.* [30] Shiovitz *et al.* [31]
Carcinoma subtype and architecture	More heterogeneous architecture, compared to LS with a high frequency of tubular architecture	Less heterogeneous architecture than FCCTX with a high frequency of mucinous and solid architecture	0.02, 0.02, 0.0003. 0.0001[13]	Klarskov *et al.* [27]
Mucin production	Low frequency of mucin production	High frequency of mucin production	0.01, 0.001[14] 0.502[11] 0.03 Not given	Klarskov *et al.* [27] Llor *et al.* [26] Valle *et al.* [29] Francisco *et al.* [32]
Dirty necrosis	High degree of dirty necrosis	Absence of dirty necrosis	<0.0001	Klarskov *et al.* [27]
Peritumorous lymphocytes	Lower rate of peritumorous lymphocytes than LS	Higher rate of peritumorous lymphocytes than FCCTX	0.02 OR[1] = 0.49, CI[5]: 0.26-0.90	Klarskov *et al.* [27] Shiovitz *et al.* [31]
Tumour-infiltrating lymphocytes	Low rate of tumour-infiltrating lymphocytes	High rate of tumour-infiltrating lymphocytes	<0.0001 0.033 Not given OR[1] = 0.14, CI[5]: 0.07-0.26	Klarskov *et al.* [27] Llor *et al.* [26] Francisco *et al.* [32] Shiovitz *et al.* [31]

(Table 3) contd….

—	FCCTX	LS	P-value, OR[1] and RR[2]	References
Crohn-like reactions	Low degree of Crohn-like reactions	High degree of Crohn-like reactions	0.003 OR[1] = 0.27, CI[5]: 0.14-0.54	Klarskov *et al.* [27] Shiovitz *et al.* [31]
Synchronous and metachronous tumours	Low rate of synchronous and metachronous tumours	More synchronous and metachronous colorectal tumours compared to FCCTX	0.017, <0.001[15] Not given	Mueller Koch *et al.* [28] Francisco *et al.* [32]
Tumour budding	High degree of tumour budding	Low degree of tumour budding	<0.0001[16]	Klarskov *et al.* [27]
Adenoma/ carcinoma ratio	Higher adenoma/carcinoma ratio compared to LS	Lower adenoma/carcinoma ratio compared to FCCTX	0.03 Not given	Mueller Koch *et al.* [28] Francisco *et al.* [32]
Invasive border	Infiltrative growth pattern	Expansive growth pattern	<0.0001[17]	Klarskov *et al.* [27]
Chromosomal stability	High degree of gains and losses (CIN+[18]) with frequently gain of 20q and loss of 18	Generally chromosomal stable (CIN-)	<0.01[19] Not given	Therkildsen *et al.* [33] Abdel Rahman *et al.* [15]

[1] Odds ratio; [2] Risk ratio; [3] *MLH1* mutation positive and *MSH2* mutation positive respectively; [4] HNPCC MSI MLH1/MSH2 mutation positive and HNPCC MSI MLH1/MSH2 mutation negative respectively; [5] Confidence Interval; [6] Standardized Incidence Ratio; [7] Extracolonic HNPCC-related cancer. Defined as cancer in uterus, ventricle, kidney, ovary, small intestine, ureter; [8] HNPCC-related cancer, not defined; [9] Extracolonic HNPCC-related cancer. Defined as cancer in endometrium, stomach, renal pelvis, ureter, ovary; [10] All endometrial cancer; [11] Not significant; [12] OR for FCCTX *vs*. LS tumours being poorly differentiated; [13] P-values for FCCTX tumours showing more heterogeneous architecture than LS, higher frequency of tubular pattern in FCCTX and higher frequency of solid and mucinous morphologies in LS, respectively; [14] P-values for intracellular and extracellular mucin, respectively; [15] P-value for CRC and extracolorectal tumours respectively; [16] P-value <0.0001 is given in Table **2**, while p-value 0.1 is given in the text. The p-value <0.0001 includes uncertain cases, while the p-value 0.1 comprises definite cases, only (personal communication); [17] P-value for FCCTX more often displaying infiltrative growth compared to LS; [18] Chromosomal instability positive; [19] P-value for 20q gain and 18 loss

Table 4. LINE-1 methylation dosage ratio [40].

—	FCCTX (I) (n=18)	LS (II) (n=43)	Sporadic MSS (III) (n=55)	Sporadic MSI (IV) (n=52)	P-value
CRC tumours	0.80	0.84	0.85	0.87	0.042 (I *vs*. IV)
Normal mucosa	0.84	0.90	0.91	0.93	<0.05 (Any group *vs*. I)

3.5. Molecular Genetic Analysis of FCCTX and Subdivision of FCCTX Based on Beta-catenin

The Wnt signalling pathway is often deregulated in CRC due to mutations in *APC* or *CTNNB1* (beta-catenin). However, in two studies of tumours from 55 FCCTX and 57 MMR mutation negative MSS (HNPCC or HNPCC-like) cases, the majority of these tumours (61-63%) showed membranous location of beta-catenin, indicating normal regulation of the Wnt signalling pathway. In tumours displaying aberrant beta-catenin, *APC* mutations were detected in about half (56% - 57%) [15, 16] (Table **5** and **8**). However, once beta-catenin localisation is disregarded, none of the examined tumours harboured *CTNNB1* mutations. LS tumours, on the other hand, showed deregulation of the Wnt signalling pathway in 25/31 (81%) of the tumours (indicated by aberrant location of beta-catenin in the cell), and a *CTNNB1* mutation was identified in 29% of the LS tumours [15] (Table **5**). After dividing FCCTX cases into two subsets based on beta-catenin, their clinical and morphological characteristics were explored. Membranous localization of beta-catenin was associated with chromosomal stability (CIN-), younger age of onset (53.7 years), dominance of right-sided tumours and infrequent *p53* mutations. Aberrant beta-catenin was associated with older age of onset (58.6 years), dominance of left-sided tumours and CIN+ phenotype associated with *p53* mutations (Table **6** and **7**) [15]. Mutations in the *GNAS* gene, or other genes related to the Wnt signalling pathway, might therefore only explain the pathogenesis in the minority of FCCTX tumours, displaying aberrant beta-catenin and being CIN-.

4. CRC SUCEPTIBILITY GENES/LOCI

So far, few published studies have searched for the germline mutations involved in FCCTX, and only a few germline mutations have been detected, and therefore the heredity in FCCTX remains unexplained. However, several studies have

Table 5. Molecular differences between CRC tumours found in FCCTX *vs*. LS patients [15].

—	Tumours from MMR Mutation Negative HNPCC Patients (FCCTX)	%	Tumours from MMR Mutation Positive HNPCC Patients (LS)	%	P-value
MSI	2 out of 24	8 %	31 out of 31	100 %	Not given
Aberrant beta-catenin (Nuclear, cytosolic)	7 out of 18	39 %	25 out of 31	81 %	0.005
Membranous beta-catenin	11 out of 18	61 %	6 out of 31	19 %	0.005
CTNNB1 mutation	0 out of 24	0 %	9 out of 31	29 %	0.007
Loss of APC expression	6 out of 7 (tumours with aberrant beta-catenin)	86% (tumours with aberrant beta-catenin)	Not given	Not given	-
Loss of *APC* locus	4 out of 7 (tumours with aberrant beta-catenin)	57 % (tumours with aberrant beta-catenin)	Not given	Not given	-
p53 protein stabilisation	8 out of 18	44 %	4 out of 31	13 %	0.04[1]
p53 mutation	5 out of 8 (tumours with p53 stabilisation)	63 %	4 out of 4 (tumours with p53 stabilisation)	100 %	Not significant
CIN+	7 out of 16	44 %	Not given	Not given	-

[1] P-value= 0.04 is given in textbox, while p-value=0.02 is given in text.

identified genes and loci that are associated with CRC in general [20, 21, 41-64] (summarized in Table **9**), and these results may aid in narrowing the search for those genes involved in FCCTX.

Table 6. Association between location of beta-catenin and CIN for FCCTX [15].

—	CIN-	%	CIN+	%	P-value
Membranous beta-catenin	6 out of 7	86 %	0 of 6	0%	0.005

5. APPROACHES TO IDENTIFYING GENES/LOCI THAT PREDISPOSE ONE FOR CRC

Different approaches are used to identify loci/genes associated with CRC. Deciding which approach will give the best results depends on whether the cause of the disease is monogenic or polygenic. LS, for example, is a monogenic disease caused by mutation in one of the MMR genes [6], whereas in a polygenic disease model, several low/moderate-penetrance risk-alleles act together to increase cancer risk [20, 65].

5.1. FCCTX – Monogenic or Polygenic Disease Model?

The fulfilment of the AC in FCCTX indicates that FCCTX have an autosomal dominant mode of inheritance. Warden *et al.* found, by analysis of 66 AC-I families from the New Foundland population (which is the population with the highest rate of familial CRC in the world), that FCCTX and LS show a similar geographical distribution, with geographical clustering of families in isolates, and that the two have a similar Family History Score (FHS)[1]. In LS cases, evidence was found for the geographical clustering of three *MSH2* mutations, two of which have been confirmed as founder-mutations, while nine other MMR mutations were randomly distributed along the coast. Furthermore, pathologic heterogeneity was observed in FCCTX cases and genealogic research failed to identify linkages between the FCCTX families. Based on these results, the authors concludes that FCCTX is a heterogeneous disease that occurs via different pathogenic pathways, and that FCCTX is most likely to be caused by different mutations in one or more CRC genes [22]. These results contribute to the hypothesis that FCCTX is a monogenic, autosomal dominant disease.

The AC were developed for research purposes, in order to select families in which CRC is likely to be caused by LS, but only about half of the HNPCC families have LS, and on the other hand only 1/3 of LS families fulfil the AC. Three possible explanations have been given for the reasons why families fulfilling the AC do not have LS [12]:

1) CRC is a common disease. There is a possibility that two siblings and a parent have CRC by coincidence and not as a consequence of an autosomal dominant disease, even though one of the family members is diagnosed before age 50. Therefore, one should be sceptical of the AC when the two other affected in the triad are diagnosed later than age 70 [12].

[1] FHS was calculated based on CRC incidences in families with CRC compared to CRC incidences in the population in general. Family covariates (age, sex and race) were included in the calculations.

Table 7. Characteristics for subgroups of FCCTX [15].

Majority (Membranous beta-catenin, 61%)	Minority (Nuclear beta-catenin, 39%)	P-value
Younger age of onset of CRC (53.7 years)	Older age of onset of CRC (58.6 years)	Not given
Dominance of right-sided CRC	Dominance of left-sided CRC	Not given
Microsatellite- and chromosomal stable (MSS/CIN-)	Microsatellite stable and chromosomal unstable (MSS/CIN+)	0.005
Infrequent p53 mutations	p53 mutations (p = 0.011) are associated with various 18q losses	Not given

Table 8. Molecular genetic analysis of MMR mutation negative tumours [16].

—	MMR Mutation Negative Tumours	%
MSS	52 out of 52	100 %
***APC* mutation**	10 out of 52	19.2 %
Nuclear beta-catenin	16 out of 44	36 %
Membranous beta-catenin	28 out of 44	63 %
***APC* mutation**	9 out of 16 (of tumours with nuclear beta-catenin)	56 % (of tumours with nuclear beta-catenin)

Table 9. Colorectal cancer susceptibility genes/loci.

Location	Suggested Genes in or Near the Loci/variant	References	Method	Material	Hypothesis/conclusion
1p36.13	*PLA2G2A*	[41]	Genotyping (*PLA2G2A* specific), sequencing and LOH (loss of heterozygozity) analysis	Sporadic CRC and FAP cases	*PLA2G2A* mutation[1] identified in one CRC patient
1	*EXO1*	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 1 associated with CRC in colon/breast cancer[3] oligopolyposis[4] and multiple cancer[5] cases
1q41	*DUSP10*	[21]	Meta-analysis of 3 GWAS	CRC cases	Common low risk variants[6] at 1q41 associated with CRC
2p25.1	*ODC1*	[43]	Genotyping, self-administered questionnaires and cell line experiments	Sporadic CRC cases	Homozygous for the A-allele[7] and use of aspirin associated with decreased risk for adenoma recurrence[8]
3p24.1	*TGFBR2*	[44]	PCR (polymerase chain reaction) and sequencing (*TGFBR2* specific)	MSS CRC cases	*TGFBR2* mutation identified in one hereditary MSS CRC patient[9]
3q29	*MFI2*	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 3 associated with CRC in oligopolyposis cases[10]
3q26.2	*MYNN*	[21]	Meta-analysis of 3 GWAS	CRC cases	Common low risk variant[11] associated with *decreased* CRC risk
4	-	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 4 associated with CRC[12]

(Table 9) contd….

Location	Suggested Genes in or Near the Loci/variant	References	Method	Material	Hypothesis/conclusion
4q31.3	*TLR2*	[45]	Genotyping/allele frequency (TLR2/TLR4 specific)	Sporadic CRC cases	Short-sized and long-sized[13] *TLR2* alleles[14] associated with CRC
5	-	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 5 associated with CRC in multiple cancer cases[15]
6p12.3	*PKHD1*	[46]	Genotyping (T36M *PKHD1* mutation specific)	CRC cases	T35M *PKHD1* mutation protects against CRC
7	-	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 7 associated with CRC[16]
8q23.3	*EIF3H*	[47]	GWAS	CRC cases[17]	Common low risk variant[18] associated with CRC[19]
8q24	*POU5F1P1*[20] DQ515897[21]	[48]	GWAS	CRC cases[22]	Common low risk variant[23] associated with CRC
	POU5F1P1[20] *MYC*	[49]	Genotyping (8q24 specific)	CRC cases (multinational)	Common low risk variants[24] associated with CRC
	DQ515897[21] DQ486513[21] CB104826[25] *POU5F1P1*[20] *POU5F1*[20] *MYC*	[50]	GWAS (confirmed by sequencing)	Familial colorectal tumour cases (replication study: CRC cases)	Common low risk variant[26] associated with colorectal adenomas and cancer
	DQ515897[21] DQ486513[21] *MYC*	[51]	GWAS	CRC cases (multinational)	Common low risk variant[27] associated with CRC
	-	[47]	GWAS	CRC cases[17]	Common low risk variant[28] associated with CRC
9q22.33	*TGFBR1* *PTCH* *XPA*	[52]	Linkage analysis (9q specific) and LOH analysis	Family with hereditary CRC	Autosomal dominant CRC linkage to 9q22.32-31.2
	SYK *PTCH* *XPA*	[53]	Linkage analysis	Familial cases with CRC and/or advanced adenomas	Autosomal dominant CRC/advanced adenoma linkage to 9q22.2-31.2
	TGFBR1	[54]	Genotyping (for germline allele-specific expression (ASE) of *TGFBR1*)	MSS CRC cases	Autosomal dominant ASE of *TGFBR1* associated with MSS CRC
	GALNT12	[55]	Sequencing (of *GALNT12* specific)	MSS colon cancer cell lines and CRC cases	Uncommon germline and somatic *GALNT12* variants associated with late onset CRC[29]
9q33.1	*TLR4*	[45]	Genotyping/allele frequency (TLR2/TLR4 specific)	Sporadic CRC cases	*TLR4* mutation[30] associated with CRC
10p14	-	[47]	GWAS	CRC cases[17]	Common low risk variant[31] associated with *decreased* CRC risk[32]

(Table 9) contd….

Location	Suggested Genes in or Near the Loci/variant	References	Method	Material	Hypothesis/conclusion
11q13.3	*CCND1*	[56]	Genotyping/allele frequency (*CCND1* specific)	CRC cases (multiethnic population)	*CCND1* 870A allele[33] associated with CRC[34]
		[57]	Genotyping/allele frequency (*CCND1* specific)	CRC cases (<60 years old)	*CCND1* 870A allele[33] associated with CRC in a recessive disease model
11q23	*LOC120376 (COLCA2) FLJ45803 (COLCA1) C11orf53 POU2AF1*	[48]	GWAS	CRC cases[22]	Common low risk variant[35] associated with CRC[36]
12	-	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 12 associated with CRC[37]
12q13.13	*LARP4 DIP2B ATF1*	[21]	Meta-analysis of 3 GWAS	CRC cases	Common low risk variants associated with increased[38] and *decreased*[39] CRC risk
12q24.33	*POLE*	[58]	Whole genome sequencing, linkage and association analysis	Large families with CRC/multiple adenomas[40]	Dominantly inherited, high penetrance variant[41] associated with MSS adenomas/CRC[42]
13q13.1	*BRCA2*	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 13 associated with CRC in breast/colon cancer cases[43]
13q31	*KLF5 KLF12 LMO7 C13orf7 - (RNF219) SPRY2 GPC5 MYCBP2 POU4F1*	[59]	Linkage analysis, LOH analysis sequencing.	Large family with hereditary CRC	Locus on chromosome 13q22.1-13q31.3 associated with CRC and adenomatous polyps in an autosomal dominant disease model[44]
14q22.2	*BMP4*	[20]	Meta-analysis of 2 GWAS	CRC cases	Common low risk variant[45] associated with increased CRC risk[46]
14q32.12	*GOLGA5*	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 14 associated with CRC in oligopolyposis cases[47]
15q	*GREM1(part of CRAC1) SCG5 (part of CRAC1)*	[60]	Genotyping (*CRAC1 specific*)	Familial/early onset CRC cases[48]	Common low risk variant[49] associated with CRC
	CRAC1	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Locus at chromosome 15 associated with CRC in oligopolyposis[50] and young CRC onset[51] cases
	FMN1	[61]	GWAS and linkage analysis	Prostate cancer families with colon cancer	Linkage to cancer at 15q11-14 in families with both prostate and colon cancer
	CRAC1	[47]	GWAS	CRC cases[17]	Common low risk variant[52] associated with CRC
16	-	[42]	Genotyping	Sib pairs with CRC (divided in subgroups)[2]	Loci at chromosome 16 associated with CRC in oligopolyposis cases[53] and CRC in all groups[54]

(Table 9) contd….

Location	Suggested Genes in or Near the Loci/variant	References	Method	Material	Hypothesis/conclusion
17p13.3	*HIC1*	[42]	Genotyping	Siblings with CRC (divided in subgroups)[2]	Locus at chromosome 17 associated with CRC in breast/colon cancer cases[55]
16q22.1	*CDH1*	[20]	Meta-analysis of 2 GWAS	CRC cases	Common low risk variant[56] associated with *decreased* CRC risk
18q21.1	*SMAD7*	[48]	GWAS	CRC cases[22]	Common low risk variant[57] associated with CRC
		[47]	GWAS	CRC cases[17]	Common low risk variants[58] associated with CRC
		[62]	GWAS supported by sequencing	Familial colorectal tumour cases	Common low risk variants[59] associated with CRC
19q13.33	*RHPN2*	[20]	Meta-analysis of 2 GWAS	CRC cases	Common low risk variant[60] associated with *decreased* CRC risk[32]
	POLD1	[58]	Whole genome sequencing, linkage and association analysis	Large families with CRC/multiple adenomas[40]	Dominantly inherited, high penetrance variant[61] associated with MSS, CIN+ adenomas/CRC's[62]
20p12.3	*BMP2*	[20]	Meta-analysis of 2 GWAS	CRC cases	Common low risk variants[63] associated with increased CRC risk[32]
20q13.33	*LAMA5*	[21]	Meta-analysis of 3 GWAS	CRC cases	Common low risk variant[64] associated with *decreased* CRC risk
21	-	[42]	Genotyping	Siblings with CRC (divided in subgroups)[2]	Two loci at chromosome 21 associated with CRC in breast/colon cancer cases[65]
22q12.1	*CHEK2* +	[63]	Allele-specific oligo-hybridization assay (for *CHEK2* mutation)	Hereditary CRC cases[66]	Low-penetrance variant[67] associated with CRC (in families with breast and colorectal cancer)
		[64]	Restriction fragment length polymorphism (for *CHEK2* – I157T variant)	CRC cases[68]	Risk allele (Il57T) for both familial and sporadic CRC and for multiple cancer types

[1] Deletion at genomic position 11119 (codon 48) in exon 3; [2] Subgroups: 1) young age of onset, 2) breast and colon cancer, 3) multiple colorectal adenomas (oligopolyposis), 4) multiple cancers, 5) severe histopathology; [3] Flanked by polymorphic dinucleotide repeat markers: D1S1588 and D1S534; [4] Flanked by polymorphic dinucleotide repeat markers: D1S549 and D1S1609; [5] Polymorphic dinucleotide repeat marker: D1S1665; [6] SNP: rs6687758 and rs6691170; [7] SNP in Intron 1 +A316G of *ODC1*; [8] The two risk factors acting independently; [9] In a HNPCC-like family; [10] Flanked by polymorphic dinucleotide repeat markers: D3S2427 and D3S1311; [11] SNP: rs10936599; [12] Polymorphic dinucleotide repeat marker: D4S2366; [13] Short sized <18 GT repeats and long sized 19-25 GT repeats; [14] 100 basepair upstream of the *TLR2* translational start site in intron 2; [15] Flanked by polymorphic dinucleotide repeat markers: D5S2500 and D5S1725; [16] Polymorphic dinucleotide repeat marker: D7S3070; [17] Phase 1: familial colorectal tumour cases, phase 2-4: CRC cases; [18] SNP: rs16892766; [19] In a dose-dependent manner (effect significantly stronger in younger cases); [20] Pseudogene; [21] mRNA, corresponding to DNA sequence located at 8q24.21; [22] Phase 1: early onset Scottish CRC cases, phase 2: Scottish CRC cases, phase 3: multinational CRC cases; [23] SNP: rs7014346; [24] SNPs: rs6983267, rs10808556 and rs7013278; [25] Non-coding RNA corresponding to DNA sequence located at 8q24.21; [26] SNP: rs6983267; [27] SNPs: rs10505477 and rs6983267; [28] SNP: rs6983267, identified in phase 1; [29] Germline *GALNT12* mutations: M1l (start codon) ATG>ATA, T491M, R297W, Y395X, R373H and R382H, somatic *GALNT1*2 mutations: C479F and E341D; [30] Asp299Gly; [31] SNP: rs10795668; [32] In a dose dependent manner; [33] Codon 242; [34] With gene-dosage effect, association stronger for advanced stage disease and for rectal cancer; [35] SNP: rs3802842; [36] Greater risk for rectal than colon cancer and significantly differences in risk observed among European and Japanese cases; [37] No marker given; [38] SNP: rs7136702; [39] SNP: rs11169552; [40] Validated in cases with familial CRC/multiple adenomas and early onset; [41] POLE l424V; [42] Multiple or very large adenoma/CRC phenotype or early onset CRC's; [43] Marker D12S1493; [44] Gain of chromosome 13q identified in selected cases; [45] SNP: rs4444235; [46] In a dose-dependent manner supporting a multiplicative model, association significantly stronger in MSS tumours compared to MSI; [47] Flanked by polymorphic dinucleotide repeat markers c14S1937 and D14S1436; [48] Stage 1: CRC cases selected for family history and/or early onset CRC, stage 2-3: CRC cases; [49] SNPs: rs4779584 and rs10318; [50] Flanked by polymorphic dinucleotide repeat markers D15S165 and D15S1012; [51] Polymorphic dinucleotide repeat marker D15S165; [52] SNP rs4779584, identified in phase 1 and 2; [53] Flanked by polymorphic dinucleotide repeat markers D16S540 and D16S539; [54] Polymorphic dinucleotide repeat marker D16S3019 and an unnamed marker; [55] Polymorphic dinucleotide repeat marker: D17S1308; [56] SNP: rs9929218, some evidence for association with gender - more common in females than males; [57] SNP: rs4939827, greater risk for rectal than colon cancer (no heterozygosity observed across study populations); [58] SNPs: rs4939827, rs12953717 and rs4464148, identified in phase 1, familial CRC cases; [59] SNPs: rs4939827, rs12953717 and rs4464148; [60] SNP: rs10411210; [61] S478N; [62] Multiple or very large adenoma/CRC phenotype or early onset CRC's, also predisposition to endometrial cancer and perhaps brain tumours; [63] SNP: rs961253; [64] SNP: rs4925386; [65] One locus flanked polymorphic dinucleotide repeat markers D21S1432 and D21S1440 and one at polymorphic dinucleotide repeat marker D21S446; [66] FAP, HNPCC-like and breast cancer cases; [67] 110delC; [68] Sporadic and familial, Finnish population

2) Clustering of low/moderate-risk genes in a family [12].

3) Known or unknown high-risk genes in a family [12]

It has been calculated that there is a 25% chance of meeting the AC-I by coincidence in FCCTX families where only one of them is affected before the age 50, indicating that the AC does not necessarily select families with an autosomal dominant disease. Therefore, the clinical diagnosis cannot stand alone, but must be supported by other clinical, pathological and molecular characteristics [66].

Ku *et al.* has pointed out that certain characteristics, such as later CRC onset and lower penetrance of CRC in FCCTX families, compared to LS, indicate that FCCTX might have a polygenic component. This polygenetic component may involve multiple low-penetrance variants interacting with non-genetic factors, such as shared lifestyle and environmental factors [67]. This hypothesis is supported by the molecular genetic similarities observed between FCCTX and sporadic CRC, such as similarities in gene expression and a high rate of gains and losses.

FCCTX, therefore, cannot clearly be determined as either a monogenic or polygenic disease, and might consist of cases in both groups, which complicates choosing the optimal approach for studying the genetic predisposition in these families.

5.2. Linkage Analysis

Genome-wide linkage analyses in combination with positional cloning and sequencing, has led to the identification of causal genes in monogenic diseases following the classical Mendelian inheritance patterns (autosomal dominant, autosomal recessive and X-linked). For example, *MLH1* and *MSH2* mutations involved in LS were identified by this method [68, 69]. With the use of linkage analysis, it is possible to detect rare variants in large families or collections of smaller families. The method is based on the observation that genes, which are localised close to each other on a chromosome, are inherited together in the meiosis (they are in linkage disequilibrium). By using DNA polymorphisms (markers) to determine the haplotype and recombination frequency in family members, the locus of the disease-causing gene can be identified. The mathematical tool LOD score (the logarithm to odds) is used as a statistic estimate of the likelihood that two loci are linked. LOD Score = log (the likelihood of the observed data, if loci are linked with the distance θ/the likelihood of the observed data if loci are not linked. A LOD score >3 indicates that the loci are significantly linked with odds 1:1000 for the observed data being a coincidence, while a LOD score <-2 indicates that it is highly unlikely that there is a link between loci. In complex, heterogenic diseases, where the genetic model for inheritance cannot be determined, it is not possible to calculate correct LOD scores. Misdiagnoses and frequent phenocopies also give misleading results in linkage analyses [70-72].

5.3. Linkage Analysis and FCCTX

Warden *et al.* points out, that the suggested genetic heterogeneity in FCCTX will weaken the strength of linkage analyses [22]. As an example, it was not possible for Skoglund *et al.* to identify loci/genes in a linkage analysis of 11 families with hereditary CRC, but when linkage analysis was restricted to a single large Swedish family, a LOD score of 2.3 was achieved on chromosome 9q22.32-31.1, an area containing several putative candidate genes (see Table **9**) [52]. This indicates that subdivision of families is needed in order to increase genetic homogeneity, but it also requires that large families are available in order to have enough cases. Two other linkage analyses have confirmed familial CRC linkage to the locus at 9q22, all in MSI negative cases, making this an interesting locus for FCCTX [42, 53, 73]. Other interesting loci for FCCTX, identified by a combination of linkage analysis, association studies and sequencing, are 12q24.33 encompassing POLE, and 19q13.33 encompassing POLD1. They are both associated with familial CRC with early onset of disease [58].

Furthermore, FCCTX cases could be subdivided based on molecular genetics, as this will increase genetic homogeneity. Subdivision could, for example, be based on CIN status or beta-catenin placement, because different molecular pathways seem to be involved in tumour genesis in the subgroups, and therefore, most likely, is also the genetic cause of the disease.

5.4. Sequencing

NGS has replaced classical approaches, such as Sanger-sequencing. DNA sequences of interest are captured and sequenced in millions of parallel reactions, and afterwards sequences (reads) are analysed. Identifying causative mutations requires comprehensive bioinformatics work, comparing sequences to published reference sequences, and extracting putative, causative variants [74]. In the filtering process, causal mutations may be discarded, for example by focusing on nonsense variations, if the causative mutation is a low/moderate penetrance missense variation, which might be important in diseases with reduced penetrance [75] such as FCCTX. The 1000 Genomes Project, a project that developed a catalogue of variants in the human genome, is a helpful reference tool in NGS bioinformatics, and the power of NGS is thought to improve as more variants are added to the catalogue and more populations are sequenced [76].

By targeted capture of DNA, carefully selected pieces of DNA can be analysed, such as single genes or the 1-2% of the genome constituted by exomes (exome sequencing). One could also extend and explore the whole genome with whole genome sequencing (WGS). It has been demonstrated that causative gene mutations in Mendelian diseases can be detected by exome sequencing, even in a small number of unrelated individuals sharing a monogenic disease. Extending to WGS enables the identification of functional non-coding variations in introns [74, 76], but the method is still comprehensive and expensive. Exome sequencing is also still relatively expensive, which is one of the limitations in using this method routinely in larger cohorts [77]. Since only a few mutations have been detected in FCCTX, explaining only a few cases, there is a possibility that the causative mutation(s) is/are to be found in introns.

5.5. Targeted Sequencing in FCCTX

Also in sequencing, genetic heterogeneity is a challenge [74]. Ku *et al.* therefore point out the need to select extreme

cases, such as those with a very young age of onset or a severe phenotype, to increase the monogenic component in FCCTX cases. Results from such studies can then be prioritized in further studies with larger sample sizes [67]. Other challenges in sequencing are the detection of large-scale structural variants, including segmentally duplicated regions, inversions and copy-number variants (CNV's) [74, 76].

Another cost-effective approach suggested by Ku *et al.* is targeted sequencing of genes/loci identified in linkage analyses or GWAS, or sequencing of causal genes in other familial cancers [67]. For example, *PALB2* germline mutations are implicated in both familial pancreatic cancer and breast cancer [78].

Guda *et al.* performed targeted sequencing of the *GALNT12* coding exons in 30 MSS colon cancer cell lines, and 2 somatic mutations were detected that inactivated the enzyme activity of *GALNT12* proteins. When extending to 272 colon cancer cases, 6 inactivating germline mutations were detected. None of the mutations were identified in controls, which indicates that rare *GALNT12* germline variants are associated with colon cancer development [55].

Based on these results, Segui *et al.* sequenced *GALNT12* coding exons in 103 FCCTX cases. No evidence was found for a high-penetrance function of *GALNT12* mutations in FCCTX, but since other studies have provided strong evidence for the linkage of 9q22-31 to familial CRC, Segui *et al.* do not exclude *GALNT12* as a moderate/low susceptibility gene in CRC, or that other genes under the linkage peak might be relevant in CRC susceptibility [79].

5.6. Mutations in Genes Identified in FCCTX Families

Using a combination of linkage analysis, exome sequencing, tumour studies and functional investigations, Nieminen *et al.* recently identified a truncating germline mutation in *RPS20* in a FCCTX family comprising of 4 generations. The alteration showed full co-segregation with MSS CRC (LOD score 3.0), and were absent in healthy controls (0/584). However, when screening blood DNA from 25 other FCCTX families, and tumour DNA from 61 primary CRC´s and cancer cell lines, no *RPS20* mutations were detected [80], indicating that *RPS20* mutations can only explain CRC in a small fraction of FCCTX families. The aforementioned family was identified in a previous study of 18 FCCTX families, where linkage analysis and sequencing led to the identification of *BMPR1A* mutations in two individuals from two different FCCTX families. *BMPR1A* mutations account for 20% of families with juvenile polyposis syndrome and 50% of families with hereditary mixed polyposis syndrome, but none of the patients from the two FCCTX families had diagnostic polyposis [81]. Therefore, screening for mutations in both *RPS20* and *BMPR1A* should be taken into consideration when screening FCCTX families. Schulz *et al.* recently identified a germline mutation in *SEMA4A*[2] in a large FCCTX family, by using a combination of linkage analysis and whole exome sequencing. The mutation was inherited in an autosomal dominant mode with incomplete penetrance, which indicates that other genetic, environmental or lifestyle modifiers are necessary to induce CRC. Furthermore, gains on 1q22, involving the *SEMA4A* locus, were observed in 2/3 of the CRC's from patients with the *SEMA4A* germline mutation, which lead the authors to the hypothesis that tumour suppressor inactivation of *SEMA4A* could be a consequence of loss of the *SEMA4A* wildtype allele combined with amplification of the mutant allele. The group then screened 53 unrelated FCCTX cases for *SEMA4A* germline mutations[3]. Two *SEMA4A* mutations and one SNP[4] were identified, with the SNP being significantly associated with FCCTX in a genetic association study, including 1,138 control cases [82].

5.7. Genome-wide Association Studies

GWAS have gained ground in genetics throughout the last couple of years. The method has a case-control design and is non-hypothesis driven (*i.e.* investigation is made without focus on a particularly locus/gene). It is based on the *'common-disease, common variant'* model, where the cause of the disease is attributed to a few or more predisposing risk-alleles with a relatively high frequency. The prevalence of the genetic markers is compared in affected and unaffected (control) individuals, and markers cover both coding and non-coding regions [83].

Several loci with association to CRC have been identified by GWAS (Table **9**). An interesting example is a meta-analysis performed by Houlston *et al.*, based on familial colorectal neoplasia cases in Great Britain. 4 novel loci associated with CRC were identified, all in close proximity to genes implicated in CRC development; 20p13.2 (*BMP2),* 14q22.2 (*BMP4)*, 16q22.1 (*CDH1)* and 19q31.1 (*RHPN2)*. *BMP2* and *BMP4* are both part of the transforming growth factor-beta (TGF-beta) family, that are signalling stem cells in the intestines through suppression of the Wnt/beta-catenin signalling pathway. *CDH1* is also involved in the Wnt/beta-catenin signalling pathway, while *RHPN2* is involved in the actin cytoskeleton and in cell motility, which can promote cancer invasiveness through adherence junction formation [20].

5.8. GWAS and FCCTX

A great challenge in GWAS is the need for stringent p-value thresholds, to ensure that the associations identified are not coincidental, and in order to achieve statistical significance, large cohorts are needed [21]. A relatively low number of CRC cases are FCCTX (HNPCCC accounts for only 5-10% of all CRC cases, and about 40-50% of these are FCCTX) [3, 4]. Very large international databases are therefore needed, in order to collect enough cases to meet the stringent thresholds. As a result, Ku *et al.* have suggested that results from GWAS, that have identified loci associated with CRC in general, should be used in targeted investigation in FCCTX families. This approach is cost-effective, and requires fewer cases to achieve statistical significance, because less SNP's are investigated. Based on the hypothesis that FCCTX is a polygenic disease, it is very likely that SNP's associated with CRC in multiple GWAS are also associated with CRC in FCCTX cases, and if this is the case, further studies can be performed in larger international GWAS of FCCTX cases [67].

[2] P.Val78Met

[3] p.Gly484Ala and p.Ser326Phe

[4] p.Pro682Ser

Targeted association studies of SNP's identified in GWAS have been performed by Middeldorp *et al.* in two studies of familial and early onset CRC cases and in FCCTX cases respectively [37, 84]. Among 995 familial and/or early onset CRC cases and 1340 controls, 5 out of 6 SNP's identified from GWAS were associated with CRC. It was also demonstrated that early onset familial cases had an increased number of risk-alleles, suggesting that low-risk variants, indeed, cluster in CRC families [84]. To investigate whether these results could be replicated in FCCTX families, 7 large families (including 112 family members) were studied. When investigating 10 SNP's identified in GWAS, the allele-frequency of two SNPs (rs16892766 at 8q23.3 and rs12953717 at 18q21.2) were significantly associated with CRC. However, there was no correlation between the number of risk-alleles and CRC status, and it was concluded, that the low-risk variants identified are insufficient to account for the familial clustering of CRC in these families. Linkage analysis of individual families revealed no clear regions of linkage or suggestive linkage, in this study, but a locus at 3q21.3, with LOD score 1.49, was identified when analysing all seven families together. These results indicate that it is unlikely that a single high penetrance gene contributes to CRC in these families, and a model based on moderate-risk or multiple low-risk factors is more likely [37]. Further, it is not possible to achieve statistical significance for risk-alleles with a frequency between 5-0,5% (minor allele frequency, MAF), and the variants do not carry sufficiently large effect to be detected by linkage analyses [20, 83]. Loci containing low/moderate-risk variants also might contain rare variants with larger effect size [83], and both common and rare variants may impact on inheritance and penetrance [75]. Sequencing of genomic regions with common variants identified in GWAS might therefore reveal rare variants of importance. Another challenge in GWAS is that frequencies of heterozygosity in SNP's differ between populations. For example, Tenesa *et al.* observed significant differences in CRC risk between Scottish and Japanese cases in SNP's at 11q23 [48].

Studying diseases with reduced penetrance is difficult, because various factors impact on penetrance. It is not known whether the identified disease-causing mutation exhibits reduced penetrance acting on its own, is dependent on other variants acting together, or is a variant with small effect size, where the real causative variant is still unknown. The importance of low penetrance variants therefore cannot be excluded, simply because they cannot explain all of the heritability [75].

5.9. Copy Number Variants

Tumour profiling has revealed that gains and losses are characteristic of FCCTX, [15, 33, 35-37], but chromosomal aberrations in the germline have not been thoroughly examined. Whole genome genotyping arrays based on SNP's make it possible to detect such copy number variants (CNV's) (deletions or duplications) that can affect gene expression. A genome-wide screen for germline CNV's has been performed in 41 MSS, nonpolyposis familial and/or early onset CRC cases and novel rare CNV's were detected in 6 patients. These involved deletions in *MFHAS1*, *CDH18* and two microRNA genes (*hsa-mir-646 and has-mir-491*), as well as duplications in *BCR* and *GREM1*. None of the CNV's were identified in control databases. However, not all carriers of the identified CNV's were diagnosed with CRC, indicative of a moderate penetrance [85].

6. DIAGNOSES AND SURVEILLANCE IN FCCTX AND LS

It is recommended that all CRC's and all endometrial cancers (EC) should be tested for LS by immunohistochemical analysis of MMR proteins or tested for MSI. Tests should be accompanied by testing for *MLH1* methylation. In LS cases, a colonoscopy should be performed with a 3-year interval, beginning between the ages of 20-25 years of age, and, if CRC is observed, this interval should drop to between 1-2 years [14, 17]. FCCTX is associated with a later age of onset of CRC compared to LS, and a less stringent protocol has been suggested for this group. Here, it is suggested that colonoscopies are initiated 5-10 years prior to the age of the earliest CRC diagnosis and with an interval of 5 years [14]. However, clinicians follow surveillance programs with shorter intervals between colonoscopies than those mentioned above, in both LS and FCCTX cases [86]. Dove-Edwin *et al.* conducted a prospective cohort study to examine the incidence of advanced neoplasia during surveillance in 68 FCCTX cases and 29 LS cases. The risk for developing high-risk adenomas was equal in the two groups, but the risk for developing cancer in between colonoscopies was significantly lower in FCCTX ($p = 0.01$) [24]. On the other hand, studies have showed significantly better prognosis for LS compared to FCCTX, based on a five-year survival rate ($p = 0.01$, $p = 0.0001$) [23, 25]. Since FCCTX seems to be such a heterogeneous group, the risk for CRC most likely will vary between families, and surveillance programs should be conducted with consideration for the individual families' characteristics. Definitive recommendations regarding surveillance programs can be developed when the genetic background for FCCTX is revealed.

CONCLUSION

FCCTX fulfilling the AC appears to be a heterogeneous group that consists of cases caused by single high-penetrance genes, cases caused by multiple low-penetrance genes acting together, and sporadic CRC cases. Molecular genetic studies indicate certain similarities to sporadic CRC, such as a high rate of CIN+ and similarities in gene expression, and that FCCTX should be subdivided into subgroups, which could for instance, be based on beta-catenin. Subdivisions would increase genetic homogeneity, which could ease the gene identification process. Other strategies in genetic studies include selecting extreme cases, such as a very young age of onset, or severe phenotype, when sequencing for susceptibility loci identified in linkage analyses or GWAS. Low-penetrance variants acting together can be revealed by GWAS, but this requires large cohorts. Therefore, focused strategies, such as investigating SNP's associated with CRC in general, is cost-effective and should be conducted. NGS is challenging because of the large amount of bioinformatic work required, but as sequencing strategies improve and more variants are catalogued, exome sequencing and WGS have become promising tools in revealing the genetic backgrounds of FCCTX. In addition, WGS makes it possible to

examine the importance of variants in the introns. Finally, the role of regulatory sequences acting as tumour suppressors or oncogenes are still not fully clarified in CRC, and as more is known about their role in cancer development, it will be interesting to explore their involvement in FCCTX.

LIST OF ABBREVIATIONS

AC = Amsterdam Criteria
AC-I = Amsterdam Criteria I
AC-II = Amsterdam Criteria II
APC = Adenomatous Polyposis Coli
ASE = Allele-specific Expression
ASIP = Agouti Signaling Protein
ATF1 = Activating Transcription Factor 1
AXIN2 = Axis Inhibitor 2
BCR = Breakpoint Cluster Region Protein
BMP2 = Bone Morphogenetic Protein 2
BMP4 = Bone Morphogenetic Protein 4
BMPR1A = Bone Morphogenetic Protein Type IA
BRCA2 = Breast Cancer 2, Early Onset
BRAF = B-Raf Proto-oncogene, Serine/Threonine Kinase
C11orf53 = Chromosome 11 Open Reading Frame 53
CCND1 = Cyclin D1
CDH1 = Cadherin-1
CDH18 = Cadherin 18, Type 2
CDH26 = Cadherin 26
CHEK2 = Checkpoint Kinase 2
CI = Confidence Interval
CIN+/- = Chromosomal Instability Positive/Negative
CNV = Copy Number Variant
CNV = Copy Number Variant
cnLOH = Copy Neutral Loss of Heterozygosity
COLCA1 = Colorectal Cancer Associated 1 *(FLJ 45803)*
COLCA2 = Colorectal Cancer Associated 2 *(LOC 120376)*
CRAC1 = Colorectal Adenoma and Carcinoma 1
CRC = Colorectal Cancer
CTNNB1 = Catenin (cadherin-associated protein), beta 1
DIP2B = Disco-Interacting Protein 2 Homolog B (Drosophila)
DUSP10 = Dual Specificity Phosphatase 10
EC = Endometrial Cancer
EIF3H = Eukaryotic Translation Initiation Factor 3, Subunit H
EPCAM = Epithelial Cell Adhesion Molecule
EXO1 = Exonuclease 1
FAP = Familial Adenomatous Polyposis
FCCTX = Familial Colorectal Cancer Type X
FHS = Family History Score
FMN1 = Formin 1
GALNT12 = Polypeptide N-Acetylgalactosaminyltransferase 12
GNAS = Guanine Nucleotide Binding Protein (G Protein), Alpha Stimulating Activity Polypeptide 1
GOLGA5 = Golgin A5
GPC5 = Glypican 5
GREM1 = Gremlin 1
GWAS = Genome-wide Association Study/Studies
HIC1 = Hypermethylated In Cancer 1
HNPCC = Hereditary Non-Polyposis Colorectal Cancer
IHC = Immunohistochemistry
KLF12 = Kruppel-Like Factor 12
KLF5 = Kruppel-Like Factor 5 (intestinal)
LAMA5 = Laminin, Alpha 5
LARP4 = La Ribonucleoprotein Domain Family, Member 4
LMO7 = LIM Domain 7
LOD = Logarithm of Odds
LOH = Loss of Heterozygozity
LS = Lynch Syndrome
MAF = Minor Allele Frequency
MFHAS1 = Malignant Fibrous Histiocytoma Amplified Sequence 1
MFI2 = Melanoma-Associated Antigen p57
MLH1 = MuTL Homolog 1
MLPA = Multiplex Ligation-dependent Probe Amplification
MMR = Mismatch Repair
MSH2 = MutS Homolog 2
MSH3 = MutS Homolog 3
MSH6 = MutS Homolog 6
MSI = Microsatellite Instability
MSI-H = Microsatellite Instability High Grade
MSI-L = Microsatellite Instability Low Grade
MSS = Microsatellite Stable
MYC = V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
MYCBP2 = MYC Binding Protein 2, E3 Ubiquitin Protein Ligase
MYNN = Myoneurin
NDUFA9 = NADH Dehydrogenase (Ubiquinone) 1 Alpha Subcomplex 9
NGS = Next-Generation Sequencing
ODC1 = Ornithine Decarboxylase 1
OR = Odds Ratio

p53 = Tumor Protein p53
PALB2 = Partner and Localizer of BRCA1
PCR = Polymerase Chain Reaction
PKHD1 = Polycystic Kidney and Hepatic Disease 1
PLA2G2A = Phospholipase A2, Group IIA
PMS2 = Postmeiotic Segregation Increased 2 (S. cerevisiae)
POLD1 = Polymerase (DNA Directed), Delta 1, Catalytic Subunit
POLE = Polymerase (DNA Directed), Epsilon, Catalytic Subunit
POU2AF1 = POU Class 2 Associating Factor 1
POU4F1 = POU Class 4 Homeobox 1
POU5F1 = POU Class 5 Homeobox 1
POU5F1P1 = POU Class 5 Homeobox 1B
PTCH = Patched Homolog 1 (Drosophila)
RHPN2 = Rhophilin, Rho GTPase Binding Protein 2
RNF219 = Ring Finger Protein 219 (*(C13orf7,* Chromosome 13 Open Reading Frame 7)
RPS20 = Ribosomal Protein S20
RR = Risk Ratio
SCG5 = Secretogranin V (7B2 Protein)
SEMA4A = Semaphorin 4A
SMAD7 = SMAD Family Member 7 (Mothers Against Decapentaplegic Drosophila, Homolog Of, 7)
SPRY2 = Sprouty Homolog 2 (Drosophila)
SRC = V-Src Avian Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene Homolog
SYK = Spleen Tyrosine Kinase
TGF-beta = Transforming Growth Factor-Beta
TGFBR1 = Transforming Growth Factor, Beta Receptor I
TGFBR2 = Transforming Growth Factor, Beta Receptor II
TLR2 = Toll-Like Receptor 2
TLR4 = Toll-Like Receptor 4
TSG = Tumour Suppressor Gene
WGS = Whole Genome Sequencing
XPA = Xeroderma Pigmentosum, Complementation Group A

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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