F．健康危険情報

特記すべきことなし。

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H．知的財産権の出願・登録状況

（予定を含む。）

なし

厚生労働科学研究委託費（難治性疾患実用化研究事業）
平成26年度　委託業務成果報告書（業務項目）

視神経脊髄炎動物モデル作成によるテーラーメード治療の確立

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新たなNMO動物モデル作成に向けた準備

新規治療ターゲットの探索～腸管免疫を利用したNMOの治療法開発

研究要旨

NMOは重篤な視神経炎と脊髄炎を主徴とする中枢神経炎症性疾患であるが、日本をはじめとする東アジアで頻度が高く、本邦で病態を解明して独自の治療戦略を立てていく必要がある。近年の研究で食餌成分により腸内細菌叢・腸管免疫の変化を介して、全身の免疫系が影響を受けることが明らかとなっており、中枢神経炎症性疾患であるNMOへの応用を本研究で試みた。我々は発酵食品に含まれる酵母に着目し、中枢神経炎症性疾患のモデル動物EAEに好影響を及ぼす酵母を探索した。その結果Candida kefyrがEAEの症状を抑制することを発見した。さらに機序として、C. kefyrの経口摂取によって変化した腸内細菌叢が腸間膜リンパ節において制御性T細胞や制御性樹状細胞を増加させ、これらの炎症制御作用によるEAEの抑制が考えられた。また腸内細菌叢解析ではC. kefyr投与群でBacteroidesの減少が重要であることが示唆された。

A.　研究目的

食餌成分により腸内細菌叢・腸管免疫の変化を介して、全身の免疫系が影響を受けることが明らかとなっている。我が国では多発性硬化（MS）の患者数が急速に増加しており、食習慣の変化が一因と考えられる。我々はこれまで発酵食品中の乳酸菌がMSのモデル動物であるEAEの症状を制御性T細胞を誘導し改善することを報告した。特定の食餌成分の摂取によりMSのみならず類縁疾患の視神経脊髄炎（NMO）

の腸管免疫を介した予防・治療に繋がる可能性がある。そこでまず発酵食品に着目し、乳酸菌以外にも全身の免疫系に好影響を及ぼしうる他の成分の探索を行う。

B. 研究方法

発酵食品に含まれて腸管免疫に影響を及ぼしうる成分として、乳酸菌以外にこれまでほとんど研究されていなかった酵母が重要であると推測される。そこでC57BL/6マウスを用いた実験的自己免疫性脳脊髄炎（EAE）を利用して各種酵母の経口摂取による効果を解析した。18種の酵母は製品評価技術基盤機構（NITE）生物遺伝資源センター(NBRC)より購入した。1次スクリーニングとして18種の酵母の死菌を腸管粘膜下層細胞のマクロファージと反応させ、IL-10とTNF-αの産生能により4群に分けた。各群より2種を選択し、マウスに経口摂取させEAEを誘導し、それぞれの効果を解析した。JAK2阻害剤については炎症機転の関与が示唆されているG93ASODTgマウスに投与し、臨床症状、免疫組織染色、脊髄由来cDNAのRT-PCRなどにより効果を判定した。

NMO患者由来血清及び血球を外来で採取し、Sema4A測定や凍結保存を行った。

（倫理面への配慮）

動物実験に使用するマウスは苦痛を十分に軽減することに配慮する。本研究は大阪大学医学部動物実験委員会で承認されている。

C. 研究結果

マウスに投与実験を施行した8種の酵母の内、唯一Candida kefyrが症状抑制効果を示した。C. kefyr投与群ではrecall assayで炎症性サイトカインの産生低下が認められ、腸管explantではIL-6の産生低下が認められた。また腸間膜リンパ節におけるFoxp3陽性制御性T細胞、CD103陽性樹状細胞の増加を認めており、これらの作用によるEAEの抑制が考えられた。

腸内細菌叢解析ではC. kefyr投与群でBacteroides/Prevotella比の減少を認めた。さらにC.kefyr投与群の腸内細菌叢（糞便）を移入することでEAEが抑制された。

JAK2阻害剤はG93ASODTgマウス脊髄のiNOSの発現を減少させたが症状を改善させなかった。

D. 考察

C. kefyr投与群の腸内細菌叢（糞便）を移入することにより、EAEが抑制された結果から、C. kefyrの摂取によって変化した腸内細菌叢が腸管免疫を介して全身の免疫系に働きEAEを抑制したと考えられる。また腸内細菌叢の中でBacteroidesの減少が重要であることが推測された。C. kefyrはMSの類縁疾患であるNMOにも有効である可能性があり、NMOのモデル動物で確かめる必要がある。

E. 結論

食品やそれにともなう腸内細菌叢の変化は代謝産物を介し、EAEの症状を変化させうる。本研究結果より食習慣

の改善が多発性硬化症や視神経脊髄炎の予防・治療法となりうることが示唆された。

F. 健康危険情報

該当なし

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H. 知的財産権の出願・登録状況

1. 特許出願

なし

2. 実用新案登録

なし

Ⅲ．学会等発表実績

熊ノ郷　淳（大阪大学　医学系研究科　呼吸器・免疫アレルギー内科学）

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
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セマフォリンによる免疫制御—ミトコンドリアDNAの病的意義の話題も含めて	熊ノ郷淳	第４４回皮膚アレルギー・接触皮膚炎学会総会・学術大会	2014	国内
Immune regulation by semaphoris and their receptors.	Atsushi Kumanogoh	University Medical Center (UMC) Invited lecture	2014	国外
Investigation for the link between amino acid-Ragulator- mTOR axis and the polarization of M2 macrophages.	Astushi Kumanogoh, Tetsuya Kimura	日本免疫学会	2014	国内

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掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
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発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
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掲載した論文（発表題目）	発表者氏名	発表した場所 （学会誌・雑誌等名）	発表した時期	国内・外の別
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発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所 （学会等名）	発表した時期	国内・外の別
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2．学会誌・雑誌等における論文掲載

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2．学会誌・雑誌等における論文掲載

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神経難病における disease-modifying therapy などの最新情報	望月秀樹	第 2 回日本難病医療ネットワーク学会学術集会 ランチョンセミナー	2014	国内

2．学会誌・雑誌等における論文掲載

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Multiple or mixed cerebral microbleeds and dementia in patients with vascular risk factors.	Mochizuki H, Miwa K, Tanaka M, Okazaki S, Yagita Y, Sakaguchi M, Kitagawa K.	Neurology	2014	国外
Echogenicity of Medium-to-Large Carotid Plaques Predicts Future Vascular Events.	Mochizuki H, Tadokoro Y, Sakaguchi M, Yamagami H, Okazaki S, Furukado S, Matsumoto M, Miwa K, Yagita Y, Kitagawa K.	Cerebrovasc Dis	2014	国外
Association between carotid stenosis or lacunar infarction and incident dementia in patients with vascular risk factors.	Mochizuki H, Kitagawa K, Miwa K, Yagita Y, Okazaki S, Sakaguchi M.	Eur J Neurol	2015	国外
Association of interleukin-6 with the progression of carotid atherosclerosis: a 9-year follow-up study.	Mochizuki H, Okazaki S, Sakaguchi M, Miwa K, Furukado S, Yamagami H, Yagita Y, Kitagawa K.	Stroke	2014	国外

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1．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
食餌成分（酵母）の腸管免疫を介した多発性硬化症病態への影響	中辻裕司、高田和城、木下允、奥野龍禎、甲田亨、富田貴之、武井雅也、萩原幸一郎、佐古田三郎、望月秀樹	第55回日本神経学会学術大会	2014	国内
食餌成分（酵母）の腸管免疫を介した多発性硬化症病態への関与	中辻裕司、高田和城、木下允、奥野龍禎、甲田亨、富田貴之、武井雅也、萩原幸一郎、佐古田三郎、望月秀樹	第26回日本神経免疫学会	2014	国内
Intestinal microflora modified by Candida kefyr reduces the susceptibility to experimental autoimmune encephalomyelitis.	K Takata, T Tomita, T Koda, T Okuno, J Honorat, M Kinoshita, M Takei, K Hagihara, Y Nakatsuji, H Mochizuki, S Sakoda.	The 30th Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS)	2014	国外

2．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Dietary Yeasts Reduce Inflammation in Central Nerve System via Microflora.	Nakatsuji Y, Takata K, Tomita T, Okuno T, Kinoshita M, Koda T, Honorat JA, Takei M, Hagihara K, Sugimoto T, Mochizuki H, Sakoda S.	Ann Clin Transl Neurol	2015	国外
多発性硬化症と視神経脊髄炎検査・診断法 臨床検査（血液・脳脊髄液マーカー）	中辻裕司	日本臨床（日本臨床社）	2014	国内

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Germline variants in the *SEMA4A* gene predispose to familial colorectal cancer type X

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Familial colorectal cancer type X (FCCTX) is characterized by clinical features of hereditary non-polyposis colorectal cancer with a yet undefined genetic background. Here we identify the *SEMA4A* p.Val78Met germline mutation in an Austrian kindred with FCCTX, using an integrative genomics strategy. Compared with wild-type protein, SEMA4A^{V78M} demonstrates significantly increased MAPK/Erk and PI3K/Akt signalling as well as cell cycle progression of SEMA4A-deficient HCT-116 colorectal cancer cells. In a cohort of 53 patients with FCCTX, we depict two further *SEMA4A* mutations, p.Gly484Ala and p.Ser326Phe and the single-nucleotide polymorphism (SNP) p.Pro682Ser. This SNP is highly associated with the FCCTX phenotype exhibiting increased risk for colorectal cancer (OR 6.79, 95% CI 2.63 to 17.52). Our study shows previously unidentified germline variants in *SEMA4A* predisposing to FCCTX, which has implications for surveillance strategies of patients and their families.

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Colorectal cancer (CRC) is the third most common cancer worldwide[1]. Approximately 5% of cases are inherited in an autosomal dominant manner with familial adenomatous polyposis and hereditary non-polyposis colorectal cancer (HNPCC) being the two major hereditary forms[2,3]. HNPCC is clinically diagnosed when Amsterdam-I or -II criteria (AC-I/-II) are met: three or more relatives affected through at least two generations by CRC (AC-I) or an HNPCC-associated cancer (AC-II), respectively, with one patient being a first-degree relative of the other two and one diagnosed before the age of 50 years[4]. However, 40 to 50% of patients with HNPCC fulfilling AC-I lack detectable germline mutations in cancer predisposition genes and are classified as familial colorectal cancer type X (FCCTX)[5–7]. In contrast to Lynch syndrome (LS)—the HNPCC entity characterized by germline DNA mismatch repair (MMR) gene mutations and somatically acquired microsatellite instability—individuals with FCCTX exhibit decreased risk for extracolonic neoplasms, that is, endometrial, stomach, small bowel and urinary tract carcinomas and tumour formation including CRC development tends to occur at a later age[5,8,9]. It is expected that single uncommon susceptibility genes transmitted in an autosomal dominant manner are responsible for a subset of FCCTX cases, which in turn implies that this syndrome is likely to be heterogeneous[2,5,8]. Here we show that germline variants in the semaphorine 4A (*SEMA4A*) gene confer susceptibility to FCCTX. This finding broadens our understanding of the biology of those malignancies and forms the basis for effective cancer detection and prevention strategies.

Results

Pedigree analysis and variant identification. In the course of a previous study focusing on pedigree analysis of patients with therapy-related myeloid neoplasms[10,11], we have identified a large Austrian kindred with FCCTX (Family K, Fig. 1a; Supplementary Fig. 1). CRCs in this family were inherited in an autosomal dominant pattern with incomplete penetrance meeting AC-I. In each affected individual, one to six colorectal adenomas and one to two CRCs were diagnosed at a median age of 62.5 years (range, 44–72). The majority of colorectal neoplasms was located in the distal colon and rectum and showed tubular histological features without evidence for an increase of infiltrating lymphocytes (Table 1).

We conducted genetic linkage analysis (LA) of five family members with colorectal neoplasms and one unaffected, putative mutation carrier (Fig. 1a), which revealed four shared regions on chromosomes 1, 3, 10 and 20 (Supplementary Fig. 2), none of them harbouring known cancer-associated genes. We next performed whole-exome sequencing (WES) on four of these individuals (Fig. 1a). A heterozygous germline variant was identified in the *MUTYH* gene (NM_001128425.1:c.650G>A: p.Arg217His, rs147754007) in the first-degree relatives K13 and K18 but not in individuals K3 and K14 (Supplementary Fig. 3). We, therefore, excluded *MUTYH* R217H as a culprit germline mutation responsible for the majority of neoplasms in this family, which is in line with the fact that *MUTYH*-associated polyposis is an autosomal recessive CRC predisposition syndrome[12]. To identify novel candidate causative mutations, we combined LA and WES and filtered heterozygous, non-synonymous protein-coding or splice-site variants with a minor allele frequency of ≤ 0.01 (Supplementary Table 1). All variants were confirmed by Sanger sequencing and analysed in two further family members with CRC (K16 and K26). Only variant p.Val78Met (NM_001193300:c.232G>A) in the *SEMA4A* gene located on chromosome 1q22 was shared by all tested individuals. However, in this approach, we included two individuals with colorectal adenomas constituting a frequent but not obligate part of HNPCC syndromes[13]. As this might constitute a potential bias, we focused in an independent analysis on variants from WES shared by individuals with CRC (K13, K18) or with an offspring with CRC (K3). Of 24 variants identified (Supplementary Table 2), two were also present in individuals K16 and K26. We excluded the p.Val212Phe variant in *ZNF763* (rs7249379) due to non-conservation because Phe212 represents the common chimpanzee allele. Only *SEMA4A* V78M segregated with all CRC cases and was also detected in individuals K9 with testicular and K14 with breast cancer, respectively (Fig. 1a). Given a mean age of 61 years of individuals with FCCTX at disease onset[5], we estimated a phenocopy rate of 0.00 and a penetrance rate of 0.56 of the *SEMA4A* V78M variant in Family K. cDNA from peripheral blood (PB) leukocytes demonstrated expression of the mutant allele (Supplementary Fig. 4).

SEMA4A is a membrane-bound class 4 semaphorin receptor with organ-specific and immunomodulatory effects as well as growth regulatory functions[14–16]. V78M lies within the SEMA domain responsible for receptor binding and Val78 is well conserved (Fig. 2a; Supplementary Fig. 5). This variant is absent from dbSNP137, the 1000 Genomes Project database and the National Heart, Lung and Blood Institute Exome Variant Server (ESP6500). Prediction tools favour consequences for its protein function (SIFT score = 0, PolyPhen-2 score = 0.987, vertebrate Phylop100 score = 7.434, vertebrate PhastCons100 score = 1, phastConsElements100 score = 407 [LOD = 65] and MutationTaster 2 = disease causing with 0.95 probability value).

Recurrent somatic mutations in CRCs of *SEMA4A* V78M carriers. We then analysed CRC specimens of mutation carriers for copy-number alterations by array-based comparative genomic hybridization and loss of heterozygosity (LOH) by Sanger sequencing, respectively. Gains on the long arm of chromosome 1 involving the *SEMA4A* locus were observed in two of three CRCs together with a homozygous *SEMA4A* V78M status (Fig. 3). We did not detect copy-number alterations in the *MUTYH* gene in any of the three analysed CRCs including the heterozygous R217H carrier K13. We also analysed four available CRCs for recurrent, somatically acquired mutations in known CRC genes by targeted deep sequencing and identified mutations in *TP53* in 3/4, *APC* in 2/4, *KRAS* in 2/4 and *PIK3CA* in 1/4 CRC cases, respectively, as possible cooperating events (Table 2). Notably, there was no predominance of C:G to A:T transversion mutations in the CRC of patient K13 characteristic for complete loss of MUTYH activity[12].

SEMA4A^{V78M} affects proliferative pathways. Compound heterozygous germline mutations in *SEMA4A* have been reported in patients with retinal degenerative diseases and studies in knock-in mice showed that one of these mutations (F350C) leads to an abnormal Sema4A localization in retinal pigment epithelial cells[17,18]. A three-dimensional protein model of human SEMA4A predicts that Val78 has no spatial relationship to residues associated with retinal disorders (Fig. 2b). In agreement with this prediction and the family's history lacking apparent ocular manifestations, the expression of a fusion gene composed of Sema4A^{V78M} and carboxyl-terminal green fluorescent protein (GFP) in human retinal ARPE-19 cells showed normal GFP signal distribution (Fig. 4a).

SEMA4A is widely expressed including normal colonic tissue (Supplementary Fig. 6) but is undetectable in 2/4 CRC cell lines analysed (Supplementary Fig. 7). It has been shown to have inhibitory effects on proliferation and migration of endothelial cells by antagonizing vascular endothelial growth factor[16].

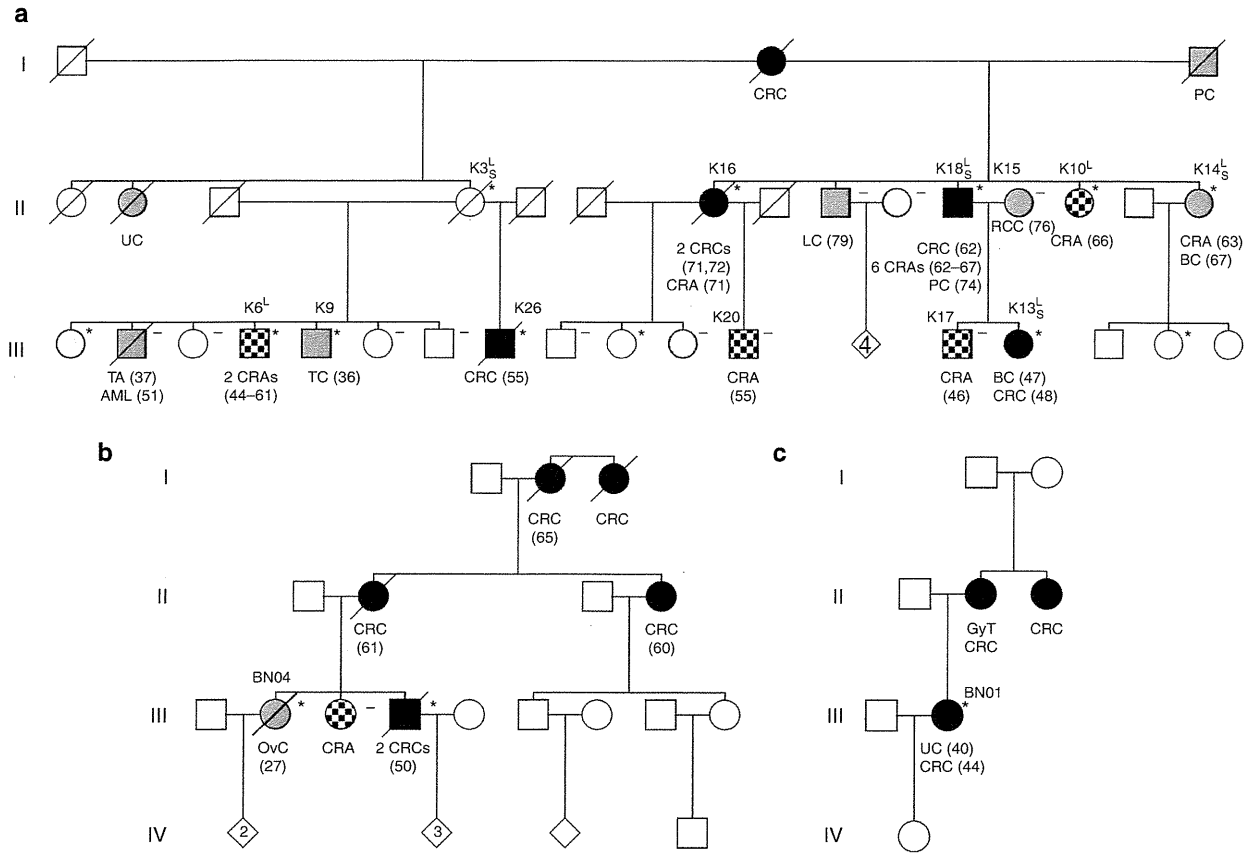

Figure 1 | Pedigrees of families with germline *SEMA4A* mutations. Families with V78M (**a**), G484A (**b**) and S326F (**c**) mutations are shown. L, individual included in LA; S, individual included in WES; asterisk, *SEMA4A* mutation carrier; minus, *SEMA4A* wild type; black symbol, CRC; checkered symbol, colorectal adenoma; dark grey, malignant neoplasm; light grey, benign neoplasm; number in symbol, number of unspecified offspring. AML, acute myeloid leukaemia; BC, breast cancer; CRA, colorectal adenoma; GyT, gynaecologic tumour; OvC, ovarian cancer; PC, prostate cancer; TA, thyroid adenoma; TC, testicular cancer; UC; uterine cancer; UT, uterine tumour. Results of mutational analyses are indicated in tested individuals only. Age at diagnosis (years) is given in parentheses. For multiple colorectal adenomas, age at first presentation or at screening colonoscopy is indicated. An extended pedigree of the family with the V78M mutation including age of the individuals is shown in Supplementary Fig. 1, histopathological characteristics of their colorectal neoplasms are summarized in Table 1.

We therefore analysed transiently transfected SEMA4A-deficient HCT-116 cells characterized by *KRAS* and *PIK3CA* mutations. We were unable to demonstrate significant differences between wild-type and mutant SEMA4A on migration (Supplementary Fig. 8). However, as compared with $SEMA4A^{wt}$, significantly more $SEMA4A^{V78M}$-transfected cells were in S phase under normal growth conditions (Fig. 4b,c). We then assessed activation of the phosphoinositide 3-kinase/Akt (PI3K/Akt), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) and Wnt/β-catenin pathways that have been shown to be important in colorectal carcinogenesis[19]. As compared with $SEMA4A^{wt}$, $SEMA4A^{V78M}$-transfected HCT-116 cells revealed significantly enhanced activation of the PI3K/Akt and MAPK/Erk pathways both mediating proliferation by increasing cells in S phase and accelerating G2/M transition (Fig. 4d,e; Supplementary Fig. 9)[20–22]. Transient transfection of 293T cells, however, showed no effect of SEMA4A on the PI3K/Akt pathway (Supplementary Fig. 10).

***SEMA4A* variants are associated with FCCTX.** To study the prevalence of *SEMA4A* germline mutations in FCCTX, we screened 53 unrelated FCCTX cases from Austria, Germany and the United States (Supplementary Table 3) and identified two further mutations located in the SEMA domain (heterozygous c.1451G4C, p.Gly484Ala, rs148744804; homozygous c.977C4T, p.Ser326Phe; Supplementary Fig. 11). These mutations affect highly conserved residues (Fig. 2a and Supplementary Figs 12 and 13) and prediction tools indicate an effect on protein function for both of them (Supplementary Table 4). The G484A variant has a global minor allele frequency of 0.001 in the 1000 Genomes Project and ESP6500 databases. It was also found in the index patient's brother affected with CRC (Fig. 1b; Supplementary Fig. 11). The novel S326F variant affects a residue predicted to be involved in homodimer formation (Fig. 2b; Supplementary Fig. 12). Furthermore, we detected the heterozygous single-nucleotide polymorphism (SNP) p.Pro682Ser (c.2044C>T, rs76381440) in six of 47 (13%) German and Austrian FCCTX patients, respectively (Supplementary Table 3; Supplementary Fig. 11). We, therefore, initiated a genetic association study using DNA from 1,138 Caucasian control subjects from Austria without a personal or family history of cancer. These specimens were collected previously during the course of a local health screening study[23]. The P682S SNP demonstrated a highly significant association with the FCCTX phenotype resulting in an increased risk for CRC (Table 3). Screening the 1000 Genomes Project data base revealed a comparable prevalence of heterozygotes among European individuals of 2.0%.

Table 1 | Clinical characteristics of colorectal neoplasms of Family K exhibiting the germline V78M *SEMA4A* mutation.

Patient	Neoplasm	Age (years)	Histology	Grading/staging	Localization	*SEMA4A* V78M
K6	CRA	44	Tubular adenoma	Well to moderately differentiated	NA	+
K6	CRA	61	Tubular adenoma	Well differentiated	Sigmoid colon	+
K10	CRA	66	Tubular adenoma	Well to moderately differentiated	NA	+
K13	CRC	48	Adenocarcinoma	pG-3, pT-4, pN-1	Coecum	+
K14	CRA	63	Tubular adenoma	Well differentiated	Rectum	+
K16	CRC	71	Tubulopapillary and mucinous adenocarcinoma	pG-2, pT-2, N-0	Coecum	+
K16	CRA	71	Tubulovillous adenoma	Well to moderately differentiated	Coecum	+
K16	CRC	72	Tubular adenocarcinoma	pG-2, pT-X	Descending/sigmoid colon	+
K17	CRA	46	Tubular adenoma	Well differentiated	Rectum	−
K18	CRC	62	Tubular adenocarcinoma	pG-2, pT-1, N-0	Sigmoid colon	+
K18	CRA	62	Tubular adenoma	Well to moderately differentiated	Sigmoid colon	+
K18	CRA	62	Tubular adenoma	Well to moderately differentiated	Sigmoid colon	+
K18	CRA	64	Tubular adenoma	Well to moderately differentiated	Ascending colon	+
K18	CRA	65	Tubular adenoma	Well to moderately differentiated	Descending colon	+
K18	CRA	66	Tubular adenoma	Well to moderately differentiated	NA	+
K18	CRA	67	Tubular adenoma	Well to moderately differentiated	Descending colon	+
K20	CRA	55	Tubulovillous adenoma	Well differentiated	Sigmoid colon	−
K26	CRC	55	Adenocarcinoma	pG-2, pT-3, pN-2	Rectum	+

CRA, colorectal adenoma; CRC, colorectal cancer; NA, not available.

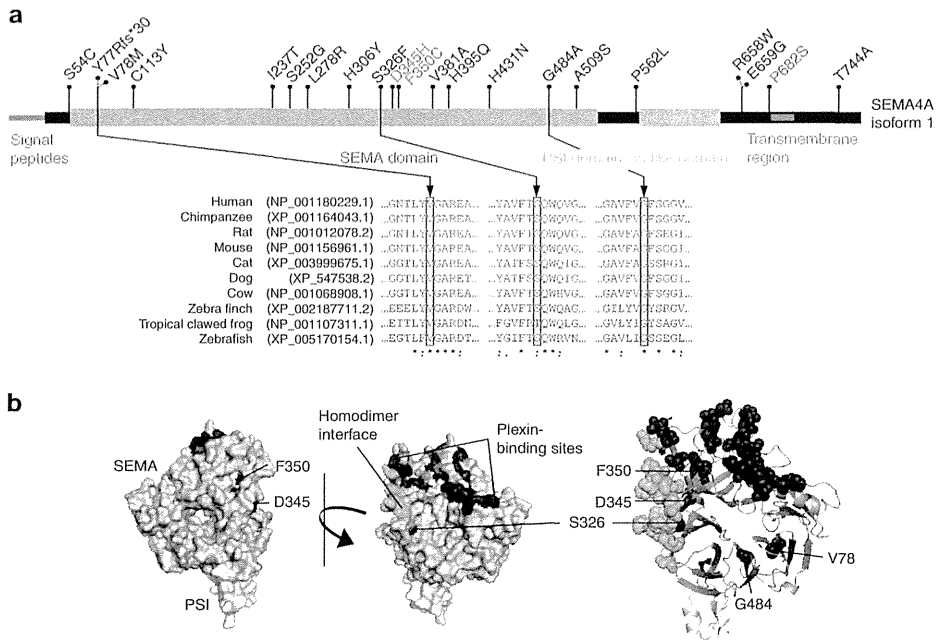

Figure 2 | Localization of germline and somatic CRC *SEMA4A* mutations at the protein level. (**a**) Germline mutations found in this study are illustrated in red, the SNP in orange, germline mutations associated with eye diseases in blue and somatic CRC mutations in black, respectively. Multiple sequence alignments of SEMA4As of selected species are shown below. Note that class 4 semaphorins can only be found in vertebrates. (**b**) SEMA and PSI domains (55-527, yellow) of human SEMA4A were modelled primarily to SEMA4D (1OLZ). Eye disease-associated residues D345 and F350 are located in the back of the protein below the plexin binding sites (magenta). V78 and G484 have no contact to the surface, are spatially distinct from D345 and F350 but are located in juxtaposition in β-propellers 1 and 7, respectively. S326 is part of the homodimer interface (cyan) having surface contact.

***SEMA4A* is somatically mutated in sporadic cancers.** Finally, we were interested whether somatically acquired *SEMA4A* mutations are prevalent in sporadic CRCs as well as other neoplasms. Analysis of confirmed mutations across different cancer types revealed that *SEMA4A* mutations occur in 2.7% (15/559) of colorectal, 2.8% (6/212) of stomach and 3.3% (8/241) of uterine cancers[24,25]. In 92% of them, they constitute missense mutations (Supplementary Table 5) scattered throughout the gene (Fig. 2a). Data from the cBioPortal for Cancer Genomics indicate that the *SEMA4A* gene is amplified in a wide range of different tumours and that deletions are only rarely seen (Supplementary Fig. 14).

Discussion

Semaphorins constitute a family of secretory or membrane-bound receptors, which were first described as regulators of neuronal axon growth[26]. They are characterized by an extracytoplasmic amino-terminal β-propeller—the SEMA

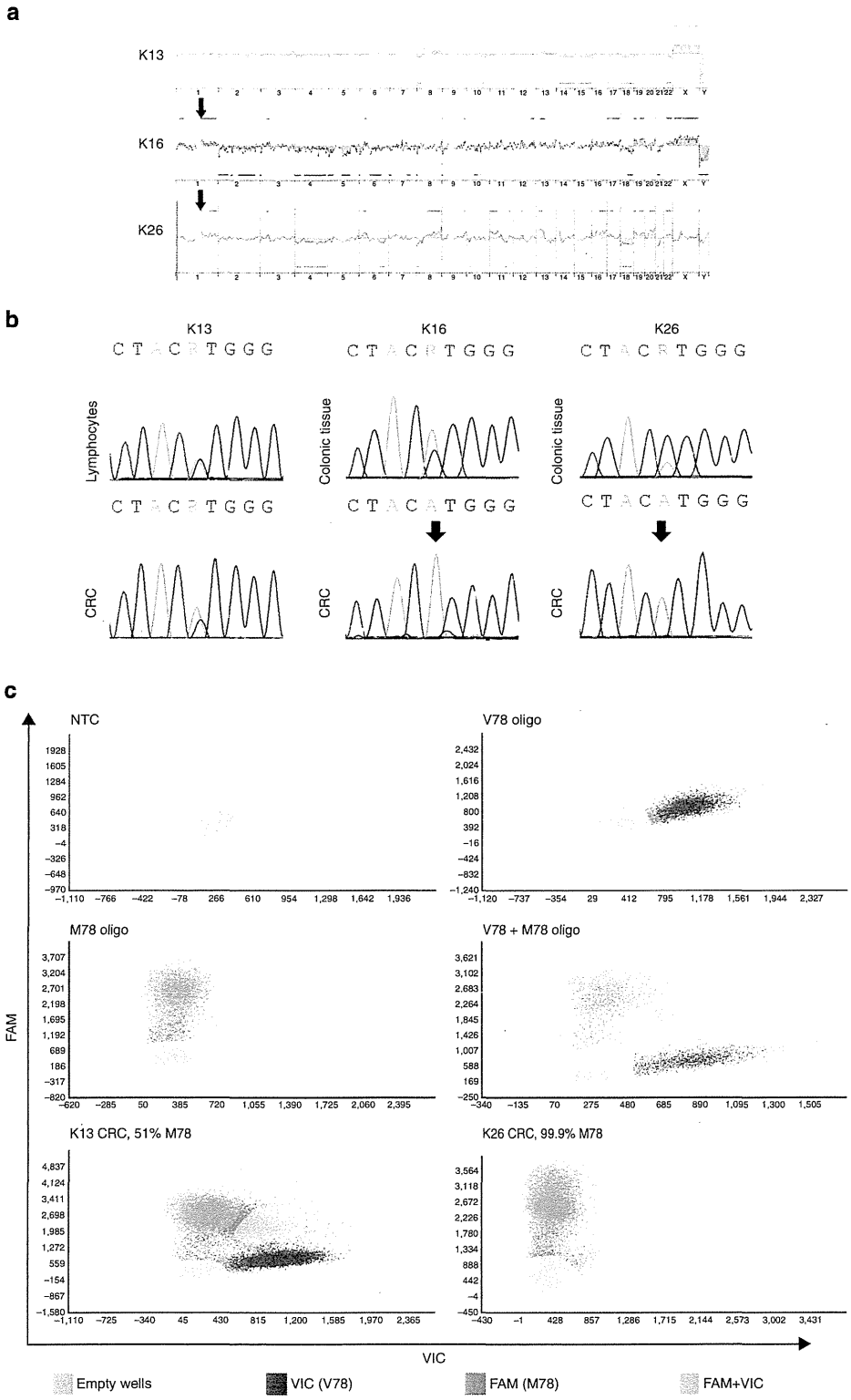

Figure 3 | LOH in CRCs of patients K16 and K26. (**a**) Array-based comparative genomic hybridization of three CRCs from Family K with germline *SEMA4A* V78M mutation. A gain in the *SEMA4A* locus is marked with an arrow. (**b**) Sanger sequencing. (**c**) Quantitative dPCR using fluorophore-coupled (VIC, FAM) TaqMan probes specific for wild-type (V78) or mutant (M78) *SEMA4A* nucleotide variants. Each dot represents a single well on a 20K chip. The performance of this assay was tested with specific oligonucleotide templates. The confidence level was set to 95% and the desired precision value was 10%. NTC, no template control.

Table 2 | Results of targeted deep sequencing of cancer hot spot regions in CRCs from Family K with the germline V78M *SEMA4A* mutation.

Patient	Somatic mutation	Protein alteration	dbSNP141
K13	*APC* NM_000038.5:c.2626C>T	p.R876X	rs121913333
K13	*APC* NM_000038.5:c.4348C>T	p.R1450X	rs121913332
K13	*KRAS* NM_004985.4:c.34_35delinsAT	p.G12I	NA
K13	*TP53* NM_000546.5:c.380C>A	p.S127Y	NA
K13	*PIK3CA* NM_006218.2:c.1633G>A	p.E545K	rs104886003
K16	None found	None found	—
K18	*TP53* NM_000546.5:c.844C>T	p.R282W	NA
K26	*APC* NM_000038.5:c.4135G>T	p.E1379X	rs121913326
K26	*KRAS* NM_004985.4:c.34G>A	p.G12S	NA
K26	*TP53* NM_000546.5:c.743G>A	p.R248Q	rs11540652

CRC, colorectal cancer; NA, not available.

domain—which is needed for plexin receptor binding[27,28]. In addition to their role in developmental and physiological processes, semaphorins and their receptors have increasingly been associated with neoplastic disorders (reviewed in refs 24,26). Interestingly, they have been found to act both, in an anti- and protumoral fashion depending on the particular semaphorin as well as the tumour context. Several tumorigenic properties are thereby influenced including cell proliferation, evasion of apoptosis, angiogenesis, oxidative stress regulation and metastasis. However, no particular semaphorin has been implicated in cancer susceptibility yet. Here we have shown for the first time that *SEMA4A* germline variants predispose to a hereditary neoplastic syndrome.

The germline *SEMA4A* V78M variant was inherited in an autosomal dominant fashion with incomplete penetrance in this family with FCCTX pinpointing additional genetic, environmental or life style modifiers necessary to establish the malignant phenotype. Individuals with this variant developed tumours at a higher age than classical LS patients[7], showed a moderate number of colorectal adenomas and had a propensity for extracolonic malignancies. Such a genetic modifier might be *MUTYH* where the heterozygous germline variant R217H was found in two *SEMA4A* V78M carriers with CRC (K13, K18). Biallelic germline *MUTYH* mutations—primarily Y179C and G396D—are the cause of *MUTYH*-associated polyposis, which is a rare autosomal recessive syndrome resembling familial adenomatous polyposis[12,29]. Monoallelic *MUTYH* germline variants are associated with a small increase in CRC risk; however, this assumption should be handled with care as different studies have come to inconsistent results[12,30,31]. The *MUTYH* variant R217H found in K13 and K18 has been previously described once in a cohort of 406 patients with more than five polyps and/or CRC from France but its predisposing role has not been established yet[32].

We were able to identify two further *SEMA4A* variants in a mutational screening of 53 FCCTX patients and studied the segregation of G484A, which followed a dominant inheritance pattern. In both pedigrees, variants were associated with extracolonic neoplasms—ovarian cancer in G484A and endometrial cancer in S326F. Homozygosity of the S326F genotype observed in the index patient could either be the result of an additional germline mutation unrelated to other familial cancer cases or may indicate an autosomal recessive mode of inheritance operational in this family. However, due to lack of DNAs from other family members, we were unable to resolve this issue.

The *SEMA4A* P682S SNP is associated with an increased risk of CRC in our association study including Austrian and German individuals. Although this finding has to be replicated in an independent cohort and might reveal ethnic differences, the data, nevertheless, suggest that P682S constitutes a risk allele for a small proportion of CRC cases probably missed by genome-wide association studies that detect mostly frequent, low penetrant susceptibility loci[2].

The compound heterozygous germline *SEMA4A* variants D345H and F350C have been described in patients with retinitis pigmentosa and cone rode dystrophy but until now this finding has not been replicated[17,33]. Sema4A-deficient mice exhibit photoreceptor degeneration and disturbed T-helper cell function but lack apparently increased tumour development[14,34]. Given the wide expression of *SEMA4A* in different tissues, it is plausible that mutations can have different effects depending on the respective tissue. In fact, only the F350C but not the D345H variant was able to recapitulate the retinal disease phenotype of Sema4A-deficient mice in a homozygous knock-in mouse model, a genotype not described in humans yet[18]. This observation stresses the special role of the F350 residue for photoreceptor function. The fact that these mice do not develop overt tumours does not necessarily argue against a potential tumour predisposing role. First, these animals have not been thoroughly investigated for tumour formation, and second, mutations in human cancer susceptibility gene homologues do not consistently result in increased carcinogenesis in mice. With respect to colorectal carcinogenesis, this has been clearly shown for the MMR gene *Pms2* as well as for *Smad4* predisposing to LS and juvenile polyposis syndrome, respectively[35,36]. For both conditions, additional germline truncating mutations in the gatekeeper gene *Apc* are needed for intestinal tumour development in mice.

SEMA4A variants found in this study were not restricted to a certain hot spot region indicating a loss-of-function mechanism[37]. This assumption is further supported by functional *in vitro* assays performed in the SEMA4A-deficient CRC cell line HCT-116. Whereas activation of mitogenic pathways like MAPK/Erk and PI3K/Akt within these cells could be diminished by transfection of a $SEMA4A^{WT}$ construct, expression of the $SEMA4A^{V78M}$ mutant failed to do so. Accordingly, re-expression of $SEMA4A^{WT}$ but not $SEMA4A^{V78M}$ inhibited G2/M-phase transition in HCT-116, again suggesting a loss-of-function of the V78M substitution. It has to be mentioned that the results of our copy-number analysis demonstrated a gain of chromosome 1q22; however, homozygosity of the V78M variant observed in two of the CRCs could nevertheless indicate that SEMA4A acts as a tumour suppressor rather than a proto-oncogene in the context of familial colorectal tumorigenesis. Middeldorp *et al.*[38] found that tumour specimens from patients with FCCTX frequently exhibit gains of different chromosomal regions including chromosome 1, which is accompanied by copy-neutral LOH. Loss of the *SEMA4A* wild-type allele accompanied by amplification of the mutant one might be one mechanism of tumour suppressor inactivation in this particular entity. Unfortunately, due to low-quality DNA obtained from formalin-fixed, paraffin-embedded (FFPE) tumour specimens as well as lack of appropriate heterozygous microsatellite loci within or adjacent to the *SEMA4A* gene, we were unable to prove the type of LOH in tumours of Family K. Whether public data indicating that the *SEMA4A* gene is predominantly amplified in diverse cancers can also be