Fordyce granules and hereditary nonpolyposis colorectal cancer syndrome

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Abbreviations
HNPPC Hereditary Non-Polyposis Colorectal Cancer
MMR Mismatch Repair
CRC Colorectal Carcinoma
FGs Fordyce Granules
Hh Hedgehog
MSI Microsatellite Instability
MTS Muir Torre Syndrome
ABSTRACT
Background: Germline mutations in Mismatch repair (MMR) genes are found in only nearly half of clinically diagnosed families, with hereditary nonpolyposis colorectal cancer syndrome (HNPPCC) (or Lynch syndrome). Early identification of gene carriers is essential to reduce cancer incidence and overall mortality.
Aims: Recent evidence indicates an increase in size and number of sebaceous glands following the activation of the Hedgehog pathway, a crucial signaling pathway for animal development that is aberrantly activated in several types of cancer. Here, we sought to assess a possible association between Fordyce granules (FGs) (i.e., ectopic sebaceous glands on the oral mucosa) and HNPPCC.
Methods: A total of 15 members of 5 different, genetically unrelated HNPPCC kindreds (MLH1 gene mutation, n=8; undetectable MLH1 protein at immunohistochemistry n=4; clinical diagnosis, n=3) and 630 genetically unrelated age- and sex-matched healthy controls were examined. Following examination of the oral mucosa surface, the subjects were categorized as either FGs-positive or FGs-negative.
Results: Evidence of FGs was significantly associated with HNPPCC (13/15 [86.7%] of the affected patients vs. 6/630 [0.95%] of controls p<0.0001), with a relative risk of 91.0 (95% CI: 40.05–206.76). The observed difference remained significant when carriers of germline mutations in MMR genes were considered (8/15 vs. 6/630, p<0.0001). The most common site for the FGs in HNPPCC patients was the lower gingival and vestibular oral mucosa.
Conclusions: Our findings suggest a previously unrecognised activation of the sebaceous glands system occurs in HNPPCC. The observation could be of help for the attending physicians in identifying the affected families and/or increase the accuracy of the currently available molecular genetics screenings.
INTRODUCTION

Hereditary nonpolyposis colorectal cancer syndrome (HNPPC) [or Lynch syndrome] is the most common form of hereditary colorectal cancer (CRC),1,2 caused by autosomal dominantly inherited, germline mutations in mismatch repair (MMR) genes. Mutation carriers have a lifetime risk >80% of developing CRC, together with an excess of extracolonic cancers at an early age.3,4 MLH1 and MSH2 accounts for almost 90% of identified mutations, while MSH6 accounts for about 10%, and mutations in PMS2, MLH3 and EXO1 have also been reported.1,2 Early identification of HNPPC gene carriers is essential to reduce cancer incidence and overall mortality.1,3,4 However, the germline mutations in MMR genes can be established in only about half of clinically diagnosed HNPPC families,1 while, with the single exception of the rare Muir-Torre syndrome (MTS) variant (OMIM #158230),5,6 no obvious phenotypical physical stigmata aiding in the diagnosis exist. As a consequence, the actual frequency of HNPPC in the general population remains unknown. Theoretical reasons indicate a prevalence of 1:350 to 1:1700, while more conservative estimates are around 2%-7% of the total CRC cases, which suggests that HNPPC is currently underdiagnosed,4 with devastating clinical consequences. Fordyce granules (FGs)7,8 (i.e., intra-oral ectopic sebaceous glands) are thought to be benign, common lesions on the oral mucosa of adults subjects. Their prevalence rates increase from childhood (0.2%)9 to adulthood (0.97%), and is slightly higher in adult males (1.77%) than in females (0.52%).10 No correlations between FGs and systemic diseases are known to date. Recent evidence indicates an increase in size and number of sebaceous glands following the activation of the Hedgehog (Hh) pathway,11 a crucial signaling pathway for animal development that is aberrantly activated in several types of cancer.12,13 Here, we sought to assess a possible association between HNPPC and FGs.

METHODS

A total of 15 surviving members of 5 different, genetically unrelated HNPPC kindreds (M:7, F:8; age at examination: 53.7 (SD:15.1) years; family 1: n=4; family 2: n=4; family 3: n=1; family 4: n=3; family 5, n=3) originating from the Taranto and Brindisi areas of southern Italy, were examined. All the families fulfilled the Amsterdam criteria, and all patients had a history of proven cancer at the time of examination (CRC, n=11 [73.3%]; other cancers n=4). Given the specific aim of the present study, only subjects who accepted to undergo HNPPC genetic molecular studies were invited to participate, while subjects with undetermined disease status were excluded. Mean age at cancer onset was 41.5 years (SD:14.1). High molecular weight DNA was extracted from frozen tissues or blood according to standard methods. Microsatellite instability (MSI) was assessed using five polymorphic markers: BAT25, BAT26, D2S123, D5S346 and D18S858. MSI was considered to be present when two or more of the five markers showed instability. The promoter regions and each exon of MLH1 and MSH2 genes from genomic DNA were individually amplified, and subsequently sequenced directly. Germline mutations in MMR genes were defined according to standard nomenclature recommendations.14 Immunoistochemical analysis was performed as previously described.15 The panel of immunostaining encompassed MSH2 (FE11, Oncogene Research products, Boston, MA, USA, diluted 1:200) and MLH1 (G168 – 728, Pharmingen, S. Diego, CA, USA
diluted 1:100). 5µm -sections containing both tumour tissue and normal colonic mucosa (as internal control) were cut. After deparaffination ad rehydration, including a step of endogenous peroxidase block with methanol-peroxidase for 30 minutes, the sections were microwaved for non-enzymatic epitope retrieval in citrate buffer solution (pH 6.0) at 750W for 5 minutes (3 times). The slides were left to cool in the buffer solution for 20 minutes. Monoclonal antibodies (MAbs) anti-MSH2 and anti-MLH1 were incubated for 60 minutes. The slides were incubated with the primary antibodies for 30 minutes. Immunostaining was carried out by using the streptavidin-biotin-peroxidase amplification system in an automatic immunostainer (Techmate 500, DAKO). CRCs were judged to be negative for expression only if they lacked staining in a sample in which normal colonocytes and stroma were stained. The results were not considered in case of lack of immunostaining for normal tissues.

A total of 630 genetically unrelated age- and sex-matched healthy controls (M: 300, F: 330, age: 54.0 [SD:16.1] years) were also recruited. The oral mucosa surface was examined by an experienced odontostomatologist, who was unaware of the clinical and genetical data of the subjects, and were categorized as either FGS-positive or FGS-negative. No cases of CRCs were present in the family history and pedigrees of controls. High-resolution photographs of the selected areas were acquired using either a Yashica Dental Eye photocamera with an automated on-axis flashbulb, emitting standard photographic white light, and a 55-mm, f 1:4 Yashica lens (Yashica-Kyocera Co., Kyoto, Japan) or a Nikon Coolpix 4500 digital camera (4Mpixels, 4x optical + 4x-digital zoom, 16Mb card; Nikon, Japan). We obtained approval from our local Human Investigation Committee and written informed consent for examination and photographic documentation from all the subjects before inclusion in the study.

**Data Analysis**

A coefficient of agreement for nominal scales (κ),[16] was applied to assess inter-observer variations in FGS detection. A κ value <0.20 was rated as a poor inter-rater agreement, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as good, and 0.81-1.0 as very good.[17]

Differences in the proportions of FGS between the HNPCC patients or carriers of germline mutations in MMR genes vs. controls were assessed using the chi-square statistics and relative risk was calculated. The effects of population size on type I (α) (i.e., probability of rejecting a true null hypothesis) and type II (β) (i.e., probability of accepting a false null hypothesis) statistical errors in the data interpretation were examined using a sampling size algorithm. Given the reported proportions of FGS in the HNPCC and control subjects, the minimal required sample size was n=5 for each group for type I/α and type II/β statistical errors of P= 0.01. A two-sided p value of <0.05 was considered to be statistically significant. The MedCalc ver. 7.5 statistical software package (MedCalc Software, Mariakerke, Belgium) was used.

**RESULTS**

Germline mutations in MMR genes were detectable in 8/15 cases (53.5%) (i.e., 3/5 families) and identified as MLH1 mutation, including c.210_213delAGAA in exon 3 (n=3 patients) and c.545+1G>A in intron 6 (n=5 patients) mutations. MLH1 protein resulted to be undetectable at immunohistochemistry in 4 patients (26.7%) and the
diagnosis was clinical in 3 patients (20%). MSI was present in 10 out of 11 examined
CRCs (90.9%).
Clinical examination was found to be reproducible in identifying FGs, with a $\kappa$ value
for inter-observer agreement (mean $\pm$ SEM) of 0.936 $\pm$ 0.045 (95% CI: 0.847 to 1.0),
indicating a high degree of concordance. FGs (Figure) were evidenced in 13 out of 15
(86.7%) of the affected patients vs. 6 out of 630 (0.95%) of the controls
difference=77.18%, 95% CI: 59.84-94.51; chi-square = 79.45, df=1; $p < 0.0001$),
with a relative risk of 91.0 (95% CI: 40.05-206.76). The difference in the FGs
proportions remained significant when only carriers of germline mutations in MMR
genes were accounted for (8/15 HNPC vs. 6/630 controls; difference=43.81%, 95%
CI: 18.46-69.16, chi-square = 25.36, df=1; $p < 0.0001$; relative risk: 56.0, 95% CI:
22.17-141.42).
Both FGs-negative HNCC patients had a clinical diagnosis, without detectable
germline mutations in MMR genes. The FGs maximum diameter was estimated to be
1.54 $\pm$ 0.64 mm (95% CI: 1.33-1.74 mm; values range: 0.8-3.0 mm), with the most
common site for the FGs being the lower gingival and vestibular oral mucosa in
HNPC patients vs. the mandibular retromolar pad and the upper lip vermilion in
controls. Sensitivity and specificity of FGs as a diagnostic test for HNPC were 86.7%
(95% CI 59.5-98) and 99% (95% CI 97.9-99.6) for cases and controls. The positive
and negative predictive values were 68.4% and 99.7%, respectively.

DISCUSSION
Our findings suggest, for the first time, that FGs may be associated to systemic
disease, and that a previously unrecognised activation of the sebaceous glands
system may occur in individuals carrying HNPC gene mutations and/or patients with
Amsterdam criteria-positive CRC. This observation may be of help for identifying the
affected families. In the present study, the frequency of recognizable FGs was found
to be about 50 to 90-fold higher in HNPC patients than in controls.
To date, FGs are considered to be benign lesions on the oral surface in healthy
individuals.[8] [9] [10] Demonstration of cancers originating from the FGs is rarely
reported in the literature.[18] The average frequency of FGs has been reported to
increase $\sim$4.85 folds from childhood [9] to adulthood.[10] The mechanisms
underlying the age-dependent changes in the frequency of FGs remain unclear. As a
consequence, the frequency of the observed FGs sign as a function of age at
examination and/or development of CRC in HNPC patients, needs further
investigation.
Moreover, the question of the biological specificity of the FGs sign in HNPC remains
to be addressed. Evidence of FGs in CRC-negative HNPC individuals and lack of FGs
in patients with sporadic (Amsterdam negative) CRCs (S. Parrini, et al, unpublished
data) seem to suggest a link of ectopic sebaceous glands to the MMR gene defect,
rather than to the simple coexistence of the CRC itself.
The biological significance of the observed clinical association remains unclear to
date. However, Hh, a signaling pathway regulating a variety of developmental
processes, including vasculogenesis, has been shown to increase the size and
number of sebaceous glands.[11] [12] Thus, a conceivable working hypothesis is
that Hh pathway activation may coexist in HNPC, leading to sebaceous glands
development, and recognizable FGs. To this regard, a specific role for the Hh
signalling pathway in colonic cancerogenesis has been previously reported.[19] [20]
[21] lending support to this speculation. In view of this clinical observation, it would be advisable to test the role of the Hedgehog signalling pathway in the HNPPC cancers. Interestingly, the only recognisable physical sign on clinical examination for HNPPC is the skin changes related to neoplasms originating from the skin sebaceous glands in MTS, a rare variant of HNPPC.[5]

A strong correlation between MSH2 mutations and MTS has been recently reported.[22] [23] MTS is a cancer-predisposing genodermatosis characterized by sebaceous gland tumours, including sebaceous adenomas, sebaceous epitheliomas, and sebaceous carcinomas, and less commonly keratoacantomas.[24] However, MLH1 mutations have also been found in at least two families.[25] [26] Interestingly, in both reports a MLH1 deletion was present and found to be similar to the one identified in our family 1. Moreover, phenotypic variation seems to be present even among carriers of the same MSH2 mutations. For instance, the splicing mutation affecting exon 5 identified in 3 MTS families is one of the most common HNPPC molecular defects,[27] while no information regarding the cutaneous tumors in the majority of the families with MSH2 mutation reported in the literature is available.[23] Taken together, the reported evidence, along with other recent studies,[28] would support the hypothesis that the molecular basis of MTS does not substantially differ from that of HNPPC.

Since the Hh pathway has been shown to play a key role in cancer-related angiogenesis,[29] [30] our previous report on increased microvascular complexity in the oral mucosa of patients with HNPPC [4] [31] further supports this hypothesis. In view of our findings, an interesting future study would be a comparison between HNPPC patients and non-HNPPC patients carrying CRCs with documented MSI, although in the latter cases both mutations in MMR genes are usually somatic. In these patients a distinct molecular pathway has been demonstrated to be caused by a defect in DNA MMR genes,[32] although the concept that the first mutation is germinal in HNPPC patients and somatic in sporadic MSI colorectal tumours should be relevant in determining the associated phenotypic features.

Further prospective research on a larger population is certainly needed to confirm the reported clinical association, and to verify the predictive value of FGs marker in detecting HNPPC. Whether this clinical observation could be helpful in increasing the rate of HNPPC diagnosis in affected families warrants further investigation.
REFERENCES


**Figure Legend**

**Top panel:** Fordyce granules (FGs) (arrowheads) in the oral vestibular mucosa of an HNPCC patient carrying the c.545+1G>A in intron 6 *MLH1* germ line mutation. FGs appear as rice-like, yellow-white papules with a maximum diameter of ~1-3 mm, surrounded by normal mucosa. Local elevation of the epithelium is clearly recognisable.

**Bottom panel:** Corresponding oral vestibular mucosa in a healthy control subject.
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On behalf of all the Co-Authors, I declare that we have no competing interests.