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Limitations of non-ceruloplasmin-bound copper in routine clinical practice

We read with interest the paper on Wilson's disease by Merle *et al* (this issue, p 115). Some aspects of the investigation profile are omitted from the paper. Although data on the prevalence of reduced ceruloplasmin levels and raised non-ceruloplasmin-bound copper levels are provided outside cut-offs, data on mean (and median) levels, SD (interquartile ranges) and any skew or kurtosis are not. This would be useful as the pattern of result distributions in patients with Wilson's disease has substantial implications for the validity of diagnostic algorithms. In addition, this cohort survey clearly showed that some patients with Wilson's disease have ceruloplasmin concentrations within the reference interval (11.8%)—an important message when devising a diagnostic algorithm for Wilson's disease.

According to Roberts and Schilsky,[1] the upper end of the reference interval for non-ceruloplasmin-bound serum copper (NCC) is 15 μg/dl (2.4 μmol/l) and, in most patients with untreated Wilson's disease, the concentration is >25 μg/dl (3.9 μmol/l). They correctly went on to state that interpretation is difficult as the NCC depends on satisfactory copper and ceruloplasmin assays. Since then, we have shown that the upper reference interval in our population was 40 μg/dl (6.3 μmol/l)[2] but that the lower reference interval was −18.4 μg/dl (−2.9 μmol/l), which is clearly not possible and thus supports the assertion by Roberts and Schilsky about the need for satisfactory copper and ceruloplasmin assays. Furthermore, 40 μg/dl is considerably higher than the cut-off of 25 μg/dl used by Merle *et al* to detect 86.6% of patients with Wilson's disease; accordingly fewer patients would be detected using a more appropriate NCC cut-off. This, with the negative values found in 20% of normal patients, means that the NCC is a poor diagnostic test for the detection of Wilson's disease.

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Heredity and DNA methylation in colorectal cancer

In their interesting review of colorectal cancer (CRC) as a model for epigenetic tumourigenesis, Wong, *et al* (in this issue, p 139) discuss the role of hereditary factors in explaining the aetiology of CRC with DNA methylation. Firstly, they assert that larger studies do not support a hereditary aetiology for the CpG island methylator phenotype (CIMP). Secondly, they suggest that *MLH1* epimutation (or germline hemi-allelic methylation) may be heritable. Both of these propositions may be challenged.

In the case of an inherited predisposition to CRCs with acquired DNA methylation or CIMP, a family cancer clinic-based study that excluded families with the Lynch syndrome, found that members with CRCs showing the CIMP had a 13-fold increased risk of having a first-degree relative with cancer (not necessarily CRC) as compared with those without CIMP-positive CRC.[1] A hospital-based study by Ward *et al*[2] could not confirm this finding, but it is pertinent that they excluded families considered to have hereditary nonpolyposis colorectal cancer (HNPCC). Ward *et al* regularly use clinical definitions for HNPCC.[3] The exclusion of ''HNPCC'' families is likely to have introduced a major bias, as not all families meeting a clinical definition for HNPCC (eg, the Amsterdam criteria) in fact show evidence of DNA mismatch repair deficiency that would be indicative of a germline defect in a DNA mismatch repair gene.[4]

The second large study cited by Wong *et al*[5] was a population-based series that assessed CIMP in 864 CRCs and defined CIMP-high as the presence of methylation in at least two of five markers.[5] This is not a stringent definition of CIMP-high as indicated by the low frequency of *BRAF* mutations (32/182, 17.6%) among CIMP-high/DNA microsatellite stable (MSS) CRCs. As Wong *et al* point out, an inherent difficulty in establishing whether genetic factors may explain CIMP is the lack of an agreed definition of CIMP. It is clear, however, that mutation of *BRAF* cosegregates with extensive CIMP[6] and may therefore be used as a surrogate for high-level CIMP. In the same large population-based study group, but now stratified on the basis of *BRAF* mutation,[7] the findings were different. In the subset of MSS CRCs, the odds ratio for having a positive family for subjects with *BRAF* mutation-positive CRCs was 4.23 (95% confidence interval 1.65 to 10.84) (as compared with subjects with *BRAF* mutation-negative CRCs). However, among subjects with high microsatellite instability (MSI-H) CRCs, a stronger family history of CRC was observed when cancers did not have the *BRAF* mutation.[7] This is only to be expected, as subjects with MSI-H CRCs that lacked *BRAF* mutations were relatively young and a proportion would have Lynch syndrome.

In 2005, a further paper described a series of 11 Lynch syndrome-like families, in which some CRCs were MSI-H but others had low-level MSI or were MSS.[8] Both CRCs and polyps in these ''MSI-variable'' families showed frequent mutation of the oncogene *BRAF* or methylation of the CIMP marker MINT31.[8] Affected subjects also had serrated polyps and two had hyperplastic polyposis. On the basis of these observations, it was suggested that an inherited predisposition to acquired DNA methylation in somatic tissues could give rise to a ''serrated pathway syndrome''.[8] Should *MLH1* be implicated, then one might observe the development of CRCs that were MSI-H. This would only apply to a subset of CRCs, but could by chance, as in the case of a similar serrated pathway syndrome family described in 1997,[9] affect all CRCs tested in a single family.

Loss of imprinting (LOI) of *IGF2* in normal colonic mucosa and normal leucocytes has been associated with a personal and family history of CRC.[10] A study from Japan has shown that IGF2 LOI was more frequent in CRCs with the features of CIMP.[11] The link between CIMP and LOI may be explained by methylation of the H19 differential methylated region.[12] These observations provide further evidence for the heritability of CIMP.

Turning to germline hemi-allelic methylation of *MLH1* (or *MLH1* epimutation), Gazzoli *et al*[13] were the first to identify an early-onset CRC, in which one *MLH1* allele showed methylation. Interestingly, the same allele was also found to be methylated in the subject's lymphocytes. By elegantly exploiting the existence of a common polymorphism in the promoter region of *MLH1*, Gazzoli *et al* succeeded in showing that the wild-type *MLH1* allele had been lost in the CRC. On this basis, they introduced the concept of germline hemi-allelic methylation of *MLH1* as a cause of ''HNPCC''.[13] However, they were unconvinced that a methylated allele could be transmitted vertically from parent to child, and concluded that the finding was likely to be both rare and sporadic. Miyakura *et al*[14] reported four more examples of early-onset MSI-H CRC associated with germline hemi-allelic methylation of *MLH1*. Although the patients were ascertained through cancer family clinics, they did not have family histories suggestive of Lynch syndrome. They were merely young and some had multiple tumours consistent with Lynch syndrome. Again, Miyakura *et al*[14] did not

imply that germline hemi-allelic methylation of *MLH1* could be transmitted vertically.

A paper from Ward's group arrived at a different conclusion with respect to germline hemi-allelic methylation or epimutation of *MLH1*.[3] They documented two additional subjects carrying an *MLH1* epimutation, who also met clinical criteria indicative of a diagnosis of ''HNPCC''. Additionally, the epimutation was present in spermatozoa of one of the affected subjects. The second finding not only fitted with a germline defect but also provided evidence for vertical transmission of the defect. The authors therefore advanced the concept of *MLH1* epimutation as a new cause of HNPCC.[3] Nevertheless, it may be questioned if epimutations can in fact be inherited. Although germline hemi-allelic methylation was indeed shown in single members of two families that met certain clinical criteria for HNPCC, this is hardly surprising as the search for the epimutation was conducted exclusively in members of families registered in cancer family clinics. This ascertainment bias aside, it is now clear (as stated above) that when a family happens to meet a particular clinical definition of ''HNPCC'' this does not automatically prove the existence of an underlying altered DNA mismatch repair gene (the basis for Lynch syndrome).[4] Although one of the affected subjects indeed showed methylation of *MLH1* in spermatozoa, this was in <1% of spermatozoa.[3] Should such an affected sperm succeed in fertilising an ovum, subsequent clearance of methylation during early embryogenesis would negate the effects of vertical transmission of the affected allele.

Ward *et al* subsequently showed the de novo origin of germline hemi-allelic methylation of *MLH1* in a male subject who was shown to have inherited the methylated allele from his mother in whom the same allele was not methylated.[15] These authors nevertheless continued to claim that *MLH1* epimutation was ''weakly'' heritable, although they also contradicted themselves in the same paper by asserting that there was no evidence that *MLH1* epimutation could be inherited. Wong *et al* now cite the four preceding reports on this topic as providing evidence for the heritability of germline epigenetic change.

In summary, the balance of evidence suggests that genetic mechanisms will be found to at least partially explain the evolution of CIMP-positive CRCs and will account for a subset of families that may mimic Lynch syndrome. On the other hand, there is no evidence to support the inheritance of *MLH1* epimutation.

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Methylene blue but not indigo carmine causes DNA damage to colonocytes in vitro and in vivo at concentrations used in clinical chromoendoscopy

Identification of mucosal abnormalities is aided by the use of dyes during colonoscopy (chromoendoscopy).[1] Two dyes that have found particular favour are methylene blue and indigo carmine.[2 3]

Methylene blue, which, unlike indigo carmine, is taken up by cells, induces cellular DNA damage in vitro via the generation of singlet oxygen when photoexcited by white light.[4] In contrast, indigo carmine appears to be photostable and to possess little potential to damage genetic material in vitro.[5 6] A recent clinical study has shown that the extent of DNA damage (particularly oxidative DNA damage) in human oesophageal cells is increased after methylene blue chromoendoscopy.[7] Additional iatrogenic oxidative DNA damage to epithelial cells is of particular concern in such precancerous tissue because of the association between oxidative DNA damage, mutagenesis and the development of malignancy.[8] We hypothesised that indigo carmine would induce less DNA damage than methylene blue both in vitro in cultured colon cells during simulated chromoendoscopy conditions and in vivo in colonic biopsy samples collected at chromoendoscopy.

We used the alkaline comet assay to determine DNA damage in the cells treated with methylene blue/indigo carmine and white light. This is a sensitive technique for analysing and measuring such damage in mammalian cells, with the percentage of DNA in the comet tail being linearly related to DNA damage.[9] The inclusion of the DNA repair enzyme, Fapy-DNA-glycosylase (FPG), in the comet assay results in the excision of oxidised guanines to yield additional DNA strand breaks that are detectable in the comet assay.[10] This allows an estimation of specific oxidative DNA damage to cells. In all the experiments described below, the cell viability exceeded 70%.

To simulate chromoendoscopy in vitro, 50 µl of either 0.1% methylene blue or 0.1% indigo carmine dye was added to a monolayer of cultured $CaCo_2$ adenocarcinoma cells for 2 min in the presence and absence of cold white light. Only low levels of DNA damage are found in cells in both the alkaline and the FPG-modified comet assay when the exposure is to white light alone (fig 1). Treatment with indigo carmine either in the light or in the dark, or with methylene blue in the dark did not result in any major change in the extent of DNA damage compared with controls. In contrast, cells treated with methylene blue in the light showed a salient increase in DNA damage compared with controls in both the alkaline and the FPG-modified comet assay ($p<0.001$).

For in vivo experiments, ethical approval and patient consent were obtained to take biopsy

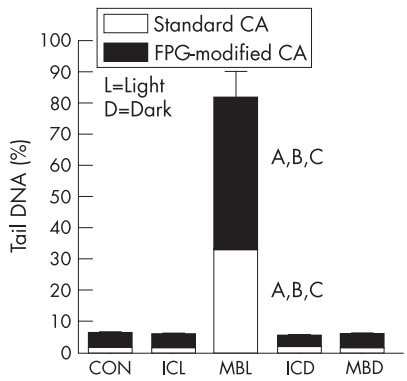

Figure 1 $CaCo_2$ cells exposed to 0.1% methylene blue (MB) or 0.1% indigo carmine (IC) either in white light or in the dark for 2 min. Control cells (CON) were treated with white light only (no dye). Data are presented as mean (SE) tail DNA (%) for three experiments. Both the DNA damage from the alkaline comet assay (CA; no fill) and the additional damage with Fapy-DNA-glycosylase (FPG; fill) are shown. (A) Significance at $p<0.001$ relative to control cells in the alkaline or FPG-modified comet assay. (B) Significance at $p<0.001$ in methylene blue versus indigo carmine cells under the same experimental conditions (either in the light or in the dark) in the alkaline or the FPG-modified comet assay. (C) Significance at $p<0.001$ in methylene blue-treated cells in the light versus in the dark in the alkaline or FPG-modified comet assay.