COLORECTAL CANCER

Abnormal oral mucosal light reflectance: a new clinical marker of high risk for colorectal cancer

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Background: A familial predisposition to colorectal cancer (CRC) has been clearly established, consisting of familial clustering in 15–20% and clear hereditary aetiology in 5–10% of overall CRC cases. Early identification of families and individuals at high risk is essential as intensive surveillance has been demonstrated to reduce cancer incidence and overall mortality. In the present study, the value of oral mucosal light reflectance in identifying hereditary non-polyposis colorectal cancer (HNPCC) carriers was investigated.

Methods: Twenty members of six different genetically unrelated HNPCC kindred and 30 genetically unrelated age and sex matched healthy controls were examined. Lower gingival and vestibular oral mucosal reflectance was measured using an imaging spectrophotometer.

Results: HNPCC carriers showed significantly lower values in the 590–700 nm wavelength range ($p \leq 0.0004$). A reflectance cut off value $\leq 47.9\%$ at the 700 nm wavelength discriminated between HNPCC carriers and controls, with 100% sensitivity and 100% specificity.

Conclusions: These findings may provide an additional phenotypic sign in HNPCC carriers, which could be used in first level CRC population screening programmes.

A familial predisposition to colorectal cancer (CRC) has been clearly established in at least 25–35% of overall CRC cases, representing familial clustering in more than two thirds (15–20%) and a clear hereditary syndrome in the remaining cases (5–10% of all CRCs).^{1 2}

Early identification of families and individuals at high risk is essential, as intensive surveillance has been demonstrated to reduce CRC incidence and overall mortality.³ Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is one of the most common hereditary cancer disorders and the commonest form of hereditary CRC.¹ HNPCC is caused by autosomal dominant germline mutations in mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, *MLH3*, and rarely *PMS1*, and *PMS2*), with gene mutation carriers having a lifetime risk >80% of developing CRC, together with an excess of extracolonic cancers at an early age.⁴ The real frequency of HNPCC in the population is unknown although reasonable estimates indicate a prevalence ranging from 1:350 to 1:1700, hence suggesting that the condition is currently underdiagnosed, with devastating clinical consequences. Recently, we have identified vascular aberrations in the oral mucosa in an HNPCC kindred,⁵ thus generating the testable hypothesis of an associated subclinical extracellular matrix (ECM) abnormality in HNPCC. Colour is an essential parameter in clinical medicine,⁶ and it is well established that the radiation emitted or reflected by an object is an essential tool for acquiring knowledge about its physical state and chemical composition.⁷ However, its potential usefulness for diagnostic purposes has not been investigated in depth. As physicochemical changes of a tissue should affect its optical properties, in the present study the value of the oral mucosal light reflectance in identifying HNPCC carriers was investigated.

METHODS

A total of 20 (11 males and nine females; age at examination 46.6 (SD 8.5) years) surviving members of six different genetically unrelated HNPCC kindred (family 1, $n = 4$; family

2, $n = 4$; family 3, $n = 1$; family 4, $n = 3$; family 5, $n = 3$; family 6, $n = 5$) originating from the Taranto and Brindisi areas of southern Italy were examined. Mean age at cancer onset was 43.1 years (SD 3.08). All HNPCC families fulfilled the Amsterdam criteria. Detailed characteristic features of the families have been reported previously.^{5 8 9} Given the specific aim of the present study, only subjects who agreed to undergo HNPCC genetic molecular studies were invited to participate. Subjects with undetermined disease status were excluded.

Immunohistochemical analysis was performed as described previously.¹⁰ The panel of immunostaining comprised MSH2 (FE11; Oncogene Research products, Boston, Massachusetts, USA; diluted 1:200) and MLH1 (G168-728; Pharmingen, San Diego, California, USA; diluted 1:100). Sections (5 μ m) containing both tumour tissue and normal colonic mucosa (as internal control) were cut. After deparaffination and rehydration, including a step of endogenous peroxidase block with methanol peroxidase for 30 minutes, sections were microwaved for non-enzymatic epitope retrieval in citrate buffer solution (pH 6.0) at 750 W for five minutes (three times). Slides were left to cool in the buffer solution for 20 minutes. Monoclonal antibodies anti-MSH2 and anti-MLH1 were incubated for 60 minutes. Slides were incubated with the primary antibodies for 30 minutes. Immunostaining was carried out by the streptavidin-biotin-peroxidase amplification system in an automatic immunostainer (Techmate 500; Dako Norden A/S, Glostrup, Denmark). 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.

Abbreviations: CRC, colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; ECM, extracellular matrix; MSI, microsatellite instability; ROC, receiver operating characteristic; FAP, familial adenomatous polyposis

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 the *MLH1* and *MSH2* genes from genomic DNA were individually amplified and subsequently sequenced directly. Germline mutations were defined according to standard nomenclature.¹¹

Thirty genetically unrelated age and sex matched healthy controls (15 males, 15 females; aged 45.0 (11.6) years), with no known inherited connective tissue or vascular diseases, were also enrolled. No cases of CRC were present in the family history and pedigrees of controls. Both approval by the research ethics committee and informed consent for examination and photographic documentation were obtained.

Photography

The lower gingival and vestibular oral mucosa was chosen as the study area because of its easy accessibility. To avoid direct mucosal contact and overlapping with blood vessels, photographs of the selected areas were acquired using a Yashica Dental Eye photo camera 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). 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. Incident illumination was approximately 10°, with a viewing pass nearly normal to the observed surface, and 8× magnified printed images were converted to digital spectral data using a standard 45°–0° viewing geometry.

Reflectance analysis measurements

Reflectance is a physical quantity measuring how well a surface reflects the light (that is, visible electromagnetic radiation). Reflectance is almost always considered as a spectral function (spectral reflectance) and it is usually referred to Lambert's surfaces (that is, those surfaces that uniformly reflect in all spatial directions the incident light). In particular, spectral reflectance of a lambertian surface is defined as the ratio of reflected to incident light intensity (that is, specific intensity of electromagnetic radiation or radiance), as estimated in a narrow spectral interval about a certain central wavelength of interest. Based on the definition, reflectance is a dimensionless quantity that must be comprised in the range 0 to 1. Accordingly, reflectance is often specified using per cent notation, being 100% reflectance the unitary reflectance (all incident light is reflected).

Oral mucosal colour was assessed from photographic prints which were scanned by means of an imaging spectrophotometer¹² calibrated on a standard white tile ($\pm 0.1\%$ shift). It is important to note that in passing from the observed oral mucosal surface to photographic prints, only the colour of the observed surface is preserved while information of higher spectral resolution may be lost. Therefore, only the colour or colour related information was employed for the subsequent analysis. Reasons for using an imaging spectrophotometer rather than a standard scanner for digital conversion of photographs were related to higher accuracy and stability. In particular, unlike the other scanners, the selected imaging spectrophotometer provided a certified colour estimation, according to CIE recommendations for colour measurement.¹³

Spatially averaged spectra acquired by the imaging spectrophotometer were used in order to estimate oral mucosal colour in the 400–700 nm spectral range of wavelength (violet region 400–450 nm; blue 450–475 nm; blue-green

475–500 nm; green 500–570 nm; yellow 570–590 nm; orange 590–610 nm; red 610–700 nm). Reflectance from 21 different artefact free and vessel free areas was measured and mean values were used for data analysis.

Derived spectral data were reproducible (intra- and interobserver coefficients of variation (mean (SD)) 1.40 (0.72)% and 3.02 (1.70)%, respectively) and qualitatively comparable ($\pm 2\%$ shift) to those obtained by direct measurements using a CM-2600d/2500d Minolta reflectance spectrophotometer (Minolta Co. Ltd, Osaka, Japan), with an absolute spectral error (δE) of ~ 0.4 (that is, spectral difference measure averaged over the visible spectral range by means of a perceptive weight function). Direct measurements were actually made on the same patients, and three independent observers were involved for interobserver variability. All patients and controls were evaluated at 31 different wavelengths in 21 different oral areas from photos and by direct measurement. All measurements were carried out by operators who were unaware of the subjects' clinical data.

Data analysis

Differences among group means were analysed by the *t* test for unpaired data. Discrimination of reflectance values at a wavelength of 700 nm was tested using receiver operating characteristic (ROC) curve analysis, and χ^2 statistics were used to assess model calibration. A two sided *p* value < 0.05 was considered to be statistically significant, and the Bonferroni corrected significance levels were used for multiple *t* tests. MedCalc version 8.1.1 statistical software (MedCalc Software, Mariakerke, Belgium) was used.

RESULTS

Germline mutations in mismatch repair genes were detectable in 13/20 cases (65%) (4/6 families) and identified as *MLH1* mutations, including the c.210_213delAGAA in exon 3 (one family, three patients), c.545+1G>A in intron 6 (two families, five patients), and c.1558+1G>T in intron 13 (one family, five patients) mutations. *MLH1* protein was undetectable at immunohistochemistry in one family (four patients) (20%) and the diagnosis was clinical in the remaining cases (one family, three patients) (15%). MSI was present in 12/13 CRCs (92.3%) examined.

Comparison of the reflectance spectra in the two groups is shown in fig 1. Equations for the HNPCC and control

Figure 1 Comparison of oral mucosal light reflectance as a function of the wavelength in the visible spectrum between hereditary non-polyposis colorectal cancer (HNPCC) patients and controls. Values are given as means (SD) (half bars). Differences in the 590–700 nm wavelength range were statistically significant ($p \leq 0.0004$).

functions were determined: given their sigmoidal shape, linear normalisation of the curve functions was achieved using a logit transformation. The oral mucosa of the HNPCC subjects showed significantly lower reflectance values in the longer wavelengths of the visible spectrum (wavelength range 590–700 nm; $p \leq 0.0004$) than those of controls. From the ROC curve analysis results, a reflectance cut off value $\leq 47.9\%$ at the 700 nm wavelength was found to discriminate HNPCC carriers from controls, with 100% sensitivity and 100% specificity. Light reflectance spectra from HNPCC and controls were described by significantly different equations:

$$y_{\text{HNPCC}}^{\text{HNPCC}} = -5.59 + 0.0075x \text{ versus}$$

$$y_{\text{Controls}}^{\text{Controls}} = -6.94 + 0.01x; p < 0.0001$$

where y = reflectance, and x = wavelength.

DISCUSSION

Risk stratification is crucial for the design of cost effective CRC screening programmes. The present findings indicate that the optical properties of the oral mucosa in HNPCC carriers differ significantly from those of controls, hence allowing the potential identification of subjects at high risk for CRC in population screening programmes.

An inherited predisposition to CRC has been implicated in approximately 5–10% of patients with hereditary aetiology and in about 15–20% with familial clustering.¹ Based on twin studies, the genetic component of the risk of developing CRC has been estimated at 35%.²

The spectrum of genetic susceptibility ranging from low penetrance mutations, such as I1307 K carrying a modest increase in the colon cancer risk, to the more dramatic phenotype (that is, multiple colonic adenomas in familial adenomatosis polyposis (FAP)) associated with an extremely high cancer risk.¹¹

Cancer genetic counselling has been energised by the recent development of genetic tests that pinpoint familial cancer risk. DNA testing for inherited colon cancer predisposition works well with families that fit criteria for cancer family syndromes, such as FAP and HNPCC. There are several potential reasons for overlooking the diagnosis of family syndromes, including: (i) difficulty in obtaining sufficiently informative familial pedigrees in daily practice; (ii) inadequate physician awareness of inherited colon cancer syndromes¹⁴; (iii) variable phenotypic expression of the syndrome due to modulation by both genetic and environmental factors (that is, smoking)¹⁵; (iv) suboptimal sensitivity and frequent ambiguity from germline testing (that is, one third of *MLH1* gene mutations have been classified as missense mutations of unknown significance)¹⁶; (v) limitations in the clinical criteria for diagnosing hereditary syndromes¹⁷; and (vi) occurrence of de novo gene mutations.¹⁸

In contrast, it should be taken into account that the relevance of such limiting factors is strictly dependent on the characteristics of the hereditary colon cancer syndrome. If we consider FAP patients (that is, approximately 1% of all CRCs), affected individuals develop multiple (≥ 100) adenomas diffusely throughout the colorectum and extensive mutation screening identifies genetic changes in up to 95% of patients.¹⁸ Other more rare hereditary conditions (that is, Peutz-Jeghers syndrome or juvenile and hamartomatous polyposis), which account for $<1\%$ of all CRCs, can also be easily recognised clinically.¹⁹

On the other hand, the diagnosis of HNPCC is not simple as the occurrence of colonic and extracolonic cancers in HNPCC are not specific, and clinical criteria are mainly based on family history.²⁰ Mutations in at least six different genes (*MLH1*, *MSH2*, in a few cases *MSH6* and *MLH3*, and rarely *PMS1*, and *PMS2*) can cause HNPCC. Considering the most commonly mutated genes (*MSH2* and *MLH1*), the

International Collaborative Group on HNPCC has identified mutations in 47 (26%) of 184 families suspected of having HNPCC.²¹ As a consequence, molecular testing can contribute towards establish HNPCC diagnosis in less than one third of all cases.

Limitations in clinical criteria and molecular testings make it difficult to establish the real prevalence of HNPCC. Hence the value of 3–5% of CRCs, usually reported in the literature, may represent an underestimation.²² The clinical implications of this under recognition can have devastating effects, due to the high probability of developing malignancies with potentially preventable fatalities.

In light of the above considerations, future challenges in HNPCC are mainly: (1) a better understanding of the genotype-phenotype relationships; (2) a better strategy to estimate the clinical subsets of patients who will most likely benefit from mutation testing; and (3) more cost effective genetic testing strategies. Specific attention should be paid to any unusual clinical features, potentially useful in identification of hereditary colon cancer syndrome families or, more generally, of families with a predisposition to CRC in order to select individuals who should be investigated with DNA specific testing and/or who should undergo appropriate clinical procedures. Our results demonstrate the clinical usefulness of this additional phenotypic marker in detecting HNPCC carriers.

The mechanisms underlying the observed experimental light reflectance differences are not understood at present, although a working hypothesis involves a possible physico-chemical abnormality of the ECM in families at high risk of developing CRC. Given the major role for the ECM micro-environment in angiogenesis and blood vessel geometry,^{23–24} the previously reported finding of an increased oral mucosal microvascular network complexity in HNPCC carriers^{3–19} is in line with this hypothesis.

The present findings provide an additional phenotypic sign in families at high risk for CRC and may possibly lead to a better understanding of the role of the ECM in CRC pathogenesis. This phenotypic marker and its use in a first level screening (that is, identification of patients/families potentially at risk of HNPCC, even in the absence of Amsterdam I/II criteria) could play a role in developing new combined algorithms for identifying the most appropriate time period in which to perform molecular screening and which gene mutation has to be tested first (that is, *MLH1* in the present research). Further studies are needed to validate this new marker in identifying susceptibility to CRC and to investigate its clinical utility in first level CRC screening programmes.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 1435

Close contrast enhanced ultrasound (CEU) examination using Levovist (SH U 508A; Schering, Berlin, Germany) depicted papillary masses in the cystic area (fig 2). Cytology via nasobiliary tube demonstrated class IV findings (Papanicolaou), suggesting adenocarcinoma. The patient hesitated about undergoing an operation as she was symptom free. However, the tumour gradually grew and the patient recently consented to radical surgery, which is scheduled for the near future.

Cholangiocellular carcinomas are grossly characterised into three types: mass forming, periductal infiltrating, and intraductal growth.¹ The clinical manifestation of the last type shows a striking homology with the recently established entity of intraductal papillary mucinous tumour of the pancreas,^{2–8} which shows a relatively better prognosis, slow growth, rarely infiltrating the surrounding parenchyma, and frequently demonstrating an adenoma to carcinoma sequence.^{9–10}

Diagnosis of this entity can be established by obtaining characteristic findings. As indicated in the present case, however, there are limitations to differentiation between mucin material and papillary tumours in the bile duct on endoscopic retrograde cholangiography (ERC) or magnetic resonance cholangiography. In addition, massive amounts of mucin material produced by these tumours frequently cause difficulty in sufficiently opacifying the bile duct lumen with contrast media due to retention of mucin, resulting in diagnostic problems on ERC.⁸ Using CEU, differentiation between mucin and papillary tumours and tumour along the bile duct can be diagnosed when enhancement is demonstrated within intracystic masses or in the bile duct.

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Figure 2 Contrast enhanced ultrasound demonstrating marked enhancement of papillary projections in the cystic mass.