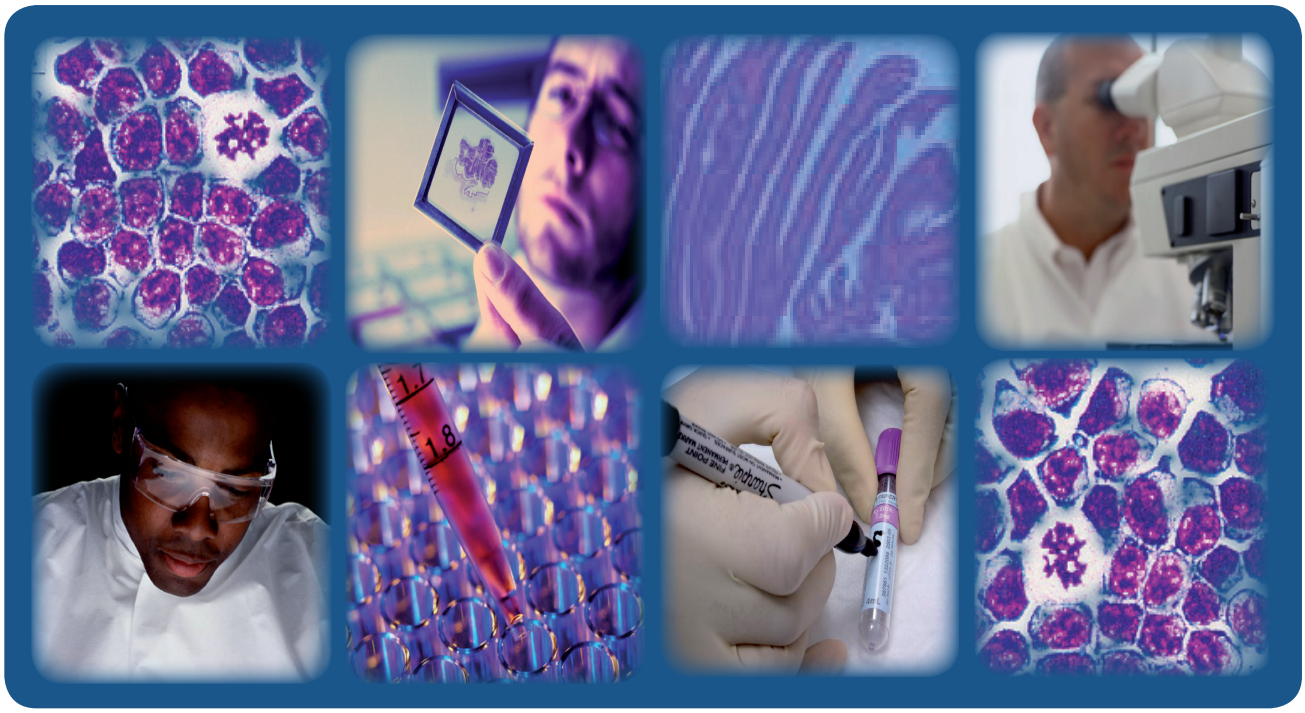
Biomarkers in familial colorectal cancer screening

Expert workshop, 14th February 2006



Workshop report & recommendations

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September 2006

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1. Background

Colorectal cancer

Cancers of the large bowel (colon and rectum, referred to collectively as colorectal cancer) are the third most common form of cancer in the UK, with a total annual incidence of around 35,000. Colorectal cancer accounts for some 16,000 deaths annually, making it the second leading cause of cancer deaths in the UK.

Although the majority of colorectal cancer cases are classed as sporadic (arising in individuals in whom there is no significant family history of the disease), it is thought that as many as a quarter of cases may involve some hereditary predisposition. A small proportion of these are familial colorectal cancers, in which a very strong genetic predisposition to develop colorectal cancer is passed on within affected families.

Familial forms of colorectal cancer

There are several familial forms of colorectal cancer, of which the most common are hereditary non polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP), which account for around 1-5% and 1% of all colorectal cancer cases, respectively. FAP is caused by inherited mutations in the APC tumour suppressor gene, although a small number of familial polyposis cases have been found to involve germline mutations in the *MYH* gene, and this sub-group of disease has been termed MYH-associated polyposis (1). Other inherited syndromes associated with an increased risk of colorectal cancer, such as juvenile polyposis (JP) and Peutz-Jehgers syndrome (PJS), are very rare.

HNPCC, the most common form of inherited colorectal cancer predisposition, was originally described by Alder Scott Warthin in 1913 (2), and first recognised as being clinically distinct from FAP and other familial colorectal cancers in the 1960s by Henry Lynch; it is therefore also known as Lynch Syndrome (3).

HNPCC / Lynch Syndrome

The true incidence of HNPCC is unknown; estimates of about 2% of all colorectal cancer cases are widely quoted, which would represent around 700 colorectal tumour cases in the UK each year, but estimates vary between 1 and 10%. However, although up to around 10% of all cases in the general population may occur in individuals with significant familial predisposition to colorectal cancer, evidence suggests that only around 1% are due to HNPCC (4-11), Unlike FAP patients, individuals affected by HNPCC develop few colorectal polyps, but these polyps are adenomas and tend to have aggressive features, often progressing very rapidly to cancer within 1-2 years (12). HNPCC colorectal cancer patients are not only generally younger than sporadic colorectal cancer patients, with an average age at tumour onset of 40-50, but also tend to show divergent clinical features. More cancers are found in the proximal part of the colon (caecum, ascending colon and transverse colon) compared with sporadic cancers, and there is an increased risk of developing additional, multiple primary colon tumours (13).

The estimated lifetime colorectal cancer risk in individuals with HNPCC is high, at least 70-80% (14); in affected women there is some evidence that the risk of colorectal cancer is lower (15), but the lifetime risk of endometrial and ovarian cancers are 30-61% and 12%, respectively (16), and as with colorectal tumours the average age of onset is significantly lower than usual. In some individuals, susceptibility is also increased to other types of cancer, including cancer of the small bowel, stomach, biliary and pancreatic system, kidneys and urinary tract.

Due to the increased incidence of non-colorectal cancers, it has been proposed that the name HNPCC is misleading, and that Lynch Syndrome is a preferable term to use for the condition. Historically, HNPCC within families was subdivided into two groups, Lynch Syndrome I and Lynch Syndrome II, based on the absence or presence of other forms of cancer (extracolonic cancers) in addition to colon cancer (17). Rarely, brain and skin tumours occur in association with HNPCC, and these clinical combinations have

been previously described as the Turcot and Muir–Torre syndromes, respectively (14).

International Collaborative Group on HNPCC criteria (11)

1. Familial clustering of colorectal and/or endometrial cancer[a]
2. Associated HNPCC-related cancers (HRC): gastric, ovary, ureter/renal pelvis, brain, small bowel, hepatobiliary tract and skin (sebaceous tumours).a
3. Development of cancer at an early age.
4. Development of multiple cancers.
5. Features of colorectal cancer: (a) predilection for proximal (right-sided) colon; (b) improved survival; (c) multiple colorectal cancers; (d) increased proportion of mucinous tumours, and tumours with marked host-lymphocytic infiltration and lymphoid aggregation at the tumour margin.
6. Features of colorectal adenoma: (a) the numbers vary from one to a few; (b) increased proportion of adenomas with villous histology and (c) high grade dysplasia, (d) probably rapid progression from adenoma to carcinoma.
7. High frequency of Microsatellite Instability (MSI).
8. Immunohistochemistry: loss of MLH1, MSH2, or MSH6 protein expression in tumours.
9. Germline mutation in MMR gene: MSH2, MLH1, MSH6, PMS2.

Any combination of 1) to 9) may be present, but 9) alone defines HNPCC.
[a] *Endometrial cancer is classed as an HRC*

Diagnosis of HNPCC is based on fulfilment of clinical criteria, as per the table on the following page:

The original Amsterdam research criteria developed in 1990 have been followed by less stringent criteria: modified Amsterdam (18) and Amsterdam II (19). The so-called Bethesda (20) and revised Bethesda (21) criteria are an attempt to help in deciding clinically which patients should have tumour testing.

Genetics of HNPCC

HNPCC is inherited in an autosomal dominant manner (that is, children of an affected parent have a 50% probability of inheriting the condition) and is caused by mutations in mismatch repair (MMR) genes. The DNA mismatch repair system plays an important role in maintaining the integrity and normal function of DNA. Faulty DNA repair can lead to cancer by allowing the accumulation of various mutations within a cell that may subvert normal control processes (by inactivating tumour suppressor genes or activating oncogenes) and permit unregulated cellular proliferation (growth).

Multiple different gene products from the *mutS* and *mutL* gene families are involved in mismatch repair and mutations in several have been associated with susceptibility to colorectal cancer: *MSH2*, *MSH3*, *MSH6*, *MLH1*, *MLH3*, *PMS1* and *PMS2*. Up to 90% of HNPCC cases are caused by germline (inherited) mutations in the *MSH2* (60%) or *MLH1* (30%) genes; currently, standard genetic testing is based on sequencing these two genes, and generally reveal mutations in about 85% of HNPCC families.

There is some evidence to suggest that a subset of HNPCC patients may have *MSH6* mutations, associated with generally later onset colorectal or endometrial cancers that often do not fulfil classic criteria for HNPCC (22) and these mutations may also be tested for (23).

Mutation testing for HNPCC

Genetic testing for HNPCC is a technically complex process; not only is there more than one gene commonly involved, but there are also more than 200 known mutations of different types spread throughout the genes. If a mutation is identified in an affected family member, testing for the presence of the same mutation in other family members is relatively straightforward, and it is then possible to inform family members whether or not they have HNPCC and are at risk of colorectal and other cancers based on whether or not they possess the familial mutation.

The *presence* of a familial mutation would indicate a need for increased levels of medical surveillance (regular colonoscopy or sigmoidoscopy) or preventative treatments (prophylactic colectomy) in order to avoid, or ameliorate the impact of, future cancers. The *absence* of the mutation would absolve other relatives from concern, or the necessity for frequent and ongoing surveillance measures for the early identification of tumours.

However, failure to identify a mutation in a family considered to be at high risk of HNPCC based on clinical criteria does not mean that a mutation is not present. For example, the mutation may be in one of the other, less common MMR genes. Mutation testing using methods based on DNA sequencing does not detect large deletions or duplications in the MMR genes; since these may account for 5-10% of mutations on the genes *MLH1* and *MSH2* it has been suggested that routine mutation detection should therefore include gene dosage analysis (24).

Further, it is worth noting that although the names HNPCC and Lynch Syndrome are often taken to be synonymous with the presence of hereditary defects in MMR genes, there are nevertheless families that fulfil the clinical criteria for HNPCC but do not have detectable MMR mutations.

It is necessary for individuals to undergo appropriate genetic counselling before undergoing testing, in order to make sure they understand issues such as the probability of finding a mutation, the clinical utility of genetic testing results, and the wider implications of the process both for the individual and for their relatives. The negative psychological factors surrounding the potential identification of a mutation that may, if present, have a major effect both the individual and family members are significant and mutation testing is not a decision to be taken lightly.

Although detection of a mutation is very useful for directing the identification and clinical management of affected family members, the technical process of testing for mutations in the MMR genes is expensive, and for these reasons pre-screening to identify the highest risk patients is often considered to be desirable.

There are two current techniques of pre-screening tumours: testing for microsatellite instability (MSI), and immunohistochemical (IHC) analysis of colorectal tumour samples from individuals who are at increased risk of having HNPCC based on their family history and/or clinical features. These techniques look for the presence or absence of *biomarkers*, indicators of disease or disease susceptibility; in this instance, indicators of the presence of MMR mutations associated with familial colorectal cancer.

The presence of abnormal results from MSI and/or IHC testing is generally taken to be a strong indication of the presence of MMR mutations, suggesting that patients should go forward to consider full mutation testing. Negative results may suggest that mutation testing is not warranted in the absence of additional factors, because the probability of identifying an MMR mutation in these patients is low. For example, one study found that over 90% of patients with an *MLH1* or *MSH2* mutation showed abnormal MSI or IHC results (25). Unfortunately due to the cost of mutation testing there are no published data comprehensively looking at large numbers of patients meeting the more sensitive (less specific) clinical criteria to detect mutations where a pre-screen has not been done. However, the prevalence of mutations in this group (IHC normal and MSI stable or low) is likely to be low based on current knowledge.

Microsatellite instability (MSI)

Errors can arise in DNA during cellular replication, and mismatch repair is one of a number of mechanisms to correct different types of error that may occur, in this instance those that create mismatched base pairs or mispaired loops in the DNA. These loops arise from faulty replication of regions of DNA known as microsatellites. Also known as short tandem repeats (STRs) or variable number tandem repeats (VNTRs), microsatellites are widely distributed throughout the human genome and comprise multiple tandem repeats of a short (1-6 nucleotide) sequence. In the absence of normal mismatch repair, the presence of mispaired loops within microsatellites means that if the DNA replication apparatus 'slips' and produces a different number of sequence repeats in the daughter DNA from the original parent DNA (for example, $(CA)_{14}$ instead of $(CA)_{15}$), these errors are not corrected and the number of repeats can vary, a phenomenon known as microsatellite instability, or MSI.

This gradual accumulation of abnormally long or short microsatellite sequences is present in 10-15% of sporadic colorectal cancers, but in around 90% of those from individuals with HNPCC. Testing a colorectal tumour sample for microsatellite instability is therefore a useful way of assessing whether full mutation testing is appropriate.

MSI testing typically analyses at least five different DNA microsatellite regions (26) from the 'Bethesda panel' of markers of an individual's normal and tumour cells, and the tumour's microsatellite instability status is classified as microsatellite stable (MSS), low (MSI-L), or high (MSI-H), depending on how many loci (positions) show high-frequency changes.

An MSI-H rating means that instability was observed at more than 40% of markers used (*i.e.* ≥2/5 markers). If instability is seen at a single locus, testing of a further five markers is recommended; if abnormal loci comprise less than 40% of all markers tested, the tumour is classed as MSI-L (20). High levels of MSI are suggestive of the presence of HNPCC related mutations in the tumour, some of these will be acquired in the tumour only (somatic), and some will be due to an inherited mutation and second somatic mutation in the normal (wild type) allele.

Mutations in the *MSH6* gene can be associated with MSI-L. There is some degree of genomic instability in most cancers so if enough markers are examined one or more is likely to appear unstable, so MSI-L may have no particular clinical significance.

Immunohistochemistry (IHC)

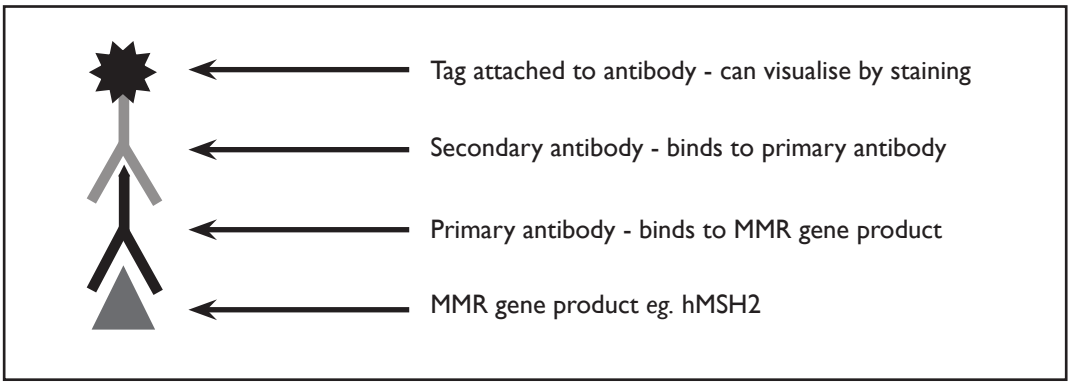
Immunohistochemistry is a technique for testing tumour samples for the loss of expression of specific genes, in this instance the MMR genes associated with HNPCC.

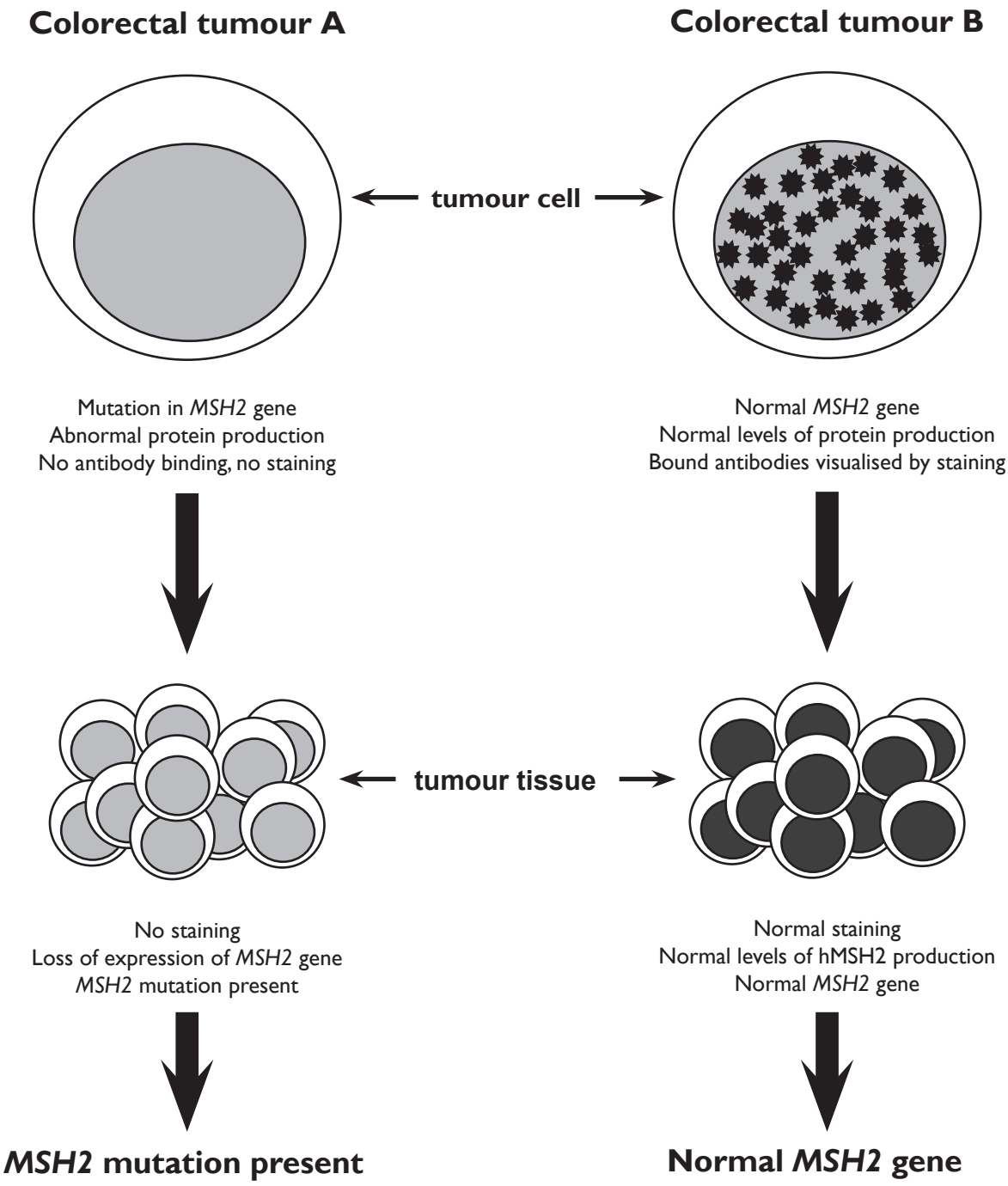
Antibodies against the MMR proteins (hMSH2, hMLH1, hMSH6 and hPMS2) are applied to paraffin-embedded tumour tissue samples and visualised by staining. In some cases secondary antibodies carrying a tag that can be visualised are bound to the primary antibodies against the MMR protein. Areas that take up the stain show that the antibody has bound to the corresponding protein. Where tumour cells fail to show staining (but surrounding normal cells do), there is considered to be a loss of expression of the protein in question.

IHC demonstrating loss of expression of one of the MMR proteins in tumour correlates very closely with MSI however MSI is less specific and a proportion of MSI-H and certainly MSI-L tumours will have normal IHC. IHC when abnormal indicates which gene is likely to carry the mutation (and hence when used as the first test prior to full genetic analysis can direct testing to one gene rather than testing both), although accurate interpretation of immunohistochemically stained tissue slides is not simple and requires an experienced eye.

There have also been reports of IHC testing indicating a loss of expression despite no mutation having been identified by sequencing, which could be indicative of a large MMR gene deletion, or sporadic methylation of the *MLH1* gene promoter.

Simplified representation of IHC for hMSH2 protein expression





Identification of HNPCC in colorectal tumour patients

The decision process for genetic testing for HNPCC in high–risk individuals is not clear-cut, and varies between different countries and centres. The guidelines have altered over time to take account of the increasing knowledge about the mutations involved in HNPCC, and the recognition that families existed which failed to meet the strictest definitions for HNPCC but nevertheless showed a strong familial predisposition to colorectal cancer that was highly suggestive of the condition.

The latest, revised Bethesda guidelines (21) for HNPCC (Lynch Syndrome) propose that tumours should be tested for MSI (and if found to be MSI positive, tested for HNPCC mutations) in the following situations:

1. The patient is younger than age 50.
2. The patient has multiple HNPCC-associated tumours in the colon or other areas associated with the same mutations, either at the same time or occurring over a period of time.
3. A patient younger than age 60 has colorectal cancer that has microscopic characteristics that are often indicative of MSI.
4. A patient has one or more first-degree relatives who had an HNPCC-related tumour at age 50 or younger.
5. A patient has two or more first- or second-degree relatives who had HNPCC-related tumours at any age.

There has already been considerable research into the relative merits of MSI and IHC as pre screening strategies, although there is no consensus. Lindor and co-workers found that IHC for hMLH1 and hMSH2 proteins was a rapid, cost-effective, sensitive highly extremely specific alternative to MSI for screening colorectal tumours for DNA mismatch repair defects, but noted that it would fail to identify some MSI-H tumours if performed alone (27).

A later study found that 100% of MSI-H tumours showed loss of expression of the hMLH1, hMSH2, or hMSH6 proteins, and proposed that hMSH6 should be included in routine IHC screening (28), whilst another advocated the additional inclusion of hPMS2 staining, reporting that the use of four antibodies confirmed MSI results in 93% of cases (29).

Hampel *et al.,* while looking at the effect of screening over 1000 unselected US colorectal cancer patients for the presence of HNPCC (specifically, for germline mutations in the *MLH1, MSH2, MSH6,* and *PMS2* genes) concluded that the effectiveness of screening with IHC analysis would be similar to that of genotyping for MSI (30).

In a similar multi-centre Spanish study of over 1200 newly diagnosed colorectal cancer patients, Pinol *et al.* performed both IHC for hMSH2/hMLH1 and MSI testing on all tumour samples, with germline MSH2/MLH1 mutation testing performed for all cases of microsatellite instability or lack of protein expression. They found that 287 of the patients (287) fulfilled the revised Bethesda guidelines; of these, 91 displayed MSI and/or IHC results suggestive of *MSH2*/*MLH1* mutations, and eleven were found to have such mutations. They concluded that the use of either pre-screening strategy would be equally effective strategies to combine with the Bethesda guidelines for the identification of patients who should receive full mutation testing.

Estimated costs per mutation detected of €11989 (£8250) and €10644 (£7324) for MSI and IHC respectively were reported, with the authors proposing that the latter might help to identify a larger proportion of patients with HNPCC on the basis that immunostaining is *"more available than DNA analysis in a clinical setting".* This study did not include any of the less common HNPCC-associated MMR mutations, inclusion of which in IHC analysis might have made it even more effective. MSI had initially showed an MSI-L result in two tumours where germline mutations were detected after abnormal IHC results were obtained and retesting for MSI in another part of the tumour then showed MSI-H results. Unfortunately, the study does not report clearly whether all germline mutations had abnormal IHC plus abnormal MSI, as

samples were tested sequentially as opposed to comprehensively (31).

A German study of 1119 colorectal cancer patients meeting the Amsterdam II criteria or the classical Bethesda guidelines, which sought to identify the most efficient strategy for the use of clinical criteria, IHC and MSI to identify HNPCC, concluded that IHC could not fully replace IHC. Although it was found to be highly predictive and specific, and valuable for indicated the gene likely to be affected, it missed a number of mutations detectable by MSI. The authors proposed a sequential application of both techniques (32).

An earlier paper outlining some of the potential pitfalls in molecular screening for HNPCC called for the use of a combination of clinical data, IHC and microsatellite analysis, making the additional point that these techniques, whilst not requiring the most sophisticated equipment, depended on accurate interpretation of results. They emphasised that results from MSI and IHC analysis had to be interpreted in the context of clinical data, and that IHC should be preformed by *"a pathologist with experience in molecular genetics"* (33).

A UK study of 138 families referred to a Regional Genetics Service with possible HNPCC attempted to determine the optimal strategy for pre-screening prior to *MLH1* and *MSH2* mutation analysis. They concluded that the full Bethesda guidelines were the most sensitive criteria for identifying families with MMR mutations but these showed poor sensitivity, and that IHC and MSI were useful pre-screening tools. It was proposed that the most cost-efficient approach was to use inclusive selection criteria followed by IHC and then MSI, with abnormal results from either or both tests indicating that full mutation testing should proceed. IHC was found to be more cost effective than MSI analysis if only one pre-screening technique was used, but the addition of MSI analysis improved sensitivity with only a moderate increase in cost. The authors noted that in some cases, access to these technologies or to suitable tumour blocks might not be available, in which case more restrictive selection criteria might be the best way of deciding which families should have mutation testing (34).

The workshop

While debate over the best technique and screening strategy continues, most experts agree that either MSI and/or IHC testing should ideally be used prior to germline mutation testing, on grounds of cost and accuracy of results. There are also other factors that support the use of these molecular techniques, for example MSI-H status having been associated with variations prognosis and therapeutic response (35). Within the UK, clinical practice varies between different centres.

It was decided to hold an expert workshop, *Biomarkers in Familial Colorectal Cancer*, to bring together professionals with an interest in the diagnosis of HNPCC (including molecular and clinical geneticists, genetic counsellors, pathologists, colorectal surgeons and policy experts), in order to evaluate current evidence for and against different approaches, and move towards development of a consensus optimal strategy for national implementation. A steering committee (see Appendix 1) was convened to plan the content of the day, and to identify key issues to be addressed by participants.

Over sixty invited delegates attended the workshop, which was held in London on the 14th February 2006, at London House, WC1N 2AB. This report outlines the presentations and discussions from the workshop, along with the conclusions and recommendations developed as a result of these proceedings.

2. The workshop

Welcome and introduction

Dr Ron Zimmern, Director of the Public Health Genetics Unit and the Cambridge Genetics Knowledge Park, opened the workshop and welcomed delegates.

Professor Diana Eccles then set the scene for the day by outlining the difficulty in making the clinical distinction between hereditary non-polyposis colorectal cancer (HNPCC) and other cases of colorectal cancer that show similar features in terms of family history and tumour features. This is much more complex than identifying the rare familial polyposis syndromes.

The purpose of the workshop was to examine the use of testing biomarkers to indicate the likely presence of mutations associated with HNPCC as a screening tool to most appropriately channel cases for full mutation screening. Professor Eccles noted that the responses and conclusions from the expert workshop would form a strong basis for funding proposals for national implementation of the most appropriate system or systems.

PRESENTATIONS

Epidemiology of colorectal cancer

Mr Adam Butterworth
Public Health Genetics Unit / Cambridge Genetics Knowledge Park

The first presentation gave the descriptive epidemiological background of colorectal cancer in the UK, reporting on the overall incidence (almost 30,000 new cases and over 14,000 deaths in 2003 in England and Wales alone) and relevant statistics. Colorectal cancer is the third leading cause of cancer in men and the second in women, with a combined lifetime risk of 4% for the general population. It is also the third leading cause of cancer death, although mortality has been decreasing in recent years.

Risk factors for colorectal cancer were outlined; these included both environmental factors (such as alcohol and dietary factors, low physical activity and the use of non-steroidal anti-inflammatory drugs or NSAIDs) and genetic factors. These fell into two broad groups: low penetrance susceptibility genes (such as specific variants of the *CYPIA*, *GSTM1* and *GSTT1* genes), and the high penetrance susceptibility genes associated with familial forms of colorectal cancer.

The appropriate terminology for individuals with a potential genetic predisposition to cancer was given:

Inherited genetic

Individual with an alteration in a cancer susceptibility gene inherited through the germ line

(a) high risk Mendelian inheritance
(b) lower risk susceptibility interacting with environment

Familial

Individual with a family history, both with and without high-risk alleles

Inherited colorectal cancer syndromes

The most common colorectal cancer syndromes are familial adenomatous polyposis coli (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), accounting for less than 1% and 1-2% of all CRC cases, respectively. FAP is caused by mutations in a single gene (APC), which have a penetrance of virtually 100%, meaning that the lifetime risk of colorectal cancer for individuals with APC gene mutations is almost 100%. HNPCC, in contrast, is caused by mutations in a number of different DNA mismatch repair (MMR) genes – most commonly *MSH2* or *MLH1*, but in some cases *PMS1, PMS2* or *MSH6*. The penetrance of these mutations is somewhat lower, with a lifetime risk of colorectal cancer of 80-85% in affected males and 40-65% in females, although the risk of the associated cancers of the uterus and ovaries among women are around 45% and 10% respectively.

Besides the inherited cancer syndromes, a further 20% of colorectal cancer cases arise in individuals with a significant family history of the disease; these cases may be considered to be 'familial', probably involving shared genetic and environmental factors, but unlike FAP and HNPCC they are not directly hereditary.

Familial risk of colorectal cancer

Data from a recent paper (36) on the relative risks of colorectal cancer based on family history was presented; risk is higher at younger ages and with multiple affected relatives:

One first degree relative	3.2 for people under 50 1.9 for people above 50
Age of affected relative	3.6 for relatives diagnosed under 50 2.2 for relatives diagnosed above 50
Two first degree relatives	5.4

Data from a prospective population cohort study (37) on the prevalence of family history of colorectal cancer was also presented:

At least one first degree relative	6.8%
Two or more first degree relatives	0.3%
One first degree relative under 45	0.3%

Developing absolute risk estimates

For population-level decision making, absolute risk is a more useful measure than relative risk.

Work on developing absolute risk estimates based on published incidence and mortality rates combined with relative risk information derived from literature review was reported; the methodology takes into account the diminishing cohort size due to colorectal cancer incidence and mortality. Results were presented based on age:

10-year absolute risk of developing colorectal cancer by age (%)						
Age	**20**	**30**	**40**	**50**	**60**	**70**
General population	0.01	0.04	0.15	0.51	1.31	2.27
At least 1 FDR *(first degree relatve)*	0.04	0.12	0.46	0.97	2.47	4.27
At least 2 FDRs	0.05	0.15	0.59	2.01	5.10	8.72

Of note, the absolute 10-year risk of colorectal cancer was found to be very low in young people, even those with a strong family history of the disease, and it was suggested that for access to population screening programmes, a certain minimum 10-year risk cut-off would be suitable; for example, a cut-off point of 2% risk would mean that the general population would be eligible for screening from the age of 70, whilst those with two affected first degree relatives would be eligible from the age of 50 (see table, above).

QUESTIONS

1) Is there evidence that relative risk of colorectal cancer remains uniform throughout life?

Mr Butterworth explained that the absolute risk for both the general population and those with a family history of colorectal cancer increases with age, but the relative risk decreases with age.

2) How would population screening for faecal occult blood to detect early colorectal cancer refine risk?

Screening effectively highlights cases for medical attention and intervention, which affects overall prevalence and therefore risk also.

Selection of Families for Mutation Analysis

Dr Hans Vasen
Hereditary Cancer Registry and Department of Gastroenterology
Leiden University Medical Centre (Netherlands)

The second presentation began with an overview of the key features of HNPCC, including multiple or early age onset colorectal tumours, the presence of associated tumours (uterine, brain, small bowel, stomach and urinary tract), the mismatch repair genes involved (MSH2 or MLH1, PMS2 and MSH6) and the frequent presence of microsatellite instability (MSI) in colorectal tumours. Next, Dr Vasen showed data on the colorectal cancer risks for men and women with HNPCC, stratified by mutation carrier status for MSH2, MLH1or MSH6 (38). He also presented data on mortality rates for colorectal cancer, showing a significant decline from 32.3% in the period 1960-75, to 10.1% in the period 1990-2004 due to nationwide surveillance of HNPCC families in the Netherlands (39).

Diagnosis of HNPCC: methods and costs

Moving on to the diagnosis of HNPCC, the 'gold standard' of molecular diagnosis via identification of an MMR gene mutation was outlined. Over 850 different mutations have been registered on the International Society for Gastrointestinal Hereditary Tumours (InSiGHT) database (see http://www.insight-group.org). Of the pathogenic mutations associated with HNPCC, 53% are in the MLH1 gene, 39% in the MSH2 gene and 5% in the MSH6 gene. The costs of genetic testing for these mutations in the Netherlands were given as €600 (around £400) for each gene, with an additional €2300 (around £1600) for genetic counselling, making this 'gold standard' form of testing prohibitively expensive for use in all possible cases of HNPCC.

This being the case, some form of selection of families for full molecular diagnosis is necessary, and the tools that could be used in making this selection were said to be family history, clinical criteria, the use of a predictive model, and forms of genetic tumour testing. A predictive model developed using three key variables (the mean age at development of colorectal cancer, the presence of endometrial cancer, and fulfilment of the Amsterdam criteria for HNPCC) was presented (40).

The relative costs of MSI and IHC in the Netherlands were presented; MSI was said to cost €500 (around £340) compared with only €52 (around £35) for MSI, but the latter was noted to require expert interpretation.

Loss of expression patterns seen in IHC

The significance of the HNPCC-associated MMR mutations in terms of their functional effect was explained; during normal mismatch repair, bulges in the DNA strand where there are mismatched bases are recognised by an MSH2/MSH6 protein complex, which in turn recruits an MLH1/PMS2 protein complex as part of the repair process. For this reason, a loss of expression of MSH2 protein (due to a mutation in the

MSH2 gene) is often accompanied by a loss of expression of its binding partner, MSH6, and so on. These 'loss of expression' patterns (see below) are those observed using IHC analysis of colorectal tumour samples.

Mutation	Loss of expression			
	MLH1	*MSH2*	*MSH6*	*PMS2*
MLH1	+			+
MSH2		+	+	
MSH6			+	
PMS2				+

The Bethesda guidelines

The Bethesda guidelines (21) for identifying possible cases of HNPCC were outlined (see introduction for details) and four key questions were then addressed:

1. What proportion of colorectal cancer cases meet Bethesda guidelines?

The Spanish study of Pinol *et al* (31) found that 24% of a 1222 colorectal cancer patient cohort fulfilled the Bethesda criteria; of these, 7.4% were IHC/MSI positive and 0.9% had mutations in *MLH1*or *MSH2*.

2. What is the yield of MSI/IHC/mutation-analysis in such patients?

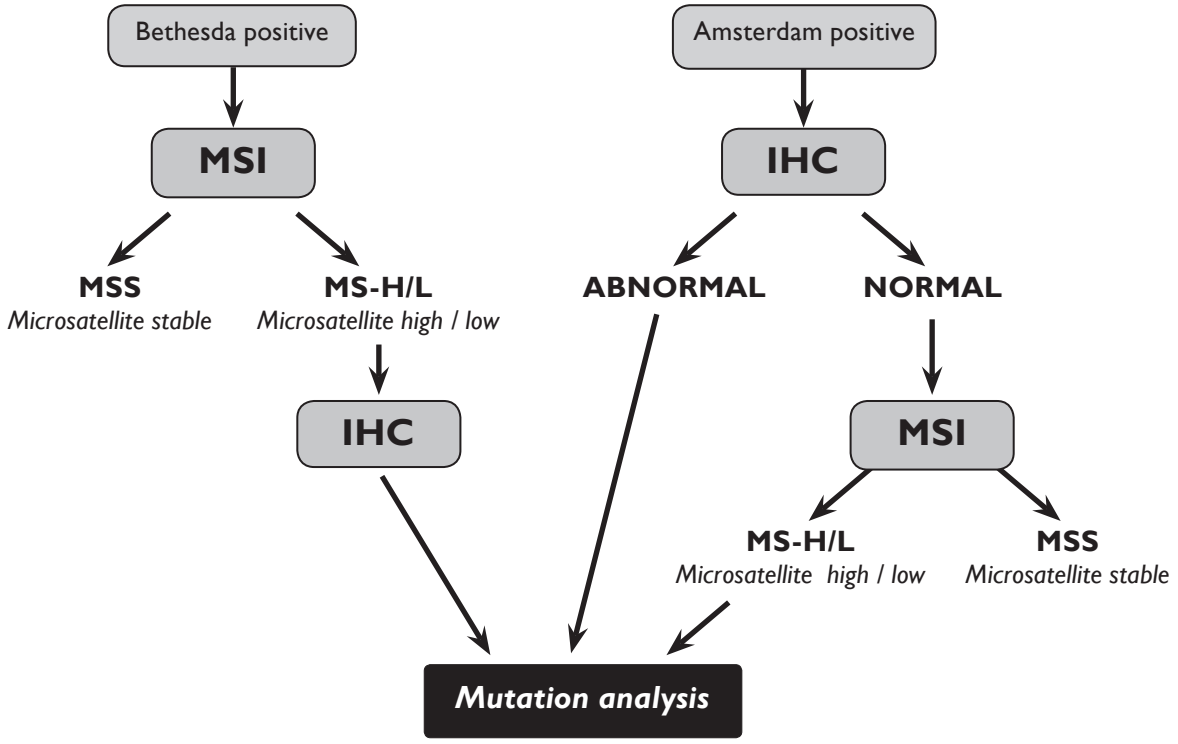
The German study by Engel *et al* (32) found that 20% of a cohort of 1119 families fulfilling Bethesda had mutations in *MLH1*or *MSH2*.

3. What is the sensitivity of MSI/IHC?

Pinol *et al* (31) reported 100% sensitivity for both MSI and IHC; a similar US study by Hampel *et al.* (30) found a sensitivity of only 91% for the techniques, although this study used MSI and IHC testing sequentially and extended analysis to mutations in the MSH6 and PMS2 genes, identifying MMR mutations in 2.2% of the study population, as opposed to 0.6% for the Pinol study. Engel *et al* reported a sensitivity of 94% for IHC and 100% for MSI testing (32).

4. Which is the best protocol?

The strategy being used by Dr Vasen's group in Leiden was outlined (below), along with unpublished results obtained using this strategy.



Conclusions

MSI and IHC analysis of tumours are both reliable, effective tools to select families for mutation testing; the sensitivity of MSI equals or exceeds that of IHC.

For families that meet the Amsterdam criteria for HNPCC, IHC is the best first-line testing strategy because its results may direct any subsequent mutation analysis (screening of the gene or genes found by IHC to show loss of expression). For cases that meet the less stringent Bethesda guidelines, it is better to use MSI as the initial test.

Implementation in clinical practice

The alternative approaches of colorectal cancer patients to mutation testing and screening for HNPCC were outlined; they might be referred via an oncologist or surgeon, on the basis of their family history and/or fulfilment of the Bethesda guidelines, or alternatively they might be referred via a pathologist, on the basis of clinical features (age of onset under 50, multiple colorectal tumours, specific pathological features suggestive of HNPCC). However, it was proposed that there was a general lack of awareness of the Bethesda guidelines, and that even obtaining an accurate family history was a greatly neglected area of clinical practice.

It was also noted that the identification of the MSI status of a colorectal tumour had a prognostic value and could be used to dictate the choice of chemotherapy provided. Notably, MSI-H tumours tend to respond poorly to chemotherapy. This being the case, there is an argument in favour of performing MSI testing on all CRC cases to detect MSI-H tumours (15-20% of the total), which would also increase detection of HNPCC.

As a final point, it was explained that, given the high costs of chemotherapy for advanced colorectal cancer, it is likely that all efforts to increase detection of HNPCC families could be said to be a cost-efficient strategy.

QUESTIONS

1) The MSI test price seemed high in comparison with mutation screening – is this related to problems in obtaining suitable samples from tissue blocks?

Dr Vasen said that it was actually very hard to estimate costs and that there was a high degree of variation between different centres in the Netherlands.

2) Have there been any studies on the ability of pathologists to accurately identify MSI positive tumours?

The answer was, not at the present time.

3) The studies mentioned were all performed in cohorts highly selected for HNPCC on the basis of their family history; could the testing strategies presented be applied to a wider cohort, *ie.* **all CRC cases?**

Dr Vasen said that there had been no systematic analysis of the approaches for this wider group.

4) Would not a risk model be useful so that risk of HNPCC could be assessed prior to surgery for colorectal cancer – for instance, to allow total colectomy as opposed to partial resection of the colon if the risk was found to be very high?

It was Dr Vasen's opinion that total colectomy (removal of all or part of the colon) was only appropriate in definite cases of HNPCC, and that therefore in the majority of cases, even those showing features of possible HNPCC such as early age of onset, it was better to wait for the results of genetic analysis before considering additional surgical interventions.

Microsatellite instability

Dr Ian Frayling
Insititue of Medical Genetics, Cardiff

The third presentation began with the ICG (International Collaborative Group on HNPCC) definition of HNPCC / Lynch syndrome (11) This is based on the presence of any combination of nine clinical features (see page 5). of which the last, the presence of germ-line MMR gene mutations, defines the condition.

Microsatellite instability testing

A microsatellite is a repetitive sequence, with a repeat unit of generally one to six bases. Mononucleotide repeats, such as $(A)_5$ - AAAAAA -or dinucleotide repeats, such as $(TG)_7$ - TGTGTGTGTGTGTG - are present throughout the genome. Microsatellite instability refers to the accumulation of multiple small mutations that affect the length of these sequences. MSI arises as the result of loss of DNA mismatch repair function, due either to point mutations in, or promoter methylation of key genes; it is present in over 95% of HNPCC colorectal tumours, but also in around 15% of 'sporadic', non-HNPCC tumours.

Specifically, MSI is defined by the presence of extra alleles (sequence repeats) in a certain minimum proportion of a set of specific microsatellite markers tested. Some of these markers were discussed, with one example given of a set of 10 markers including three mononucleotide markers (BAT-25, BAT-26 and BAT-40) and seven dinucleotide markers (D5S346, ACTC, D17S250, D13S153, D5S406, D5S107 and D2S123) (41). A typical example threshold of 29% was given, whereby for a panel of ten markers, samples showing instability at three or more markers would be classed as MSI (42;43) and referred to as being MSI-H (high). Where microsatellite instability was detected at lower levels (in this instance, in only one or two out of ten markers), it would be referred to as MSI-L (low), which is not associated with HNPCC.

Examples were shown of microsatellite stability (MSS) and instability (MSI) at different markers (44).

Technical requirements

The material required to perform MSI testing was outlined; tissue requirements are much less stringent than for IHC, with fresh, frozen or paraffin-embedded tissue being suitable along with very small adenomas. In practice, an experienced histopathologist is needed to prepare and select the most suitable tissue blocks, and cutting the blocks and extracting DNA for MSI analysis is also a skilled procedure.

MSI as a predictive test of HNPCC

Data were presented on the performance of MSI, which tends to improve as the number of tumours tested increases (45). Of note, the negative predictive value of MSI for HNPCC is excellent, typically in excess of 99%, whereas the positive predictive value is much poorer. This means that MSI is a very good test for excluding non-HNPCC cases (MSS or MSI L tumours are very unlikely to be HNPCC), but less good for including HNPCC cases (not all MSI-H tumours will be HNPCC cases).

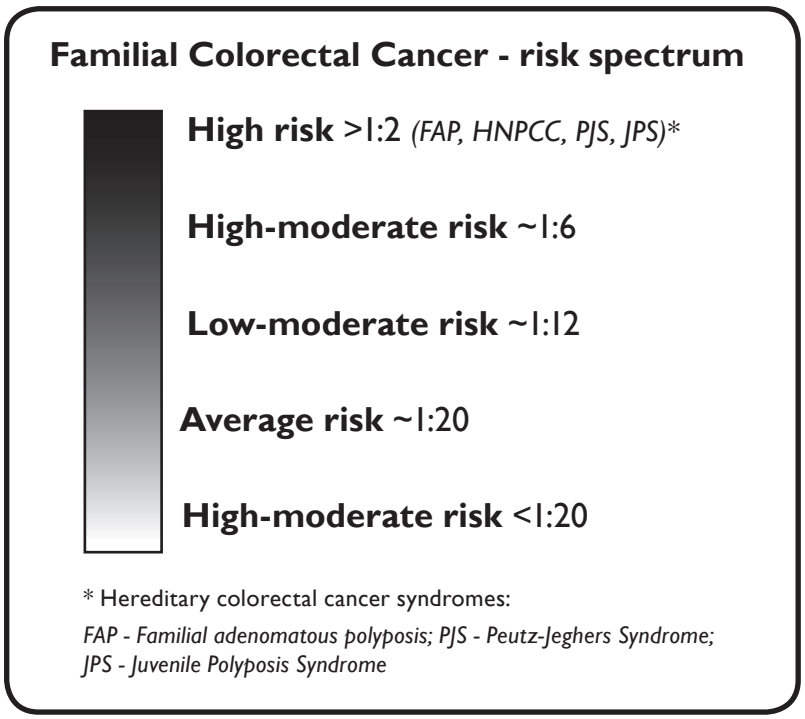
Diagnosis of HNPCC

Although detection of a mutation in one of the key MMR genes is the diagnostic 'gold standard' for HNPCC, it is not always possible. Mutation detection is an expensive procedure that cannot of itself exclude HNPCC even if not mutations are identified; HNPCC may arise from a wide range of different mutations in a number of different genes, only the most common of which are screened for.

The spectrum of familial colorectal cancer was presented (following page); one difficulty in diagnosing HNPCC is that there are other familial forms of colorectal cancer (all individuals with a family history of the disease have a greater than average risk) and that there is significant overlap between different risk categories. The key diagnostic question, therefore, was said to be not *"What is the mutation?"* but rather, *"Has this family got HNPCC?"*

The optimal use of MSI testing was proposed to be as an excluder of HNPCC where another test (such as IHC testing) cannot give definitive information to this effect, and as an includer of HNPCC where the

risk of the condition is *a priori* low. It was also stated that MSI testing must be used as part of a diagnostic process involving other stages of selection.



Integration of mismatch repair immunohistochemistry into the HNPCC diagnostic process

Dr Mark Arends
Department of Pathology, University of Cambridge / Addenbrookes Hospital

The fourth presentation began by outlining key pathological features (pan-colorectal \ proximal colonic location, showing mucinous or poor differentiation, expansive growth, intra-epithelial & peri tumoural lymphoid reactions) and genetic features (microsatellite instability, abnormal DNA mismatch repair protein expression) of HNPCC colorectal cancers. Slides showing examples of specific pathological features were shown. These histopathological features are not specific to (but are associated with) HNPCC; for example, many (~15%) sporadic, non-HNPCC colorectal tumours show MSI.

Analysis of mismatch repair proteins by immunohistochemistry

The process by which key MMR proteins correct DNA mismatches and insertion-deletion loops (in repetitive sequences) was outlined; of note, the protein MSH2, with a binding partner of MSH3 or MSH6, recruits the protein MLH1, with a binding partner of PMS1 or PMS2. The binding partners for the key MSH2 and MLH1 proteins depend on the nature of the DNA fault to be repaired. Immunohistochemistry (IHC) can show loss of expression of the primary MMR protein (due to mutation in the corresponding gene) and this is often accompanied by a corresponding loss of expression of its binding partner.

IHC can be performed to analyse altered MMR protein expression in suspected HNPCC tumours. Methods are well established for MSH2 and MLH1, and there has been a recent trial for MSH6. Antibodies to PMS2 are also available. One technical issue raised was that the antibodies used in the process are sensitive to the degree of fixation of the tissues, which affects preservation of the antigenic epitope recognised by the antibodies used for IHC. Poorly fixed tissue specimens may be a particular problem

for colorectal tumour samples because there is often a delay before fixation and some tumours are insufficiently fixed prior to processing. It was proposed that the use of an automated immunostaining process (using either the DAKO TechMate or the Vision BioSystems BondMax autostaining machines) was essential to produce acceptable reproducibility between IHC assays. Key features and brief protocols for these alternative systems were outlined.

An extensive series of IHC stains of different tumours were presented, along with explanations of their interpretation and the difficulties posed by poor fixation. When there is an absence of staining observed in tumour cells, it is only possible to conclude that there is loss of expression of the protein in question if the adjacent (non-tumour) stromal or lymphoid cells show positive nuclear staining; poor fixation can prevent clear positive staining of these surrounding cells, making the IHC results inconclusive.

Another problem presented was that of a positive but weak and patchy immunostaining pattern seen in some cases; a consistent pattern of patchy/weak immunostaining is sometimes observed in different individuals and tumours from within a particular HNPCC family. Where patchy immunostaining occurs, IHC for the corresponding binding protein can be useful to confirm whether the apparent loss of expression of the primary protein is genuine (in which case the binding partner is likely to show lost or abnormal expression too) or the result of technical/fixation problems.

It was noted that around 20-30% of HNPCC (MSI-positive) cancers were apparently normal for MMR IHC and this is a significant limitation of IHC as a test for HNPCC tumours, along with the similarity in appearance of sporadic cancers that do not express MLH1 (due to promoter methylation) with HNPCC cancers with mutations in the hMLH1 gene that lead to loss of expression of MLH1 protein.

Conclusions

Effective IHC analysis of tumour samples was concluded to require a consultant histopathologist with a particular interest in and understanding of both HNPCC and the role of genetics services in diagnosis of the condition. Specifically, the interpretation of MMR immunostains and review of histopathological and other features is a highly skilled process, and this has implications for the training of future histopathologists. The best approach to identifying cases of HNPCC is for pathologists and geneticists to work together.

IHC itself was concluded to be a good screening method for suspected HNPCC, especially as an 'includer', to identify the presence of HNPCC (as opposed to exclude it). Around 70-80% of MSI positive colorectal tumours are found to show abnormal IHC for MMR proteins (ie. loss or abnormal expression). It is hypothesised that the in remaining 20-30% of tumours that show normal IHC results despite the presence of microsatellite instability, an MMR mutation is likely to be present that destroys the mismatch repair function of the corresponding protein, but which leaves the overall protein structure intact including the antigenic epitope, so that it remains detectable by immunohistochemistry. Hence, IHC detects the presence of structural abnormalities in the key MMR proteins, whilst MSI detects functional abnormalities of the MMR system.

It was therefore proposed that the two forms of analysis were complementary and should both be included in the diagnostic process for HNPCC as an intermediate stage of selection between the identification of a significant family history of colorectal cancer and full mutation screening. The presence of abnormal MMR IHC is sufficient to warrant referral for mutation screening; normal IHC results do not necessarily exclude the possibility of HNPCC, so these cases should be tested for MSI and referred for mutation screening if they are positive for MSI.

IHC can also inform the process of downstream mutation detection, not only by directing the choice of MMR gene to begin screening (based on which MMR protein/s showed loss of expression – thus making the most efficient use of limited genetic screening resources), but also in allowing interpretation of some DNA sequence changes, where it is uncertain whether or not they are pathogenic mutations (causative of HNPCC) or merely polymorphic changes (normal variants within the gene that do not actually cause HNPCC).

Tumour testing: IHC or MSI?

Following the presentations on MSI and IHC, Dr Frayling addressed the question of which technique should be used in greater detail. It was concluded that both techniques were needed, their use depending which of two particular clinical situations was relevant:

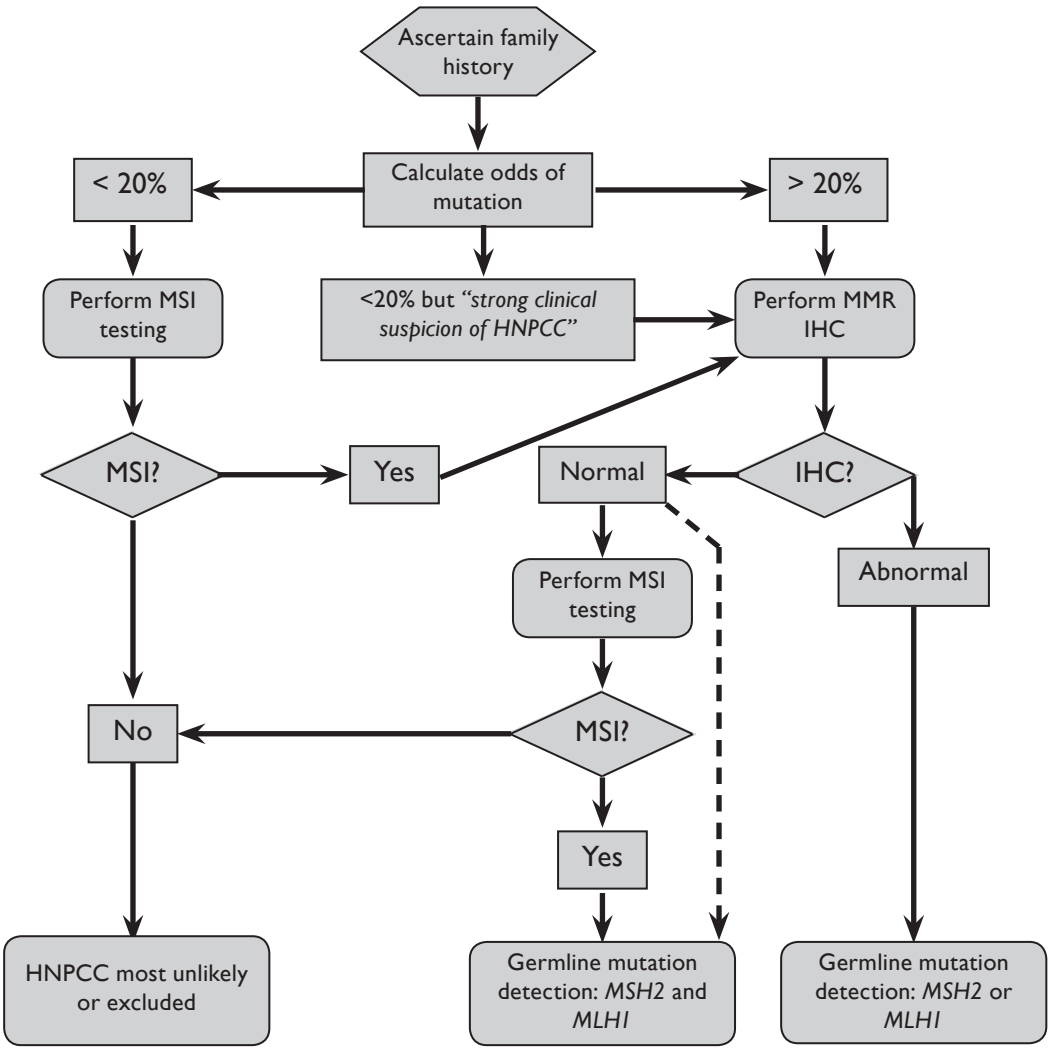
1) Clinical HNPCC (greater than 20% familial risk) – which gene is affected?

2) High-moderate familial risk – is it HNPCC?

In cases of colorectal cancer where there are clear indicators of a high level of familial risk, there is a high probability that it is HNPCC (and even if no causative mutation is identified, the family members of the individual with cancer would be considered at high risk). The key question in this situation is therefore which gene is affected, and what is the mutation? Abnormal IHC results from the tumour would effectively confirm the diagnosis of HNPCC and allow targeted mutation detection and interpretation. The presence or absence of MSI would effectively include or exclude the possibility of HNPCC, respectively, independent of the IHC result.

Where the level of familial risk is clearly raised, but falls below a suggested 'high-risk' cut-off of 20% HNPCC probability, the absence of MSI essentially excludes the possibility of HNPCC (and in the vast majority of samples tested), whilst the presence of MSI is strongly suggestive of HNPCC, irrespective of IHC results. As before, an abnormal MMR IHC result allows a confident diagnosis of HNPCC, targeted mutation detection and interpretation. BRAF mutation testing was also mentioned; a specific mutation in the *BRAF* gene (V600E) is present in around 40% of 'sporadic' colorectal tumours that show MSI, but is not present in HNPCC tumours (46). This form of testing may therefore have utility in discriminating between HNPCC and non-HNPCC MSI tumours.

A diagnostic algorithm for the identification of HNPCC (44) was presented (below):



Requirements for molecular analysis of tumours in HNPCC

Performing molecular tests as part of the process of identification/exclusion of HNPCC was concluded to represent a superior direction of resources, resulting in savings in both time and money.

Requirements for carrying out these tests were then outlined, centred on the need for a multidisciplinary team comprising both pathologists and geneticists. Key roles identified within such a team were histopathologists and genetic pathologists, immunohistochemists and molecular or clinical cancer geneticists.

Additional issues mentioned were the need to obtain tissue blocks for testing, with appropriate consent; sharing of information and experience (including use of the DmuBD, Diagnostic Mutation Database, at **http://www.dmudb.net**), and sources of potential help and advice. These included the Clinical Molecular Genetics Society (CMGS) best practice guidelines for HNPCC and the national External Quality Assessment Scheme (NEQAS) for IHC; there is no equivalent quality assurance process for MSI in the UK at present. Above all, the necessity of securing suitable funding for molecular testing was emphasised.

QUESTIONS

1) With respect to tumour tissue blocks, how much positive internal control (*i.e.* unstained, non-tumour tissue) is required for a reliable IHC result? Where the quality of tissue blocks is low, this may be as low as 5% of the total.

Dr Arends said that if there is a clear area showing good fixation, even as small as 5%, this is sufficient to have confidence in the IHC result.

2) Is the proportion of tumour tissue within a block and/or fixation quality a factor for MSI testing?

Dr Arends said that a pathologist should always request as many blocks as possible and select the best one, containing the most tumour tissue, for testing. Dr Frayling agreed with this and said that it was indeed a factor for successful MSI testing, but was also partly addressed by analysing more than the minimum (5) microsatellite markers suggested by the ICG-HNPCC at Bethesda.

3) What is the situation with MSI-low positive cases – do they ever differ from MSI-high tumours, in terms of probable mutation detection?

Dr Frayling agreed that the distinction between MSI-L and MSI-H tumours was a subtle one, affected by the choice of markers used for testing and open to interpretation, however, two large studies had shown that MSI-L was not associated with HNPCC (42,43), though this was not to say it wasn't associated with other forms of familial colorectal cancer.

4) What is the cost of the commercially available 5-marker MSI testing kit?

The answer to this question was not known, but generally the cost of reagents does not have a great bearing on the overall cost of such tests.

5) Is it known what proportion of cases carrying MMR mutations are excluded from mutation screening on the basis of molecular testing, when using diagnostic algorithms such as that presented?

The answer to this question was not known, although there was some discussion around the issue. It was agreed that more work needs doing on this, and it might form part of an HTA assessment. However, this was not a reason to delay implementation of tumour testing.

Mutation detection in HNPCC

Miss Jennie Bell
West Midlands Regional Genetics Service

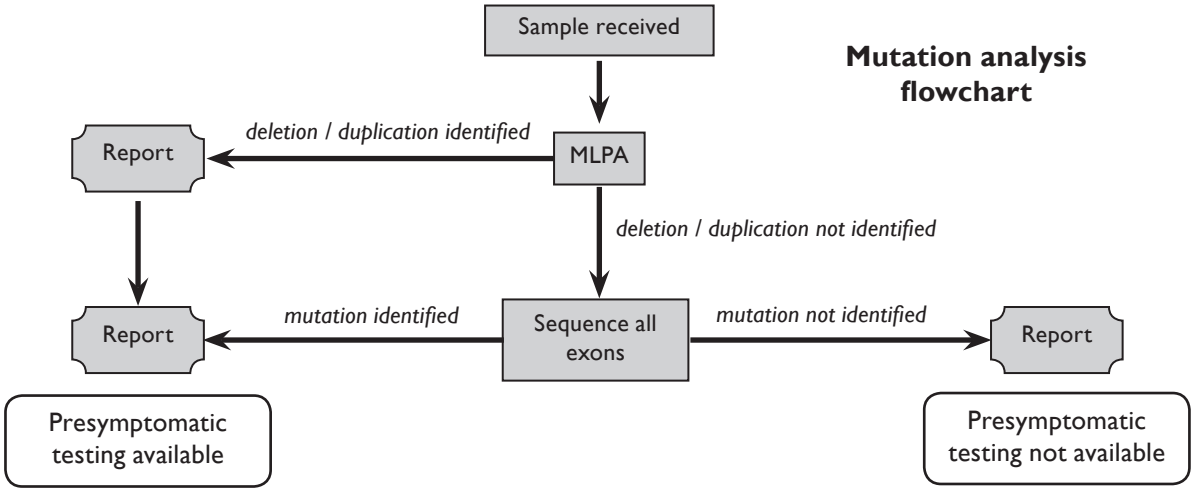
Miss Jennie Bell presented her work with Dr Yvonne Wallis on mutation detection in HNPCC, reporting that the West Midlands Regional Genetics Service laboratory in Birmingham laboratory presently offered sequencing of the *MLH1* gene (19 exons) and *MSH2* gene (16 exons). Since a range of different types of mutation may be present in the gene (from single base change point mutations to large insertions, deletions or duplications), some form of pre-screening is often used to identify the types of mutation that may be present. Techniques included single-strand conformation polymorphism (SSCP), heteroduplex analysis (HA), denaturing high-pressure liquid chromatography (dHPLC) and Multiplex Ligation-Dependant Probe Amplification (MLPA).

Evolution of HNPCC diagnostic testing

A brief history of HNPCC testing in Birmingham was given; mutation analysis has been performed since 1996. The laboratory originally used some primitive automation but relied mostly on labour-intensive and time-consuming manual techniques to deal with large numbers of patients referred for mutation analysis. Referrals were for patients who fulfilled or were close to fulfilment of the Amsterdam criteria; a mutation detection rate of around 20% was achieved. Diagnostic MSI services were introduced in 2000, although prior to this it was used in a research setting. IHC is also available now.

In 2001, Department of Health funding allowed the switch to a semi-automated processing laboratory, with the use of a dHPLC WAVE machine and Beckman CEQ 8000 genetic analyser. MLPA testing for deletions and duplications in *MLH1* and *MSH2* has been introduced since 2002. The mutation detection rate for the period 2002-2005 rose to around 35%. In 2005, funding made available via the Genetics White Paper of 2003 allowed a switch to sequence based analysis using robotics (Beckman / ABI) and commercial mutation detection software (Mutation Surveyor).

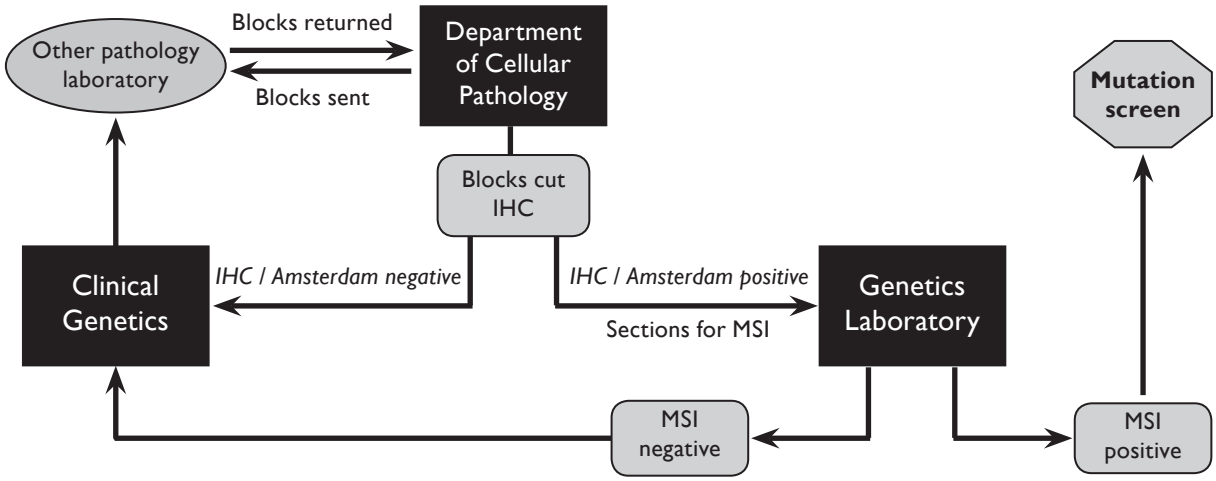
The nature and extent of mutations detected in the two MMR genes analysed for HNPCC were outlined. These were spread throughout the gene coding regions (exons); for the *MSH2* gene they included deletions, duplications, nonsense, missense and splice site mutations, whilst for the *MLH1* gene, missense mutations dominated the picture. This sort of mutation is notoriously difficult to interpret.



Evolution of MSI and IHC testing

Originally, tissue blocks were prepared for testing by a histopathologist on a contractual basis, but recently the laboratory has received two year funding to employ a dedicated part-time histopathologist in the Department of Cellular Pathology at University of Birmingham Medical School, and this move has made an outstandingly positive impact on testing.

Integration of MSI / IHC with mutation detection



A comparison was presented of the impact of automation on the laboratory time (measured in work load units, or WLUs) for HNPCC testing. Of note, the introduction of fully automated mutation screening has reduced the burden from 6959 WLU to 839 WLU, although even so mutation screening remains considerably more time consuming than MSI, which accounts for just 149 WLU per test. Reporting times have fallen correspondingly, at present, MSI test results are available in 4 weeks, and mutation screening results, which used to take around six months, will soon be available in just 8 weeks.

The advantages of using MSI / IHC

A number of real-life scenarios were presented to illustrate how the use of mutation testing can enhance the HNPCC diagnostic process:

1. An early onset (age 32) colorectal tumour was found to be MSI positive and to show loss of expression of *MSH2*. Analysis of the *MSH2* gene identified a nonsense mutation, allowing pre-symptomatic testing of family members.

2. A missense mutation in the *MLH1* gene of uncertain pathogenic significance was identified in a tumour by SSCP. MSI and IHC testing were performed to provide additional information; the tumour sample was found to be MSI positive and to show loss of expression of *MSH2* by IHC. A repeat screen for mutations using dHPLC identified a frameshift mutation in the *MSH2* gene. It was then possible to conclude that the MLH1 mutation was likely to be benign, but that a pathogenic *MSH2* mutation was present, allowing pre-symptomatic testing of family members for this causative HNPCC mutation.

3. A rectal tumour was found in a patient with onset at age 45; there was inadequate family history to fulfil the Amsterdam criteria for HNPCC, but IHC of the tumour revealed a loss of expression of *MSH2*.

The overall aim of mutation detection for HNPCC is to identify at-risk individuals for whom pre-symptomatic testing can be offered (and in those found to carry HNPCC associated mutations, to offer surveillance and/or interventions to prevent advanced cancers). MSI and IHC testing, used in combination with family history / pedigree information, improves the efficiency of mutation detection, by directing screening towards the most appropriate families. In addition, the techniques can provide supporting evidence to aid the interpretation of missense mutations.

In the future, it was envisaged that novel functional assays would be of use in defining the effects of missense mutations, and that RNA analysis could identify additional mutations that affect splicing.

QUESTIONS

1) Does the Birmingham laboratory plan to offer *MSH6* mutation screening?

Miss Bell agreed that this would be desirable and it was hoped that it would be available in the future; at present samples showing loss of expression of *MSH6* were outsourced to other laboratories.

2) Are any mutations missed during screening?

Miss Bell said that inevitably, some mutations would be missed, but they sought to minimise these by moving towards a complete sequencing strategy.

3) What is the WLU for IHC testing?

No figures were available for IHC as there were for MSI. The histopathologist performed both block preparation and staining in an efficient manner, and the value of having a highly motivated histopathologist with an interest in HNPCC was underlined again. It was also noted that having this individual available locally was a further advantage, allowing direct discussion of cases.

4) How does the previously quoted figure of €1200 (around £750) for full mutation screening of two genes compare with the Birmingham costs?

The cost to analyse one gene sequence corresponds to around £600, making the approximate cost of screening both the *MLH1* and *MSH2* genes around £1200, apparently much more expensive than the Netherlands figures. However, NHS costings for genetic testing are very simplistic, the same for all genes whilst in fact genes vary enormously in size and complexity, so they are not very useful for comparison.

5) What happens when mutations of unknown significance are found?

This was said to be problematic; if a mutation could not be concluded to be pathogenic then no pre-symptomatic testing was offered, but there was potential for the functional assays mentioned in the presentation to reduce the frequency of this situation.

There was some discussion of how best to share emerging knowledge about mutations and polymorphisms, with Dr Ian Frayling mentioning a new Human Gene Mutation Database. It was agreed that the need to contribute to and access such databases needed to be built into the funding of HNPCC screening services.

Human Gene Mutation Database: http://www.hgmd.cf.ac.uk

Economics of colorectal cancer screening

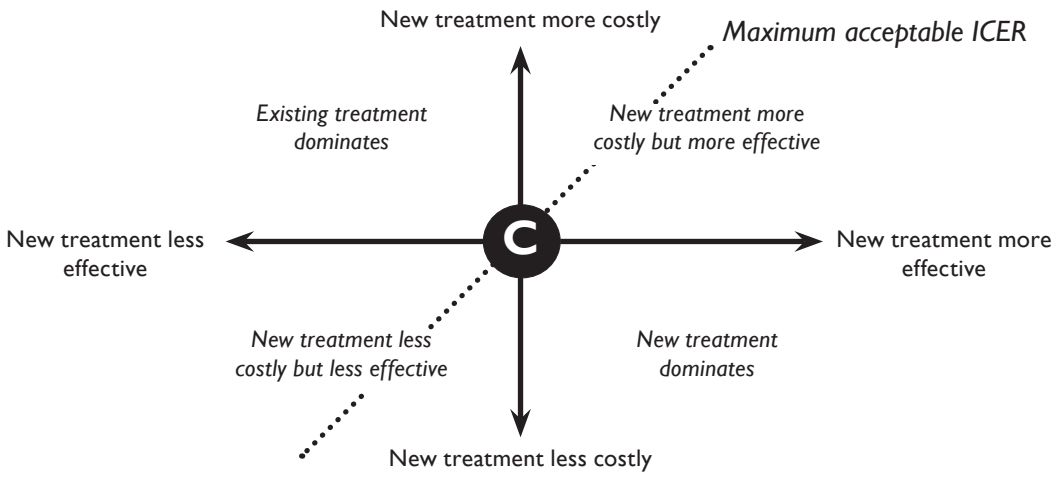
Dr Sarah Wordsworth
Health Economics Research Centre, University of Oxford

Sarah Wordsworth began by introducing the increasing necessity to include economic evaluation along with measures of efficacy (in terms of inputs and outputs) in decision-making about health procedures or programmes. Economic evaluation was said to provide a coherent and explicit measure of comparative costs and effects for at least two alternative approaches. A number of different types of economic evaluation were outlined, of which cost-effectiveness analysis was said to be the most common for measuring health outcomes; health economics also uses cost-utility analysis, which considers an additional 'quality-of-life' dimension.

Cost-effectiveness analysis

The concept of cost-effectiveness was outlined, including the incremental cost-effectiveness ratio (ICER) a relative measure that can be illustrated by a cost-effectiveness plane (following page); ICERs were noted to

be time-dependent, since both costs and outcomes for a given intervention may change over time.



Economics and colorectal cancer

Any form of screening for colorectal cancer assumes that costs can be offset by improved health outcomes (and to some extent reduced future treatment costs), by earlier detection of cancers. The key to an economically feasible screening programme will depend on an acceptable ICER. The identification of HNPCC is already accepted as a worthwhile aim, for the early detection and/or prevention of cancer in affected family members, so that the starting point for a health economic evaluation is how best this can be accomplished (in terms of technical efficiency), as opposed to whether it should be done at all.

Previous research was outlined; a cost-effectiveness analysis (47) looked at the use of the Bethesda guidelines to direct patients for MSI tumour testing, full mutation searching and lifelong screening for mutation carriers, in terms of life years gained as an outcome. For colorectal cancer patients alone, the cost of screening was found to be $42,210 per life year gained, but when the siblings and children of patients were included in the screening process the cost fell substantially to $7,556 per life year gained. Key factors affecting performance were the prevalence of HNPCC mutation carriers among colorectal cancer patients, and the accuracy of MSI testing, whereas the cost of germline mutation scanning had relatively little impact.

A later study (48) compared the cost-effectiveness of four alternative strategies for patients with newly diagnosed colorectal cancer, in terms of cost per life year saved (LYS):

1. Bethesda guidelines: clinical and family history followed by microsatellite instability testing and germline testing ($11,865)
2. Universal microsatellite instability testing ($35,617)
3. Germline testing of those who meet clinical and family history criteria ($49,702)
4. Universal germline testing ($267,548).

The clear conclusion was that the Bethesda guidelines are the most cost-effective approach to HNPCC screening.

The third published study compared the effects of alternative selection strategies for investigation of 138 families with possible HNPCC on the cost of mutation scanning, in addition to looking at their sensitivity and specificity (34). The authors found that where IHC and/or MSI were available (and tumour blocks to provide samples for analysis), it was most cost-efficient to use them as an initial screening strategy, proceeding to full mutation analysis if the result of either was abnormal; if not, the use of more restrictive clinical criteria to select patients for mutation analysis was recommended. In general there was said to be a dearth of publications on this subject, with many of those purporting to be cost-effectiveness analyses failing to include any information on costs, and others providing only inexact measures. Still fewer studies

include any consideration of patient expenses. There is therefore an outstanding need for more research in this area, in particular in the comparative costs of different testing options, to provide economic evidence in support of the optimal strategy for national implementation.

HNPCC economic decision model

Collaborative work between Sarah Wordsworth, Ian Tomlinson (Molecular and Population Genetics Laboratory, Cancer Research UK) and Ian Frayling (Institute of Medical Genetics, Cardiff) on developing an economic decision model for HNPCC diagnosis was presented. This work compares alternative strategies for HNPCC diagnosis for two groups, high and moderate risk:

High risk: (1) Mutation analysis
(2) MSI (followed by mutation analysis for abnormal results)

Moderate risk: (1) MSI (followed by mutation analysis for abnormal results)
(2) IHC (followed by mutation analysis for abnormal results)

Outcome measures will be recorded both in terms of sensitivity and specificity, and of cost per life year saved, for each of the testing strategies.

QUESTIONS

1) Will the new model include a measure of the financial savings arising from the early exclusion of individuals who would otherwise have received full mutation analysis?
Dr Wordsworth said that they were indeed attempting to incorporate measures of reduction in unnecessary interventions and their cost implications into the decision model.

Current UK practice

Dr Clara Gaff
Medical Genetics Service for Wales, Cardiff

The final presentation by Dr Clara Gaff outlined her work in surveying current UK clinical practice for HNPCC screening and diagnosis (via IHC and MSI tumour testing), an area for which there is no previously published literature. She approached 24 NHS clinical cancer genetics service centres, nine regional genetics laboratories and one pathology laboratory for information via digitally recorded interview, and all bar one genetic laboratory responded.

Immunohistochemistry (IHC)

A total of eight different laboratories were performing IHC testing of colorectal tumour samples.

Proteins tested for	Number of laboratories
MLH1, MSH2, MSH6, PMS2	4
MLH1, MSH2, MSH6	1
MLH1, MSH2	3

Microsatellite instability (MSI)

A total of five laboratories were performing MSI testing of colorectal tumour samples; of note, no two centres were using exactly the same set of markers. A range of 15 different mononucleotide and dinucleotide markers were in use, with sets of between 2 and 10 used be each laboratory.

Use by clinical genetics services

20 of the 24 clinical genetics services offered tumour testing for HNPCC:

Testing offered	MSI and IHC	IHC only	MSI only
No. services	**14** *via 5 different strategies*	**5**	**1**

The remaining four services were unable to offer access to testing due to a lack of funding; this represents a total population of approximately 6.7 million people who have no access to tumour testing even though germline HNPCC testing is provided.

A major challenge to providing services appeared to be sufficient resources; although providers all wanted to maximise detection rates, testing strategies seemed to be influenced primarily by staff and pathologist availability and test turnaround time. It was noted that obtaining tumour tissue blocks for analysis was time-consuming, and that input of an experienced pathologist was a crucial element.

Clinical protocols

Where family history alone is considered to confer a sufficiently high risk of HNPCC to warrant mutation scanning (*ie.* the Amsterdam criteria are fulfilled), at least fifteen services use tumour testing as a preliminary procedure. Where the family history is not necessarily adequate to justify germ-line mutation testing, all twenty services use some form of triage process to determine which patients are eligible. The precise criteria applied vary between services, and include the Bethesda guidelines (8/20), other documented protocols (7/20), 'moderate risk' (3/20) and 'near Amsterdam' (2/20). The role of clinical judgement in this decision-making process was emphasised by respondents.

Surveillance of at-risk individuals

18/20 study respondents said they might modify their recommended surveillance strategies of at-risk family members on the basis of tumour testing results. All of these would consider increase surveillance of moderate risk families if the results of IHC and/or MSI were consistent with the presence of HNPCC, and four might decrease surveillance if the results were not consistent with HNPCC.

Consent

The general ethos with respect to issues of counselling and consent for tumour testing was generally similar for all services; three always provided some form of counselling before testing, but most had a more flexible approach than that for full germline mutation screening. A variety of means were used to obtain some type of limited consent, including forms delivered via relatives, letters, telephone conversations and medical referrals.

One respondent was quoted as saying of the patients:

"I think a lot of them don't want to know in that sense and they basically take on board this idea that it is a preliminary test that gives a better idea of whether something is likely to be genetic or not and that we use that to decide whether we go on to testing."

Of a total of nineteen consent forms obtained, fifteen were to obtain release of the relevant tumour samples from pathology departments. Nine obtained consent for testing, eight stated or implied that the results of testing had potential implications for family members and five named the condition (eg. cancer predisposition or colorectal cancer). There was no consistency in practice when testing a tumour sample from a person who had died after the tumour sample was collected (as opposed to sample collected at post mortem). Eight services obtained written consent before testing and four would attempt to do so. When consent was sought, it was generally from the 'next of kin' but this was broadly and variably interpreted; the potential inappropriateness of the spouse in this role was noted, given that they would not be directly affected by any eventual diagnosis of HNPCC.

It was noted that in the future, referrals for tumour testing might form part of routine pathological examination of a certain subset of colorectal tumours.

Implications and proposals

The substantial degree of variation between different laboratories across the UK in terms of eligibility criteria for and methods of tumour testing was observed to pose a number of problems in terms of evaluation and audit. Most notably, the inequity of access to testing services was highlighted as a serious cause for concern; not all centres offer tumour testing (thereby restricting access to germline genetic testing for the high-moderate risk HNPCC patient group) and those that do, use different eligibility criteria. Although somatic tumour testing is not part of the remit of the UKGTN (Genetic Testing Network), inequity of access to genetic testing is, and it was proposed that this issue should be referred to them for consideration.

Another point raised was that although the services have fairly consistent expectations of the level of patient understanding with respect to tumour testing, these expectations are different from those for germline genetic testing for cancer, and this is an area where research into levels of understanding and preferences among patients could be of value.

Overall, it was proposed that a consistent tumour testing service for HNPCC should be offered throughout the UK, in terms of access and eligibility criteria, educational materials and an optimised testing strategy. The Human Tissue Act (2004) may require consent to test samples from people who have died since the sample was collected and refining and standardising consent forms would be of value. Finally, documenting the differences in counselling and rationale for these differences would form a standard for pre-test counselling by non-genetics health professionals.

Suitable bodies to support funding to devise and implement these changes were suggested to be the UKGTN and the Genetics Commissioning Advisory Group (GenCAG).

QUESTIONS

1) Was there any feel for the normal turnaround time for tumour testing results?

The interviews had not included any specific question on this issue, but several respondents were said to have made reference to frustrations over the time taken.

2) If the influence of tumour testing results on subsequent surveillance strategies is relatively minor, is the process justified?

Currently, any families who meet the Amsterdam criteria are offered high-risk level surveillance, but recent publications suggest that families who meet the Amsterdam criteria but whose tumours do not display mismatch repair defects have a lower risk of colorectal cancer, and therefore may not require the highest level of surveillance. By identifying these families, MSI has the potential to have a more significant impact, by both increasing surveillance intervals and the age at which surveillance starts, and also by identifying non-Amsterdam criteria HNPCC families. In addition, perhaps the greatest current benefit of tumour testing is as a means to assist triage for genetic testing. Dr Gaff felt that these three factors justified the process.

3) Do any patient education materials exist?

Two services were said to provide fairly comprehensive materials and others shorter, one-page versions, but there was unanimous interest in national educational material to be provided for use across the UK.

4) Is the variability in services the result of an inability to agree on a single approach?

Dr Gaff said that rather it was her impression that the underlying reasons for differences between services were essentially pragmatic, with different centres trying to offer the best service given the available resources.

3. Discussions

Following the presentations, a plenary session was held for workshop participants to discuss key questions. The first issue raised was whether subsequent discussion would refer to HNPCC or Lynch Syndrome; there is some debate over whether or not Lynch Syndrome is a more appropriate term than HNPCC, since the spectrum of familial cancers associated with the condition includes a high proportion of endometrial and some other non-colorectal cancers. It was decided that for the purposes of discussion, HNPCC would continue to be used.

Should MSI and/or IHC be available as a useful adjunct to, or component of, genetic investigation of individuals at high risk of HNPCC?

The workshop delegates were unanimously agreed that these forms of tumour testing should be available across the UK. It was noted that in a subset of cases it might be appropriate to proceed directly to full mutation screening without the use of preliminary tumour testing by either technique.

If MSI and/or IHC testing is to be available nationally, where should such testing be performed?

Workshop delegates agreed that the ideal situation for IHC testing would be within a CPA accredited histopathology laboratory colocalised with, a molecular genetics laboratory where MSI testing (and in some cases mutation screening) could be performed.

Examples were cited of centres where an IHC testing facility is adjacent to the regional genetics centre, allowing excellent communication between the two. There was some debate as to whether IHC testing needed to be performed directly by a clinical histopathologist, or if an experienced scientist with an interest in HNPCC could perform the tests, under the direction of an histopathologist. The general agreement amongst the pathologists present was that a clinical histopathologist must be involved in the final interpretation of the IHC slides and the construction of the report.

It was noted that with respect to MSI testing, with 20 out of 24 regional genetics centres already offering the service, there was not necessarily any need to encourage the remaining centres to do so, provided that samples from these centres could be outsourced to the others for testing.

A query was raised as to what would happen if (presuming equitable nationwide access to testing) a tissue blocks from a distant relative of the presenting individual needed to be tested for HNPCC – which location should handle testing of this sample? It was suggested that this might be done locally, with the result made available to other centres as required. This led to agreement between delegates that results of MSI and/or IHC testing should be made available nationally for information sharing between genetics centres, with appropriate safeguards as for any clinical information.

There was some discussion on the likely demand for tumour testing, if it were to become available nationally. Estimates of between 6500-7000 cases per year were put forward, but it was noted that although this would represent an increase on current levels, it would be expected to reduce the overall demand for full mutation screening.

Is one technique superior over the other in terms of specificity, sensitivity and cost-efficacy?

The workshop delegates agreed that the two techniques are complementary and of equal importance for tumour testing, and that ideally both should be available throughout the UK. The choice of which technique

to use was said to be dependent on the clinical situation, and currently influenced by accessibility to testing.

It was agreed that both techniques should be available, and that the ideal strategy for delivery would be for the pathology laboratories holding the tumour tissue block could perform any IHC testing, with MSI testing performed at the regional genetics centres. However, this would require appropriate levels of technical expertise and time allocation from histopathologists.

What are the barriers to implementation of national MSI and IHC testing for HNPCC?

Delegates agreed on two key barriers, both of limited resources:

1) Staff levels and training
2) Running costs and other expenses

The absence of pathologists with suitable training in genetics was noted. It was agreed that formal training in this area for medical pathologists and histopathologists, and in the longer term for clinical/biomedical scientists, was required. A Masters course in General Pathology was reported to be in development, which might include this.

The current situation in Birmingham, where HNPCC testing is shared between the West Midlands Regional Genetics Service laboratory and a dedicated (part-time) histopathologist in the Department of Cellular Pathology at University of Birmingham Medical School, was agreed to be an example of good practice. It was suggested that this position might be promoted during training as a useful sub-specialism for new histopathologists, perhaps as the sort of additional role endorsed by the NHS Agenda for Change. Alternatively, it was thought that a multi-disciplinary biomedical scientist could also co-ordinate the collection and fixation of tissue blocks, even if they did not actually perform IHC tests.

The need for these conclusions to be circulated to appropriate recipients, including the National Pathology Oversight Group and the Healthcare Scientist Career Framework was agreed upon.

Additional issues

There was some further discussion of the resource implications of widening access to MSI and IHC tumour testing; the running costs and need for increased capacity were said to be the major obstacle, as opposed to capital expenses.

The importance of retaining flexibility in testing for HNPCC was noted, including access to both types of testing and to investigate and monitor exceptional families in whom HNPCC is strongly suspected despite negative test results.

It was agreed that ideally, data should be obtained on the true proportions of false positive and false negative results obtained using mutation testing via the two techniques. It was proposed that this could form the basis of a translational research study, but workshop delegates favoured the timely introduction of both techniques nationally, with a proper auditing system that could generate this information. Effective auditing would involve additional expenses.

The colorectal surgeons present emphasised their requirement for clear referral guidelines to form part of the testing strategy.

4. Conclusions

The long-term target of screening for HNPCC is the identification of at-risk, asymptomatic relatives in order to prevent advanced disease and mortality in these individuals, and to exclude relatives who are not at risk from unnecessary clinical surveillance.

The Workshop allowed clarification of a number of matters, and the definition of issues for further research and discussion, which are outlined in this section of the report.

Case Definition

HNPCC requires an accurate case definition. We suggest that it may be useful to use two separate conceptual definitions, one phenotypic and the other genotypic.; and that in the first instance they be defined as follows:

1) Phenotypic HNPCC: **Case meets Amsterdam criteria**

2) Genotypic HNPCC: **MMR gene mutation is present**

Amsterdam and Bethesda Criteria

What proportion of colorectal cancer cases meet the Amsterdam criteria? 1-2%

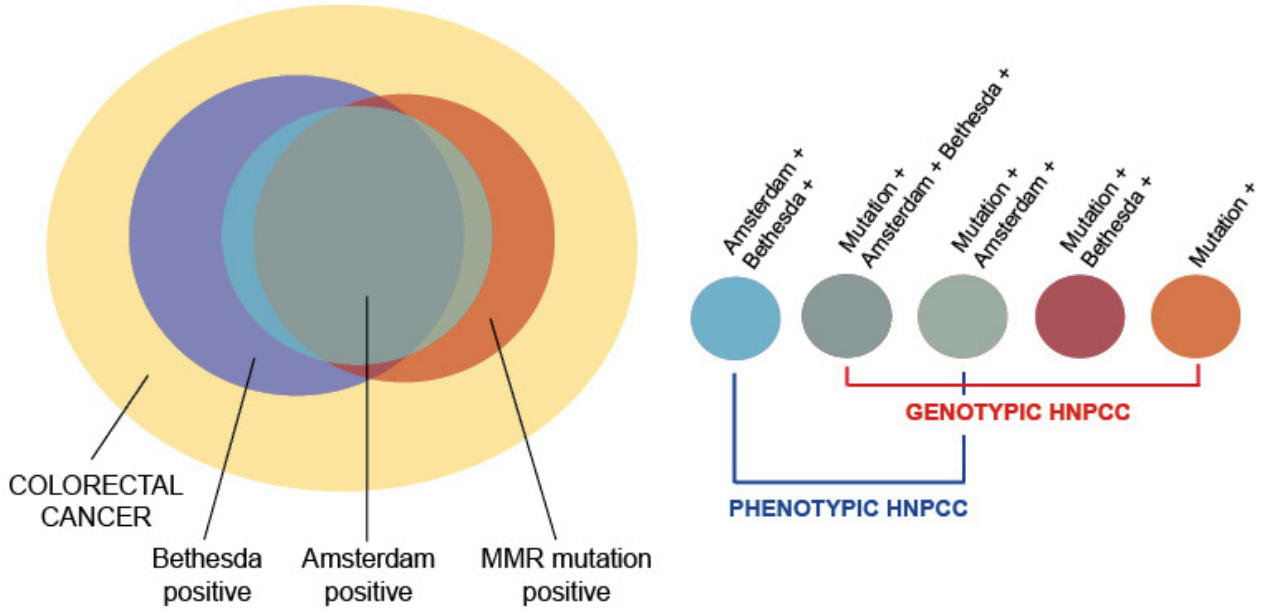
What proportion of colorectal cancer cases meet the Bethesda criteria? 20-25%

Figures based on the UK colorectal cancer (CRC) annual incidence of 34,000 suggest that around 8500 cases would meet the Bethesda criteria, and that around 300-700 cases would meet the Amsterdam criteria.

The Bethesda guidelines may be regarded as a screening tool to identify those cases of colorectal cancer that may be genotypic HNPCC. If no mutation is identified, the cases will not fulfil either the phenotypic or genotypic definitions of HNPCC and patients may then be excluded from high levels of future surveillance. If, however, a mutation is identified then family members may also be tested for the presence or absence of the mutation; those without it may then also be excluded from high levels of future surveillance

Amsterdam positive patients are defined as having the HNPCC phenotype; some form of familial cancer is thus likely to be present. The implication of mutation positive findings is the same as for the Bethesda positive cases discussed above; but if no mutation is found, by contrast to the Bethesda patients, the patients would continue to qualify for high levels of surveillance because the family fulfil the phenotypic definition of HNPCC.

The two concepts may be illustrated diagrammatically (following page):



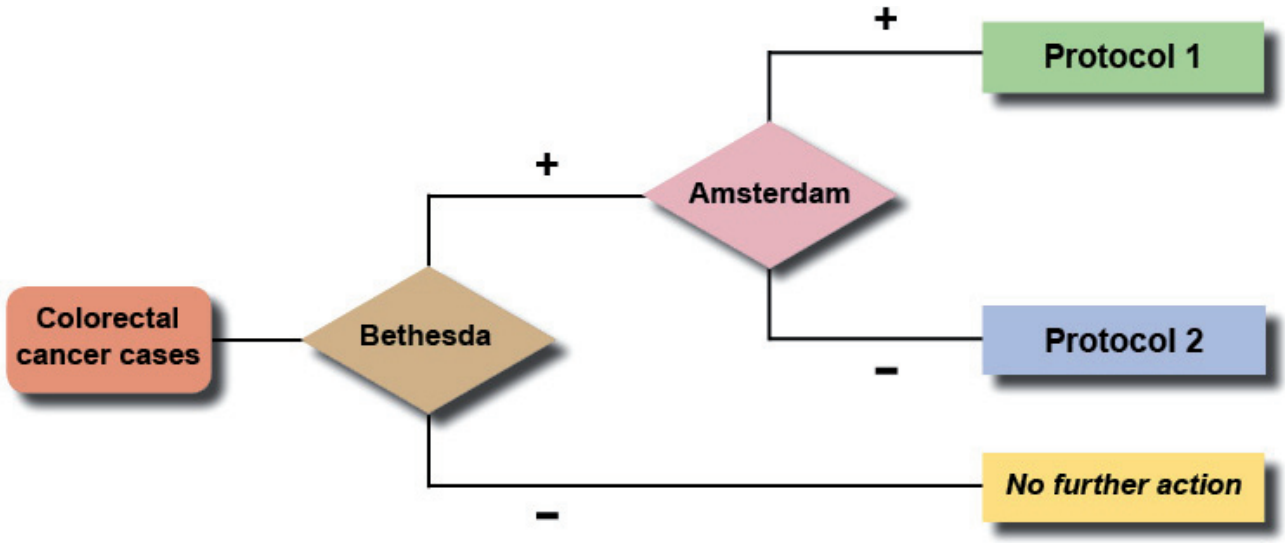
*Note: This figure is a **conceptual** diagram, and does not accurately represent the relative proportions of cases in each category.*

Use of IHC and MSI

Mutation scanning may be performed on either Amsterdam or Bethesda positive individuals to detect MMR gene defects, but there is evidence to suggest that this strategy is inefficient, and that pre-testing using microsatellite instability (MSI) or immunohistochemistry (IHC), alone or in combination, may be used as a further tool to identify those individuals for whom full mutation scanning is most strongly warranted.

The protocols for handling the two different types of cases will differ. The workshop delegates agreed that it was better to use IHC as an initial test in Amsterdam positive cases, where the prior risk is already high, in order to 'rule in' mutation testing (*Protocol 1*). The IHC test had another advantage in that it was able to indicate which MMR gene might be affected. For Bethesda positive (but Amsterdam negative) cases it was more appropriate first to 'rule out' those unlikely to be mutation positive by using MSI as the initial test (*Protocol 2*). This is because MSI is present in almost all cases of HNPCC but only in around 15% of sporadic colorectal cancers.

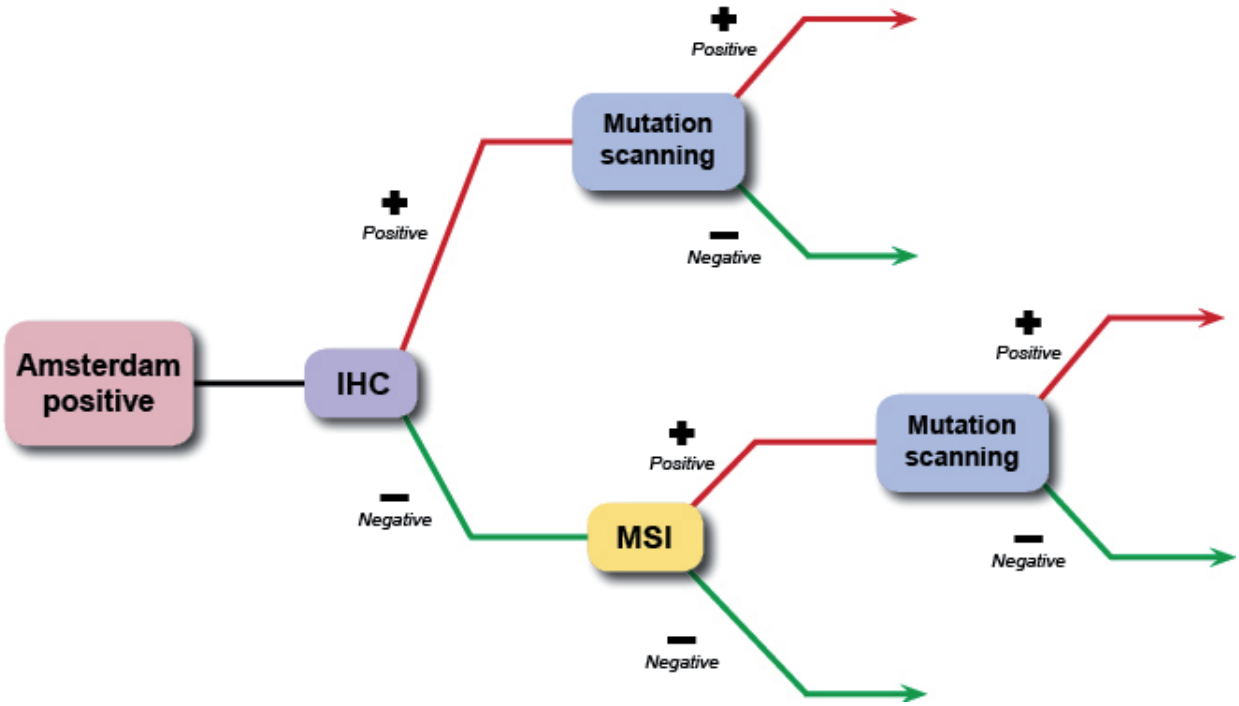
A key point to remember is that the families of patients who fulfil the Amsterdam critera may be considered to be at high risk of colorectal cancer on the basis of their family history; even though in a minority of cases, no MMR mutation may be identified in that family, this would not exclude them from long-term surveillance measures.



Uncertainties

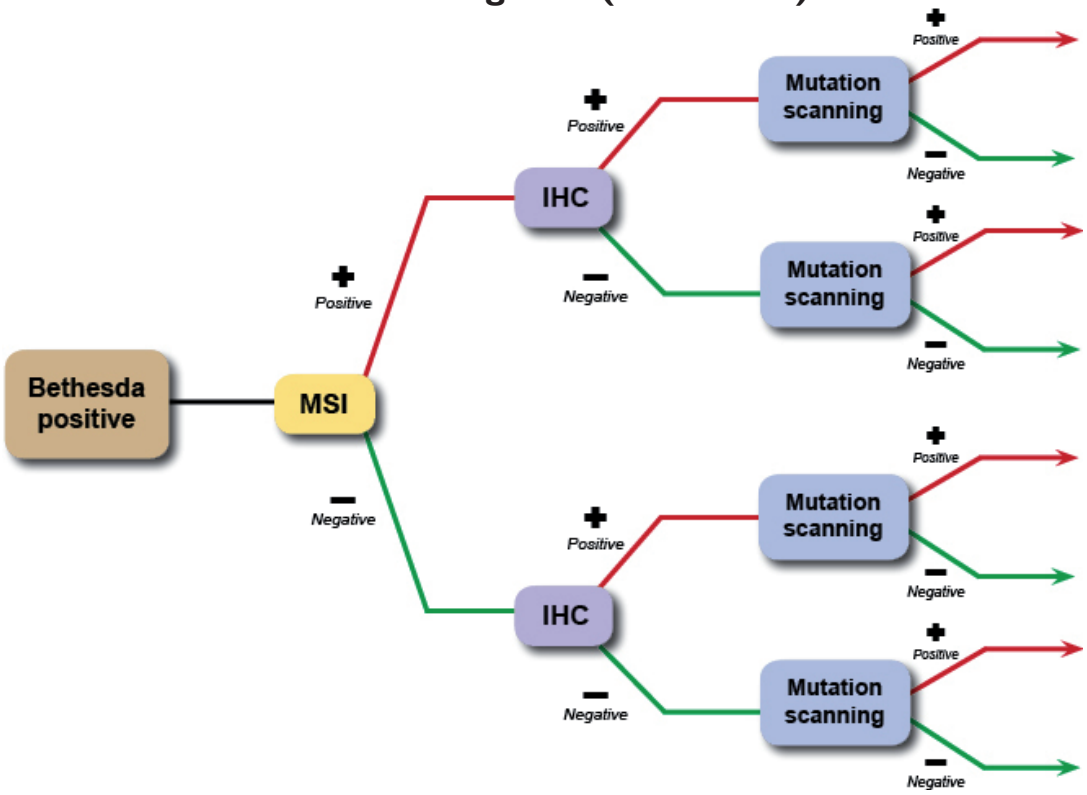
The evidence is clear that the initial pre-test for Amsterdam positive cases should be IHC and for Bethesda positive (Amsterdam negative) cases MSI. What is unclear is the protocol thereafter.

Amsterdam Positive (Protocol 1)



The evidence is clear that all IHC positive patients should have mutation scanning. The uncertainty concerns IHC negative patients, and whether it is advantageous, effective or cost effective to carry out MSI to be followed by mutation scanning in the MSI positive group or whether no further testing of any sort should be carried out on IHC negative patients.

Bethesda Positive Amsterdam Negative (Protocol 2)



It is clear that all MSI positive patients should be investigated further. The area of uncertainty is whether all such patients should go on directly to mutation scanning OR whether it is advantageous, effective and cost effective for the mutation scanning to be preceded by IHC, and if so whether this should be confined to the IHC positive group only. The second area of uncertainty is whether MSI negative patients can be discharged without further testing OR whether it is advantageous, effective and cost effective to carry out an IHC test and then to mutation scan all IHC positive patients.

It is desirable to produce a rough idea of the numbers of CRC cases that would fulfil each of the two scenarios, and to calculate the sensitivity / specificity of testing for each route as well as the negative and positive predictive values. Because prior probability of being mutatio positive in Amsterdam positive cases is higher than in those who are just Bethesda positive, predictive values will not be the same in the two scenarios.

RECOMMENDATIONS

1. **Both MSI and IHC testing for colorectal tumours should be available across the UK.**
2. **Current evidence should be used to agree on a national testing strategy, to include clear referral guidelines for colorectal cancer patients and protocols for investigation using MSI, IHC and mutation screening as appropriate.**
3. **Results of MSI and/or IHC testing, wherever that testing is done, should be reported back to the hospital where the tumour was diagnosed and should be included in the patient's tumour pathology reports, including electronically wherever possible.**
4. **An appropriate auditing system for the national testing programme should be put in place; this will generate the necessary data to determine the most cost-efficient use of the two techniques for the identification of HNPCC (and potentially refine the national strategy).**
5. **A quality assurance process should form part of the national strategy.**
6. **Assessment of MSI and IHC test results should involve a multidisciplinary team including clinical and laboratory geneticists and histopathologists, irrespective of which laboratory performs the tumour block preparation, DNA extraction and testing.**
7. **IHC and MSI testing of tumours should be performed in CPA accredited laboratories that have staff with the appropriate skills and expertise; implementation will require additional investment of time and resources.**
8. **A co-operative approach and excellent communication between different testing locations is essential to optimise the identification of familial colorectal cancer cases.**
9. **Access to tumour tissue blocks and related issues, such as consent, must also be addressed as part of the national strategy.**
10. **The UKGTN should, as with other novel genetics laboratory services, provide oversight of national strategy implementation via selected laboratories, and through the system of gene dossiers incorporate future technological developments.**

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6. Appendices

Appendix 1: Workshop steering group

The workshop was run by the Public Health Genetics Unit, and developed by a steering group comprising the following members:

Dr Mark Arends	Department of Pathology, University of Cambridge
Dr Philippa Brice	Public Health Genetics Unit, Cambridge
Prof Diana Eccles	Wessex Clinical Genetics Service, Southampton
Dr Ian Frayling	Medical Genetics Service for Wales, Cardiff
Dr Paul Pharoah	Department of Oncology, University of Cambridge
Dr Ian Tomlinson	CRUK Molecular & Population Genetics Laboratory, London

The final recommendations were produced by the steering group, based on the discussions and conclusions from the workshop.

Appendix 2: Workshop organisers

The **Public Health Genetics Unit** was originally established in Cambridge in 1997 with local NHS funding. In 2002, further funding from the genetics knowledge park programme enabled the Unit to expand its multidisciplinary team, which now includes specialists in public health genetics, policy development, knowledge management, law and ethics, epidemiology and education. The team works on projects that support development of public policy on genetics and health; development and evaluation of genetics services; translation of advances in genetics into clinical practice; communication and stakeholder engagement; promotion of genetics literacy for health professionals and provision of education and training.

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Public Health Genetics

The PHGU is a core facility of the Cambridge Genetics Knowledge Park **http://www.cgkp.org.uk.**

Appendix 3: Workshop delegates

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Dundee

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West Midlands Regional Genetics Service
Birmingham Women's NHS Trust
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