SHORT REPORT

Abnormal vascular network complexity: a new phenotypic marker in hereditary non-polyposis colorectal cancer syndrome

C De Felice, G Latini, G Bianciardi, S Parrini, G M Fadda, M Marini, R N Laurini, R J Kopotic

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ereditary non-polyposis colorectal cancer (HNPCC)1–2 (Lynch cancer family syndrome I (LCFS1)) and II (LCFS2)) is a paradigmatic example of a dominantly inherited cancer syndrome. Germline mutations in five different mismatch repair (MMR) genes (MSH2, MSH6, MLH1, MLH3, and PMS2) are linked to HNPCC.4–6 Genomic deletions in MLH1 and MSH2 are believed to account for approximately 70–90% of causal mutations in HNPCC families;1,4 although over 400 different MMR gene mutations and approximately 100 intragenic polymorphic variations have been reported to date (International HNPCC mutation database, http://www.nfhdht.nl).4 HNPCC gene carriers have a high risk (60–85%) of developing colorectal cancer (CRC), and other cancer types, primarily endometrial cancer (30–50%), at an early age.4–7,8 With the exception of the cutaneous signs of cancer risk relevant to the Muir-Torre syndrome in a subset of LCFS2 patients,9–10 no distinguishing phenotypic stigmata of cancer susceptibility in HNPCC are available to date.11 In the present study, we tested the hypothesis that an increased vascular network complexity is a phenotypic marker for LCFS2.

METHODS

Subjects
Fourteen surviving members from a large Southern Italy LCFS2 kindred (gene carriers, n = 5; non-carriers, n = 9) and 30 controls were examined. Fractal dimension (D) at two scales (D(1–46), and D(1–15), tortuosity (minimum path dimension, Dmin), and relative Lempel-Ziev complexity (L-Z) of the vascular networks from the lower gingival and vestibular oral mucosa were measured.

Results:
LCFS2 networks exhibited a significantly increased overall complexity at both larger (D(1–46): 1.82 (0.04) v 1.68 (0.08); p < 0.0001) and smaller (D(1–15): 1.51 (0.11) v 1.20 (0.09); p < 0.0001) scales, increased destructured randomness (L-Z: 0.77 (0.09) v 0.56 (0.03); p < 0.0001), and decreased vessel tortuosity (Dmin: 1.02 (0.03) v 1.07 (0.04); p = 0.0005) compared with control patterns. The vascular networks of LCFS2 gene carriers showed higher complexity at the smaller scale (D(1–15): 1.59 (0.12) v 1.47 (0.07); p = 0.034), and higher destructured randomness (L-Z: 0.85 (0.11) v 0.73 (0.05); p = 0.013) than those of non-carriers.

Conclusions:
Increased oral vascular network complexity is a previously unrecognised phenotypic marker for LCFS2, and is related to gene mutation carrier status.

Background: Hereditary non-polyposis colorectal cancer (HNPCC) (Lynch cancer family syndrome I (LCFS1) and II (LCFS2)) is one of the most common hereditary cancer disorders. HNPCC results from dominantly inherited germline mutations in mismatch repair (MMR) genes, leading to genomic instability and cancer. No predictive physical signs of HNPCC are available to date.

Aims: Increased complexity in tumour associated vascular growth has been reported. Here, we tested the hypothesis that an increased vascular network complexity is a phenotypic marker for LCFS2.

Methods: Fourteen subjects from an LCFS2 kindred (gene carriers, n = 5; non-carriers, n = 9) and 30 controls were examined. Fractal dimension (D) at two scales (D(1–46), and D(1–15), tortuosity (minimum path dimension, Dmin), and relative Lempel-Ziev complexity (L-Z) of the vascular networks from the lower gingival and vestibular oral mucosa were measured.

Results: LCFS2 networks exhibited a significantly increased overall complexity at both larger (D(1–46): 1.82 (0.04) v 1.68 (0.08); p < 0.0001) and smaller (D(1–15): 1.51 (0.11) v 1.20 (0.09); p < 0.0001) scales, increased destructured randomness (L-Z: 0.77 (0.09) v 0.56 (0.03); p < 0.0001), and decreased vessel tortuosity (Dmin: 1.02 (0.03) v 1.07 (0.04); p = 0.0005) compared with control patterns. The vascular networks of LCFS2 gene carriers showed higher complexity at the smaller scale (D(1–15): 1.59 (0.12) v 1.47 (0.07); p = 0.034), and higher destructured randomness (L-Z: 0.85 (0.11) v 0.73 (0.05); p = 0.013) than those of non-carriers.

Conclusions: Increased oral vascular network complexity is a previously unrecognised phenotypic marker for LCFS2, and is related to gene mutation carrier status.

Oral vascular network analysis
The lower gingival and vestibular oral mucosa was chosen as the study area due to its high vasculature pattern visibility and easy accessibility. A 704 mm² size (32 x 22 mm) area of the lower gingival and vestibular oral mucosa was photographed for each subject (1:1 ratio, orthogonal projection). All photographs were taken by a single operator using a...
Yashica Dental Eye photocamera with an automated on-axis flashbulb and a 55 mm f 1:4 Yashica lens (Yashica-Kyocera Co., Kyoto, Japan). Kodak Elite Chrome 100 ISO/21 DIN films (Kodak-Eastman Kodak Co., Rochester, New York, USA) were used and developed according to the standard E-6 procedure. Images were digitised using a Canon Canoscan FS2710 (Canon Inc., Tokyo, Japan) scanner (colour resolution: 680 dpi, 6.67× magnification) with a Windows '98 operating system (Microsoft Co., Redmond, Washington, USA).

The obtained images were converted into binary skeletonised form for geometric pattern analysis. Manual outline of the two dimensional trajectories of the vascular network was performed using Adobe Photoshop (Adobe Systems Inc., San Jose, California, USA) on a Sony 19’ Trinitron Multiscan G420 screen (16 μm/pixel resolution) (Sony Co., Tokyo, Japan) by two operators who were unaware of the subject’s category. The two dimensional lattices were analysed using the Image Pro-Plus version 1.3 image analysis software (Image Pro-Plus-Media; Cybernetics Inc., Silver Spring, Maryland, USA). Non-readable areas were <5% of the total area. After enlargement to 1.4 × magnification, images were processed to threshold the vessel network without background interference and the networks were subsequently converted into an outline of single pixels.

The oral vascular networks were characterised by analysing their complexity (fractal dimension (D), at two scales), tortuosity (minimum path fractal dimension (Dmin)), and randomness (relative Lempel-Ziv complexity (L-Z)) of the vascular loops. Fractal dimensions of the two dimensional skeletonised images were measured with the box counting algorithm, using the relation N(L) = L−D, where L is the box size and N(L) is the number of squares. As natural fractals show upper and lower limits, the local fractal dimension (D) was determined for two regions of box lengths, <740 μm (pixels 1–46, D(1–46)) and <140 μm (pixels 1–15, D(1–15)), respectively. The measuring procedure was calibrated against shapes of known fractal dimension with an inaccuracy of ±2%. The fractal dimension of the minimum path, Dmin, was computed for each vascular cluster from the power law l = Dmin, where Dmin is the exponent that governs the dependence of the minimum path length between two points (l) on the Pythagorean distance (r) between them in a fractal random material. After enlarging the image to 2.4× magnification, thinning to 1 pixel, and discarding all areas with a diameter <3 pixels, the half perimeter (x_i) and the maximum diameter (y_i) of either the vessel loops or vessel free areas in the single pixel two dimensional lattice were measured using an automated procedure (Image Pro-Plus version 1.3 image analysis software; examined areas for each sample: 500–1000). The slope of the log/log plot x_i/y_i represented Dmin.

The method was validated with the original one by Herrmann and Stanley, with a maximum shift of ±3%. To determine the algorithmic complexity of the vascular patterns, relative Lempel-Ziv (L-Z) values were calculated according to the Kaspar and Schuster algorithm using the Chaos Data Analyzer version 2.1 (1995) software package (CDA: Pro; The Academic Software Library, North Carolina State University, Raleigh, North Carolina, USA). Vascular network lattices from a 251×251 pixels window of the original image were transformed into 16 732 points containing one dimensional vectors, and each datum point was converted into a single binary digit according to whether the design is touched ( = 1) or not ( = 0). Relative L-Z values may range from near 0 for a deterministic equation to approximately 1.0 for totally destructured random phenomena. Using the described procedure, the relative L-Z values for the sinus function and white noise were 0.0044 and 1.047, respectively.

The vascular network analysis was reproducible, with mean intra and interobserver coefficients of variation of

![Figure 1](http://gut.bmj.com/)

**Figure 1** Lynch cancer family syndrome II (LCFS2) associated vascular network abnormalities. (A) Control oral vascular network; (B) abnormal oral vascular density of the vascular network in an LCFS2 gene carrier patient, with multiple loops features; (C) skeletonised control vascular network, with typical dichotomic branching pattern (D(1–46) = 1.57; D(1–15) = 1.07; Dmin = 0.83; L-Z = 0.49); (D) skeletonised vascular network pattern of an LCFS2 gene carrier patients showing increased density of trajectories and multiple loops aspects (green arrows) (D(1–46) = 1.79; D(1–15) = 1.68; Dmin = 0.99; L-Z = 0.92). Note (C) and (D) represent ~60 × enlarged details of (A) and (B), respectively. D, fractal dimension; Dmin, minimum path fractal dimension; L-Z, relative Lempel-Ziv complexity.
CI 0.03–0.21); t = 2.9, df = 12, p = 0.013) than those of the control vascular networks. The vascular networks of the LCFS2 gene mutation carriers showed higher complexity at smaller scales and a more marked destructured randomness than non-carriers (group B).

**RESULTS**

Oral vascular networks in LCFS2 patients showed geometric pattern abnormalities (that is, increased vessel density and presence of multiple vascular loops features) compared with the typical dichotomic branching patterns of control networks (fig 1). LCFS2 associated vascular networks exhibited a significantly increased overall complexity (table 1) at both larger (D(1–46) difference: 0.14 (95% confidence interval (CI) 0.09–0.19); t = 6.39, df = 42, p<0.0001) and smaller (D(1–15) difference: 0.31 (95% CI 0.24–0.37); t = 9.96, df = 42, p<0.0001) scales, increased destructured randomness (L-Z difference: 0.21 (95% CI 0.17–0.24); t = 11.7, df = 42, p<0.0001), and decreased vessel tortuosity (Dmin difference: 0.047 (95% CI 0.022–0.072); t = 3.75, df = 42, p = 0.0005) compared with control vascular networks. The vascular networks of the LCFS2 gene mutation carriers showed higher complexity at the smaller scale (D(1–15) difference: 0.12 (95% CI 0.011–0.23); t = 2.39, df = 12, p = 0.034), and a more marked destructured randomness (L-Z difference: 0.12 (95% CI 0.03–0.21); t = 2.9, df = 12, p = 0.013) than those of the non-carrier LCFS2 individuals.

**DISCUSSION**

HNPCC accounts for approximately 3–6% of all colorectal cancers. Early identification of HNPPC gene carriers is essential as surveillance has been reported to reduce CRC incidence and overall mortality. However, in the absence of predictive physical signs, no universal consensus exists on the most cost effective strategy for detecting potential gene carriers, with population screening by mutation analysis not being a feasible option. The main findings of the present study indicate the presence of a previously unrecognised abnormal vascular network complexity in the oral mucosa of LCFS2 patients, which is related to gene mutation carrier status. This observation may provide a useful phenotypic marker of LCFS2 in both the presymptomatic detection of DNA genetic testing candidates in HNPCC families and CRC case selection for mutational analysis. The mechanisms underlying the increased vascular complexity observed in the oral cavity of the LCFS2 subjects remain unclear.

<table>
<thead>
<tr>
<th>Vascular network variable</th>
<th>LCFS2+</th>
<th>LCFS2 controls</th>
<th>F2,41 values</th>
<th>p Value</th>
<th>Significant pairwise differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene carriers (group A, n = 5)</td>
<td>Non-carriers (group B, n = 9)</td>
<td></td>
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<tr>
<td>D(1–46)</td>
<td>1.84 (0.07)</td>
<td>1.82 (0.02)</td>
<td>1.68 (0.08)</td>
<td>17.97</td>
<td>2.5 × 10⁻⁵</td>
</tr>
<tr>
<td>D(1–15)</td>
<td>1.59 (0.12)</td>
<td>1.47 (0.07)</td>
<td>1.20 (0.09)</td>
<td>71.83</td>
<td>&lt;10⁻⁶</td>
</tr>
<tr>
<td>Dmin</td>
<td>0.99 (0.02)</td>
<td>1.03 (0.04)</td>
<td>1.07 (0.04)</td>
<td>9.07</td>
<td>0.00054</td>
</tr>
<tr>
<td>L-Z</td>
<td>0.85 (0.106)</td>
<td>0.73 (0.05)</td>
<td>0.56 (0.03)</td>
<td>20.78</td>
<td>6 × 10⁻⁶</td>
</tr>
</tbody>
</table>

Data are means (SD). p values refer to one way ANOVA results.

<5.0% and <10%, respectively. Differences among group means were analysed by the t test. Differences between multiple groups were evaluated by one way ANOVA, and post-hoc pairwise differences were tested using the Student-Newman-Keuls statistics. A two sided p<0.05 was considered to indicate statistical significance, and the Bonferroni corrected significance levels were used for multiple t tests. The MedCalc version 7.0 statistical software package (MedCalc Software, Mariakerke, Belgium) was used.

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