Interpretation of p16, p53 and mismatch repair protein immunohistochemistry in gynaecological neoplasia

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Abstract

Current management of gynaecological neoplasms is underpinned by their molecular characteristics. For many neoplasms the underlying genetic abnormalities can be reliably detected using immunohistochemistry for protein expression as a surrogate. The three most widely utilized biomarkers in this regard in gynaecological neoplasms are p16, p53 and mismatch repair (MMR) proteins, and it is vital for all pathologists to be aware of the indications for their use, correct interpretation of expression patterns, awareness of technical and interpretive pitfalls as well as appropriate reporting terminology.

Keywords endometrial carcinoma; high-grade serous carcinoma; immunohistochemistry; intraepithelial neoplasia; mismatch repair; p16; p53; vulval squamous cell carcinoma

Introduction

Molecular abnormalities are increasingly becoming the basis of classification and management of tumours of the female genital tract. This is not only limited to cancers eligible for targeted treatments, but also impacts management decisions using standard treatment strategies. The 3 most common biomarkers in current usage in gynaecological pathology are p16, p53 and mismatch repair (MMR) proteins. These are available in most laboratories and it is vital that their technical performance is externally monitored, and that pathologists are familiar with their reporting patterns as well as with pitfalls in their interpretation. While technical aspects are outside the remit of this review, we will discuss the biology, normal and abnormal expression patterns, reporting terminology, and problems and pitfalls in the interpretation of p16, p53 and MMR IHC expression in gynaecological epithelial neoplasms in which these are relevant.

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p16 immunohistochemistry

Biology of p16

The 16 kD protein p16 (also known as $p16^{INK4A}$) is a protein encoded by the *CDKN2A* gene located on chromosome 9 (9p21.3). Its major function is to inhibit cyclin-dependent kinases (CDK4 and CDK6), which are required to phosphorylate the retinoblastoma protein (pRb). Phosphorylation of pRb causes it to be released from the transcription factor E2F which then is able to switch on the transcription of multiple genes, including *CDKN2A*, thereby allowing the cell to enter into the cell cycle. The prevention of pRb phosphorylation by p16 thus results in cell cycle blockade through inhibition of G1/S checkpoint traversal,[1] and also serves as a negative feedback loop for p16 production.

The role of p16 in cancer is complex. As a tumour suppressor p16 is frequently inactivated in many cancer types by various genetic or epigenetic mechanisms.[1] On the other hand, p16 overexpression is observed in certain types of tumours and premalignant lesions, notably those related to high-risk human papillomavirus[2] (HPV) infections, but sometimes in HPV-independent lesions via other mechanisms.[3]

In the context of carcinomas and intraepithelial lesions associated with high-risk HPV infections, the mechanism of p16 overexpression is based on transcriptional release from negative feedback control.[3] HPV E6 and E7 oncoproteins inactivate p53 and pRb respectively, promoting cell cycle progression. The degradation of pRb releases p16 production from its negative feedback control, thus leading to increased levels of p16 as an attempt to counteract the proliferation.[3] For diagnostic applications, p16 is most commonly utilized as a surrogate marker for transforming high-risk HPV infection in the evaluation of intraepithelial lesions of the lower female genital tract (cervix, vagina and vulva), including both squamous and glandular premalignant lesions. With increasing clinical relevance of the HPV status in the diagnosis of vulval squamous cell carcinomas[4] and endocervical adenocarcinomas,[5] interpretation of p16 immunohistochemistry has become indispensable for classification of these tumours.

p16 IHC in intraepithelial neoplasia of the lower anogenital tract

Interpretation guidance and terminology: the guidance on p16 immunohistochemistry applies equally to all lower genital tract lesions, i.e. cervical, vaginal, vulval and anal. In particular, since the recommendations by the Lower Anogenital Squamous Terminology (LAST) consensus group,[6] p16 has become the standard diagnostic marker for cases morphologically indeterminate between high-grade squamous intraepithelial lesion (HSIL) and low-grade squamous intraepithelial lesion (LSIL), or when the distinction from mimics of HSIL is difficult. The recommendations herein are based on the existing LAST

Criteria for interpretation of p16 immunohistochemistry with recommended reporting terminology

p16 expression pattern	Criteria for interpretation	Recommended reporting terminology
Normal/reactive pattern in squamous epithelium	Absent staining, or patchy staining (nuclear ± cytoplasmic) in scattered cells without continuous basal expression	Negative/Normal/Non-block
Abnormal (block positive) pattern in squamous lesions	Strong and continuous staining (nuclear ± cytoplasmic) in the basal and parabasal cells with upward extension involving at least one-third of the epithelial thickness	Block positive/Block-type/Diffuse positive
Normal/reactive pattern in glandular epithelium	Absent staining, or patchy staining (nuclear ± cytoplasmic) with scattered negatively stained glandular cells	Negative/Normal/Patchy
Abnormal (diffuse positive) pattern in glandular lesions	Strong and continuous, diffuse staining (nuclear ± cytoplasmic) in glandular cells	Diffuse positive

Table 1

recommendations; the reader is informed that these are imminently being reviewed and may be updated/amended. Squamous and glandular intraepithelial lesions are discussed separately, covering both normal and abnormal expression patterns for each. The interpretive criteria and related terminology are summarized in Table 1.

p16 expression in squamous epithelium and squamous intraepithelial lesions/neoplasia (SIL/IN).

Normal/reactive expression pattern: Normal squamous epithelium in the cervix, vagina or vulva usually shows completely negative expression for p16, but occasional scattered cells with weak nuclear and/or cytoplasmic staining may be seen (Figure 1). Immature metaplastic squamous epithelium in the cervix may exhibit considerable positive staining in the superficial layers, often sparing the basal and parabasal layers. In reactive conditions staining may be positive staining in a larger number of cells or of stronger intensity, but the staining is generally irregularly scattered without continuous basal expression (sometimes referred to as mosaic pattern)

Abnormal (block positive) expression pattern: Abnormal p16 expression in squamous intraepithelial lesions (commonly referred to as block positive or block-type staining) is recognized by the presence of strong and continuous, nuclear or cytoplasmic with variable nuclear staining, in all epithelial cells in the basal and parabasal layers with upward extension (Figure 2). It is suggested that the upward extension should involve at least the lower one-third of the epithelial thickness and the basal continuous staining should involve at least six cells across. These criteria for the horizontal and vertical extent are admittedly arbitrary but they help to ensure the specificity and achieve diagnostic uniformity.

Reporting terminology: When describing p16 expression in reports, use of the words "positive" or "negative" alone is not recommended due to potential confusion of block-type with non-block type staining. It is preferable to describe p16 expression as "abnormal", "abnormal diffuse", "block positive" or "block-type" versus "normal/reactive".

p16 expression in glandular epithelium and glandular intraepithelial lesions

Normal/reactive expression pattern: Normal endocervical epithelium is usually completely negative for p16, but occasional glandular cells may exhibit positive nuclear and/or cytoplasmic staining, often randomly scattered amongst negatively stained cells. Tuboendometrioid metaplasia of the cervix and lower segment endometrial epithelium often exhibit positive but usually patchy p16 stainingin a mosaic pattern. The p16 staining in endometrial-type glandular epithelium can sometimes be quite extensive and may be mistaken as diffuse positivity when the gland is partially sampled with scanty or no negatively stained cells included.

Abnormal (diffuse positive) expression pattern: Abnormal p16 expression in glandular lesions is recognized by strong and continuous, diffuse staining in glandular cells, which is nuclear or cytoplasmic with variable nuclear staining. There should not be intermixed negatively stained lesional cells among the positively stained cells. Weak cytoplasmic staining alone without definite nuclear positivity should be regarded as negative.

Reporting terminology: As noted above, use of the words "negative" and "positive" alone is not recommended. p16 expression should be described as "abnormal", "abnormal diffuse positive" versus "normal/reactive". The term 'block positive' should be restricted to squamous epithelium and not be used to describe the pattern in glandular epithelium where the horizontal and vertical extension criteria do not apply.

Problems and pitfalls in p16 interpretation in intraepithelial neoplasia: while the recognition of p16 expression patterns is relatively straightforward, a number of diagnostic issues may arise when combining this with the clinical and histological contexts as discussed below.

Diagnostic issues for squamous intraepithelial lesions

- **p16 should not be used to replace morphological grading of HPV-related squamous intraepithelial lesions.** In the

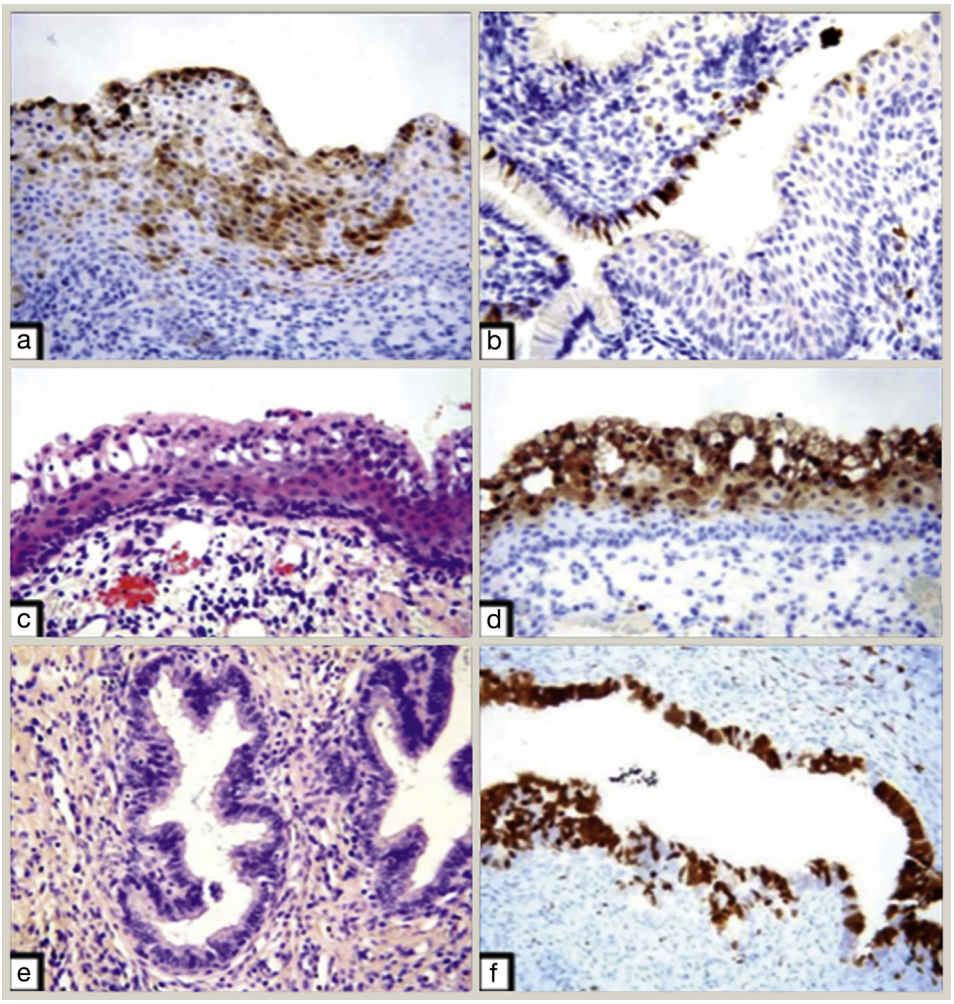

Figure 1 p16 expression in normal or metaplastic epithelium. Patchy p16 staining may be seen in normal cervical squamous epithelium (**a**) and endocervical glandular epithelium (**b**). Immature squamous metaplasia of the cervix (**c**) typically shows patchy p16 staining with sparing of the basal layer (**d**). Tuboendometrial metaplasia of the cervix can sometimes mimic adenocarcinoma in-situ with the nuclear pseudostratification and mitotic activity (**e**). The p16 staining in tuboendometrial metaplasia is sometimes quite extensive, although usually with scattered negative staining cells (**f**). (Courtesy of Professor W Glenn McCluggage.)

cervix, LSIL (or cervical intraepithelial neoplasia/CIN1) may display block positive p16 in up to about 50% of cases.[7] When the morphological findings are consistent with LSIL/CIN1, the mere presence of block positive p16 should not automatically upgrade the lesion to HSIL/CIN2, unless there are other features (such as atypical mitotic figures) that warrant such a designation upon review. Thus p16 immunohistochemistry is not recommended for morphologically unequivocal LSIL/CIN1 or HSIL/CIN2.[6]

- **The thickness of positively stained epithelium should not be used as a criterion for grading of squamous intraepithelial lesions.** Although the vertical extent of p16 staining in squamous epithelium may offer some clues to the diagnosis, this parameter does not necessarily correlate with the grade of the lesion.
- **Abnormal p16 expression in LSIL should not be used to predict the risk of progression.** Although there are many previous studies with conflicting results, the current evidence from adequately powered studies suggests that p16 cannot reliably predict the risk of progression to HSIL or cancer.[7,8]

Diagnostic issues for glandular intraepithelial lesions

Distinction between endocervical adenocarcinoma in-situ (AIS)/cervical glandular intraepithelial neoplasia (CGIN) and tuboendometrial metaplasia (TEM) can be challenging. Tuboendometrial metaplasia of the cervix may exhibit nuclear pseudostratification and mitotic activity reminiscent of AIS/CGIN, thus p16 immunohistochemistry may be performed for difficult cases. It is worth noting that extensive positive staining is not unusual for TEM, usually displaying mosaic pattern but diffuse positivity is possible especially in a small biopsy sample. Careful correlation with morphology is required for such cases; the presence of ciliated cells would favour tuboendometrial metaplasia. Other immunohistochemical markers that are of use include bcl2 (TEM displays diffuse bcl2 cytoplasmic positivity while AIS/CGIN is typically negative or only focally positive.), Estrogen

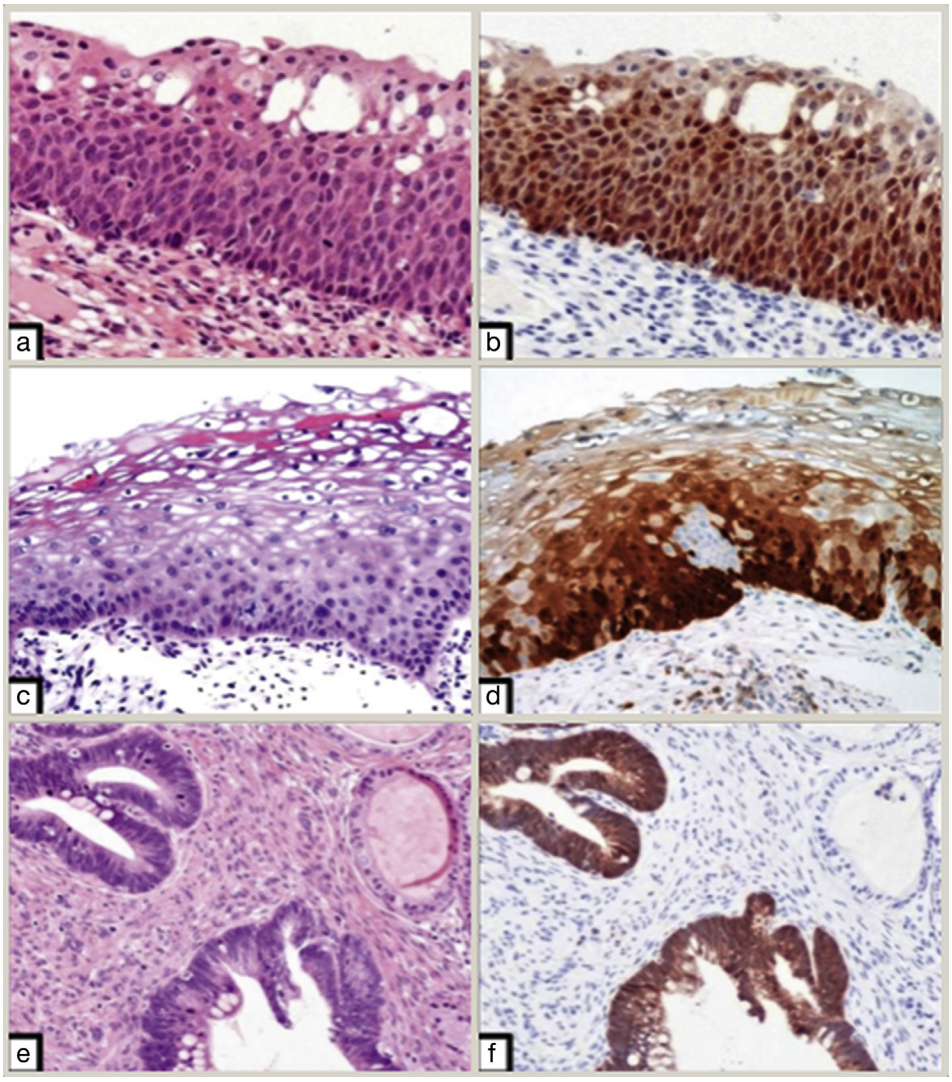

Figure 2 p16 expression in cervical squamous and glandular intraepithelial lesions. In HSIL (CIN 2) of the cervix (**a**), diffuse/block positive expression of p16 is expected (**b**). In a case with cervical squamous epithelium with maturation pattern resembling LSIL but atypical mitotic figure concerning for HSIL (**c**), the p16 staining meets the criteria of continuous basal positivity with upward extension to the lower one-third of the epithelial thickness, qualifying as diffuse/block positive (**d**). In adenocarcinoma in-situ (AIS) of the cervix (high-grade cervical glandular intraepithelial neoplasia; CGIN) (**e**), diffuse strong staining for p16 is present in the neoplastic glands, contrasting with the adjacent negative normal gland (**f**).

receptor (ER) (typically positive in TEM and negative or minimal in AIS/CGIN) and Ki67/MIB1 (typically AIS/CGIN has high proliferative index (>30% of cells) in contrast to the low MIB1 index (<10%) seen in TEM).[9]

- **Beware of gastric-type cervical glandular lesions.** When evaluating the p16 expression of a cervical glandular lesion with atypical features, the finding of negative or patchy staining is often regarded as evidence for a non-neoplastic condition. It should be remembered that while negative p16 helps to exclude HPV-related AIS/CGIN, the possibilities of HPV-independent gastric-type lesions (including gastric-type adenocarcinoma, lobular endocervical glandular hyperplasia or rarely gastric-type AIS) are not excluded. If the morphological features are compatible with any of the gastric-type lesions, further evaluation with additional immunohistochemical markers and clinical correlation would be necessary.

p16 IHC in endocervical adenocarcinoma

Interpretation guidance: while cervical squamous cell carcinomas are HPV-mediated in the vast majority of cases, it has been recognized that a significant subset of endocervical adenocarcinomas (EAC) are HPV-independent and that the distinction between HPV-associated (HPVA) and non-HPV-associated (NHPVA) EAC has major prognostic implications.[10,11] Based on this recognition the International Endocervical Adenocarcinoma Criteria and Classification (IECC) has been put forward and will replace the existing morphology-based classification of EAC.[5] The diagnosis of HPVA EAC is based on the recognition of luminal mitoses and apoptosis at scanning magnification, and then sub-categorized according to cytoplasmic qualities. It is recognized that while p16 IHC supports this classification, only 87% of HPVA EAC showed diffuse staining, and moreover 33% of NHPVA also showed similar staining in a large international study.[5] In this context the performance of RNA-based in-situ

hybridization shows superior results to p16 IHC. The role of p16 is therefore as an adjunct to morphology (Figure 3), and this should be interpreted with due regard to the pitfalls described below.

Problems and pitfalls in p16 interpretation in endocervical adenocarcinoma

- **Beware of gastric-type EAC.** A significant subset of HPV-independent gastric-type adenocarcinomas have been found to exhibit diffuse positive p16 expression,[12] which may require HPV testing for their distinction from usual endocervical adenocarcinoma.
- **Distinction between usual endocervical adenocarcinoma and endometrial carcinoma should not be solely based on p16 expression.** The morphology of HPV-related usual endocervical adenocarcinomas may significantly overlap with endometrial carcinomas of both endometrioid and serous types. When applying an

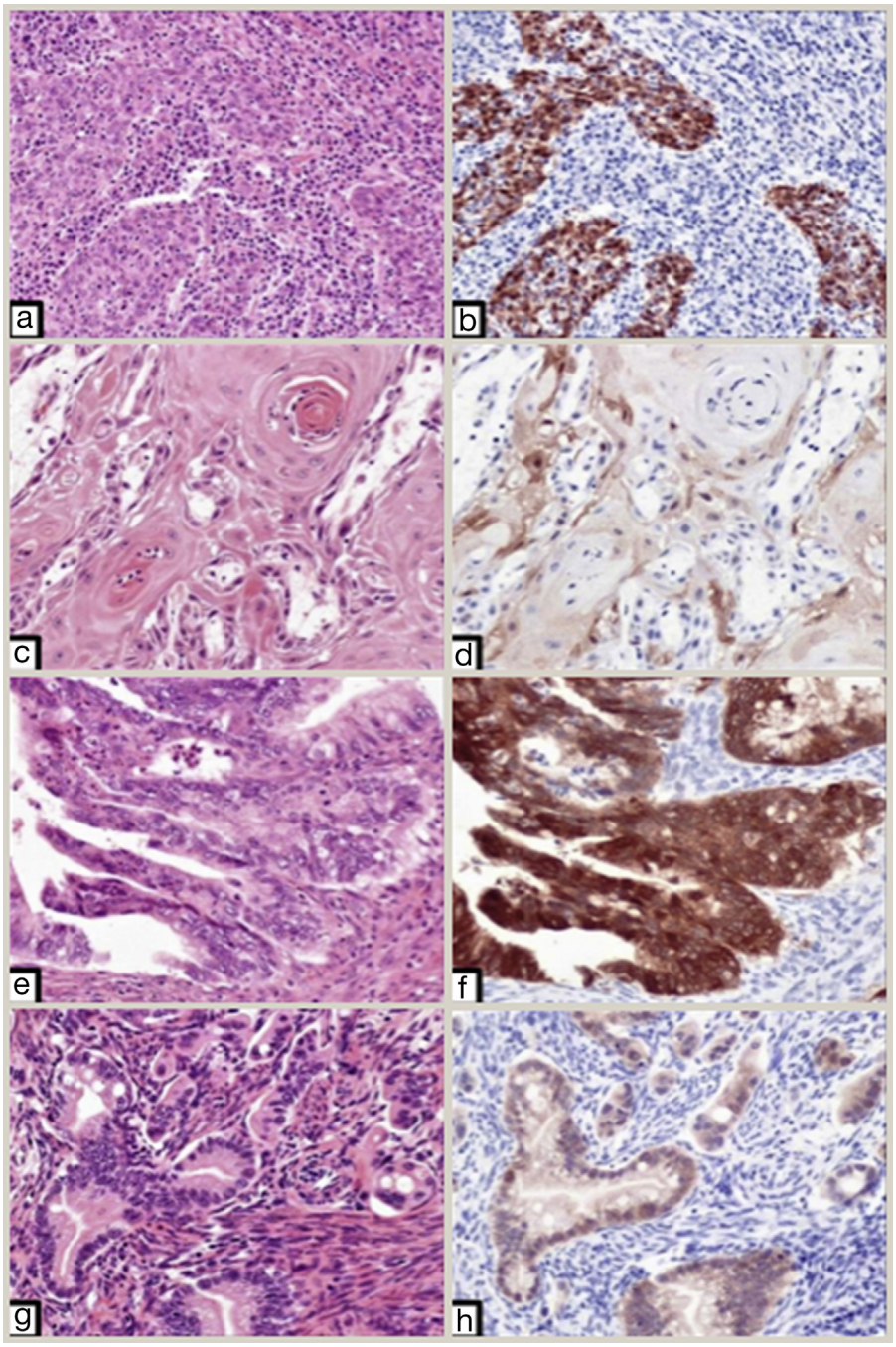

Figure 3 p16 expression in cervical and vulval carcinomas. In cervical squamous cell carcinoma, non-keratinizing type (**a**), diffuse p16 staining in the carcinoma cells are typical of HPV-associated carcinoma (**b**). In vulval squamous cell carcinoma, keratinizing type (**c**), focal non-block p16 staining in the absence of continuous basal positivity is in keeping with an HPV-independent aetiology (**d**). In HPV-associated usual endocervical adenocarcinoma of the cervix (**e**), diffuse positive p16 staining is typically seen (**f**). In gastric-type adenocarcinoma of the cervix (**g**), patchy weak p16 staining reflects its HPV-independent nature (**h**).

immunohistochemical panel to establish the diagnosis, it is crucial to note that endometrioid carcinomas often exhibit patchy positive staining for p16, which is typically of mosaic pattern but may be quite extensive[13] (Figure 4). ER may be of help but it should be noted that usual endocervical adenocarcinomas may also exhibit focal ER positivity; HPV testing may be required for difficult cases. Serous carcinomas of the endometrium also typically exhibit diffuse positive staining for p16,[13] yet these cases are characterized by mutation-type expression for p53, a finding which is extremely unusual for HPV-related carcinomas. One exception to this general rule should be noted; similar to the complete absence of basal expression of p53 in HPV-related neoplasms (discussed below in the section on p53 in vulval squamous neoplasia), EAC may also show loss of p53 expression that possibly reflects HPV E6 overexpression and resultant degradation of p53. This can result in misdiagnosis of HPVA EAC as serous carcinoma, due to erroneous interpretation as mutation-type 'complete absence' or 'null' p53 staining.[58]

p16 IHC in vulval squamous cell carcinoma

Interpretation guidance: vulval squamous cell carcinoma (VSCC) is rare, accounting for only 4% of gynaecological malignancies,[14] a fact that has made its systematic study challenging. Over recent years it has become recognized that VSCC develops along different molecular pathways, the most significant prognostic division being between those that are HPV-related versus those that are HPV-independent.[15] HPV-related VSCC occur in younger women and have a well-defined preinvasive stage that is easily diagnosed both clinically and histologically. These show a lower tendency to spread to lymph nodes, lower recurrence rates after surgery and better response to adjuvant treatment. Classification of VSCC as HPV-related or not therefore has major clinical significance, and morphology alone is not accurate enough to make this distinction.[4,16] As for EAC HPV status is most reliably determined using RNA-based in-situ hybridization techniques. p16 IHC serves as a surrogate for HPV and in head and neck tumours guidelines for p16 interpretation are well established, with >75% expression being interpreted as positive. Interpretation of p16 expression in VSCC is not as straightforward as in intraepithelial lesions; the extent of staining of the epithelium in VIN depends of the rate of maturation of the stratified squamous epithelium, so that the full thickness of the epithelium may not be stained in high grade VIN. Head and neck SCC typically shows a basaloid morphology and the high cut point of 75% may miss some cases of well-differentiated HPV-related VSCC. It is recommended that p16 in VSCC should be interpreted with due regard to the overall clinical picture and the staining of the adjacent intraepithelial component where present; the concurrent p53 expression should also be taken into account as the majority (but not all) of HPV-independent VSCC are p53 mutant.[17] In difficult cases, molecular techniques for HPV detection and/or *TP53* sequencing can help to guide treatment.

Problems and pitfalls in p16 interpretation in vulval squamous cell carcinoma

- **Rare cases of (cervical and) vulval squamous cell carcinomas may show discordant results for p16 expression and the HPV status.** While technical or fixation

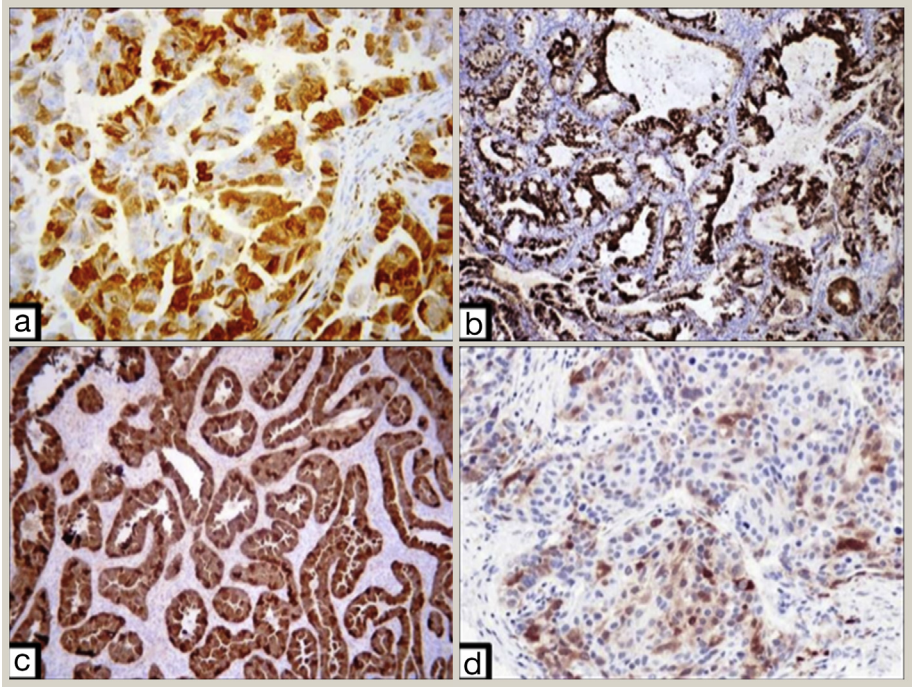

Figure 4 p16 expression in endometrial and ovarian carcinomas. Mosaic pattern of p16 staining is typically seen in endometrioid adenocarcinoma (**a**), but some cases may demonstrate nearly diffuse staining although scattered negative cells are usually observed (**b**). High grade serous carcinomas of tubo-ovarian origin often demonstrate diffuse positive staining for p16, via mechanisms unrelated to HPV (**c**), although patchy weak p16 staining may also be seen in some cases (**d**). (**a**–**c**: Courtesy of Professor W Glenn McCluggage.)

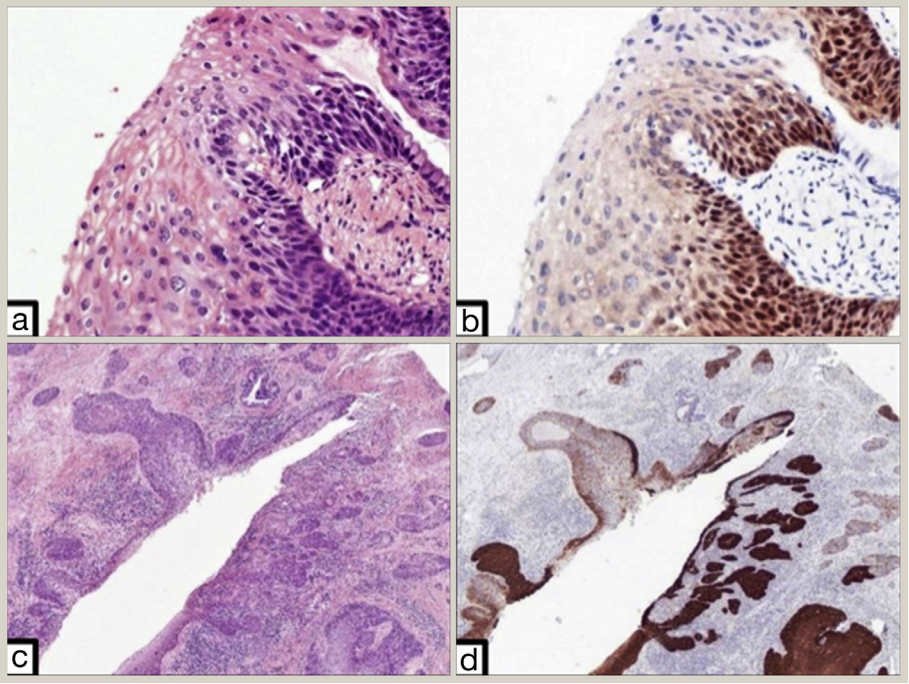

Figure 5 Caveats in interpretation of p16 IHC. In a morphologically typical LSIL (CIN1) of the cervix (**a**), block positive p16 staining may sometimes be observed (**b**). In a case of vulval HSIL (usual VIN) and squamous cell carcinoma (**c**), which was confirmed as HPV-associated by in-situ hybridization (not shown), an unusual pattern is observed featuring diffuse/block p16 staining in most areas of the HSIL with patchy absence of staining, whereas the invasive component also demonstrates patchy absence of p16 staining (**d**).

problems may account for the negative p16 expression in some cases of HPV-related carcinomas, theoretically the loss of p16 expression may occur via gene deletion or epigenetic silencing during the progression of HPV-related squamous intraepithelial lesions or carcinomas (Figure 5). Studies have demonstrated that abnormal p16 expression is rarely encountered in HPV-negative squamous cell carcinomas (proven by molecular testing) in the cervix,[18,19] as well as occasional cases of HPV-independent vulval squamous cell carcinomas.[4]

- **HPV-positive VSCC may show <75% p16 expression.** Morphologically well differentiated/'keratinizing' VSCC may show continuous 'basal' staining in invasive islands but overall <75% staining; such cases should be interpreted in the light of the staining seen in adjacent VIN, where available and in conjunction with the p53 staining pattern (see below); although some HPV-independent VSCC show widespread p16 expression, continuous strong basal expression would be unusual.

p53 immunohistochemistry

Biology of p53

p53 is a protein with tumour suppressor functions, which is encoded by the *TP53* gene on chromosome 17 (17p13.1). Its major function is to induce cell cycle arrest, senescence or apoptosis programmes in response to cellular stress signals such as DNA damage, thereby preventing the development of neoplasia from genetically damaged cells.[20] *TP53* mutations are among the most common genetic alterations in malignant tumours, present in just over half of all human cancers.[21] The detection of *TP53* mutations in premalignant lesions of various sites is also often implicated in high-grade dysplastic lesions.[21] Although *TP53* sequencing is not readily accessible to most clinical laboratories, p53 immunohistochemistry has emerged as a convenient surrogate of molecular testing for evaluating the *TP53* mutational status in histopathology specimens.[22] Originating from the observations in ovarian high-grade serous carcinomas (HGSC),[23] the immunohistochemical expression patterns of p53 have become valuable diagnostic adjuncts for various entities in the female genital tract.

The major diagnostic applications of p53 immunohistochemistry in gynaecological pathology include the following areas:-

(1) Diagnosis of tubo-ovarian HGSC and serous tubal intraepithelial carcinoma (STIC);
(2) Diagnosis of serous carcinoma (including serous endometrial intraepithelial carcinoma) in the endometrium;
(3) Molecular subtype designation for endometrial carcinoma; and
(4) Diagnosis of differentiated-type vulval intraepithelial neoplasia (dVIN) and associated squamous cell carcinoma in the vulva.

p53 IHC in ovarian carcinoma

Interpretation guidance and terminology: with recent understanding of the spectrum and significance of p53 expression patterns, a specific set of interpretation criteria and terminology has been developed in the context of ovarian carcinoma, applicable with some caveats to carcinomas of the other sites listed above .[22,24,25] Broadly speaking, p53 expression patterns may be divided into normal (wild-type) pattern and several types of abnormal (mutation-type or aberrant) patterns (Figure 6).

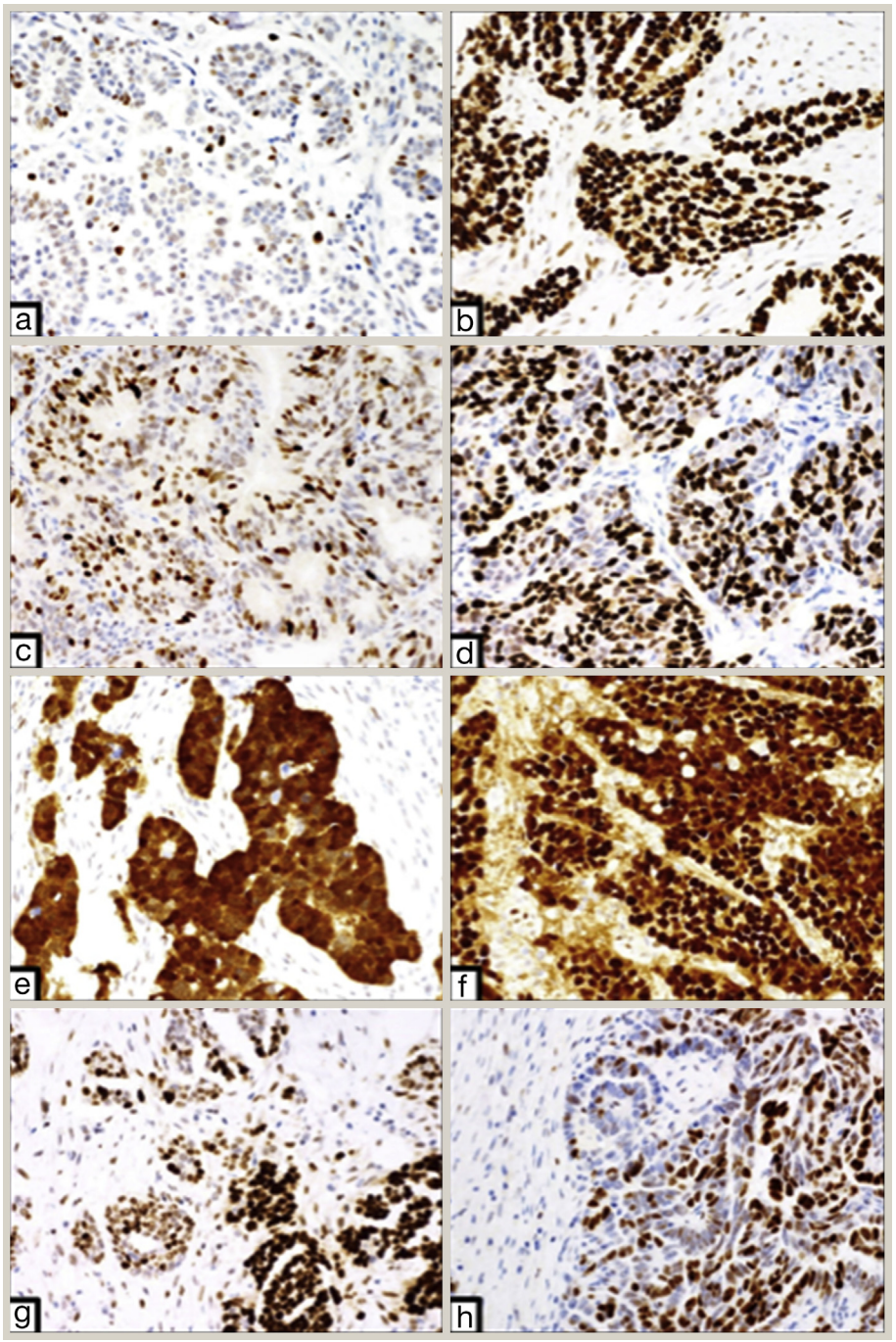

Figure 6 Wild-type, overexpression and cytoplasmic patterns of p53 expression. In a low-grade serous carcinoma with wild-type p53 pattern, many tumour cell nuclei are negative, some staining with variable weak to moderate nuclear intensity, and only few tumour cells show strong nuclear positivity (**a**). In a high-grade serous carcinoma with p53 overexpression, virtually all tumor cell nuclei show strong staining intensity compared to the intrinsic control (stromal fibroblasts) (**b**). "High" wild-type pattern, as exemplified by an endometrioid carcinoma, may demonstrate positive staining with variable weak to moderate intensity in the majority of tumour cell nuclei, while only a minority of nuclei are stongly positive (**c**). "Mosaic" pattern of overexpression in a high-grade serous carcinoma shows the majority of nuclei staining strongly but a significant proportion is negative (approaching the 80% cut-off) (**d**). Cytoplasmic p53 pattern in high-grade serous carcinoma shows moderate to strong cytoplasmic positivity in tumour cells with variable nuclear positivity (not strong and diffuse) (**e**). For comparison, p53 overexpression pattern in high-grade serous carcinoma may sometimes display extensive spurious artificial cytoplasmic staining including the stromal component (**f**), which might be confused with the cytoplasmic pattern. High-grade serous carcinoma rarely exhibits heterogeneous p53 staining (lower right – overexpression; upper left – wild-type pattern) (**g**); an area bordering on wild-type pattern depicted here (**h**).

Criteria for interpretation of p53 immunohistochemistry and associations with *TP53* mutational status

p53 expression pattern	Criteria for interpretation	TP53 status and other possible associations
Wild-type pattern	Patchy nuclear staining of variable intensity	Absence of *TP53* mutation in most cases (Exception: Some *TP53* mutations may be associated with wild-type p53 expression.)
Overexpression pattern	Strong nuclear staining in at least 80% of tumour cell nuclei	Associated with missense mutations (most common), in-frame deletions or splicing mutations in *TP53*
Null pattern	Complete absence of staining in tumour cell nuclei, in the presence of wild-type staining in the internal controls	Associated with nonsense mutations, indels or splicing mutations in *TP53*
Cytoplasmic pattern	Unequivocal predominant cytoplasmic staining in the tumour cells, in the absence of strong diffuse nuclear staining in the tumour nuclei	Associated with indels or nonsense mutations in *TP53* disrupting nuclear localization domain
Heterogeneous pattern	Presence of more than one patterns of p53 expression in a tumour	Need to exclude fixation or technical problems (For endometrioid carcinoma) May signify subclonal *TP53* mutation associated with underlying *POLE* mutation or mismatch repair deficiency

Table 2

Wild-type pattern of p53 expression generally indicates the absence of underlying *TP53* mutation (with rare exceptions). Mutation-type (or aberrant) expression of p53 comprises three distinct patterns (overexpression, null and cytoplasmic), any of which would predict the presence of an underlying *TP53* mutation. The criteria used for identifying each pattern of p53 expression and their associations are described below and summarized in Table 2.

Wild-type (normal) pattern: wild-type pattern is recognized by patchy nuclear staining of variable intensity, typically showing an admixture of negative, weakly positive and strongly positive nuclei. The extent of staining may range from only a few weakly positive nuclei ("low" wild-type expression) to the majority of nuclei being positive at variable intensity ("high" wild-type expression), generally dependent on the proliferative activity of the cells being assessed. As mentioned, wild-type p53 pattern is associated with the absence of *TP53* mutation in the vast majority of cases, but it has been demonstrated that certain nonsense or splicing mutations of *TP53* may be associated with a wild-type pattern in about 5% of tubo-ovarian HGSC,[25] due to an underlying truncating mutation resulting in a variably detectable but non-functional protein. This pattern should also be observed in the internal controls (stromal cells, lymphocytes or non-neoplastic epithelium).

Overexpression pattern*:* overexpression pattern is recognized by strong nuclear staining in at least 80% of tumour cell nuclei. This mutation-type pattern of p53 expression is usually associated with missense mutations in *TP53*, but in-frame deletions or splicing mutations are possible. These mutations are believed to interfere with MDM2-mediated ubiquitination resulting in nuclear accumulation of the p53 protein26. In tubo-ovarian HGSC this is the most common expression pattern found in about 66% of cases.[22,25] For some cases of p53 overexpression, while at least 80% of tumour nuclei are strongly positive, a significant proportion of tumour nuclei are weakly positive or negative. This has been described as the "mosaic" pattern, which is regarded as a variant of overexpression pattern, and is caused by antigen degradation, splicing mutations or overall weak staining, with potential to be confused with a wild-type pattern.

Null pattern: **n**ull pattern (complete absence or complete negative pattern) refers to the complete absence of staining in tumour cell nuclei, in the presence of wild-type staining in the internal controls (Figure 7). It should be noted that this pattern may include very faint nuclear staining (or 'blush') in a few tumour nuclei (see below in pitfalls). This mutation-type pattern is associated with nonsense mutations, indels or splicing mutations in *TP53*, which may result in a shorter mRNA that is subject to nonsense mediated decay and thus no translated protein. In tubo-ovarian HGSC this pattern accounts for about 25% of cases.[22]

Cytoplasmic pattern: cytoplasmic pattern is characterized by unequivocal predominant cytoplasmic staining in the tumour cells, in the absence of strong diffuse nuclear staining in the tumour nuclei. Nuclear staining that is weak, variable or of similar intensity as the cytoplasmic staining is acceptable. This is the least common pattern of mutation-type p53 expression, which is found in about 2% of tubo-ovarian HGSC.[25] The associated *TP53* mutations may be indels or nonsense mutations with disruption of the nuclear localization domain. Care should be taken in the distinction from non-specific weak cytoplasmic staining that sometimes accompany strong nuclear expression or spurious artefactual staining involving the background stroma.

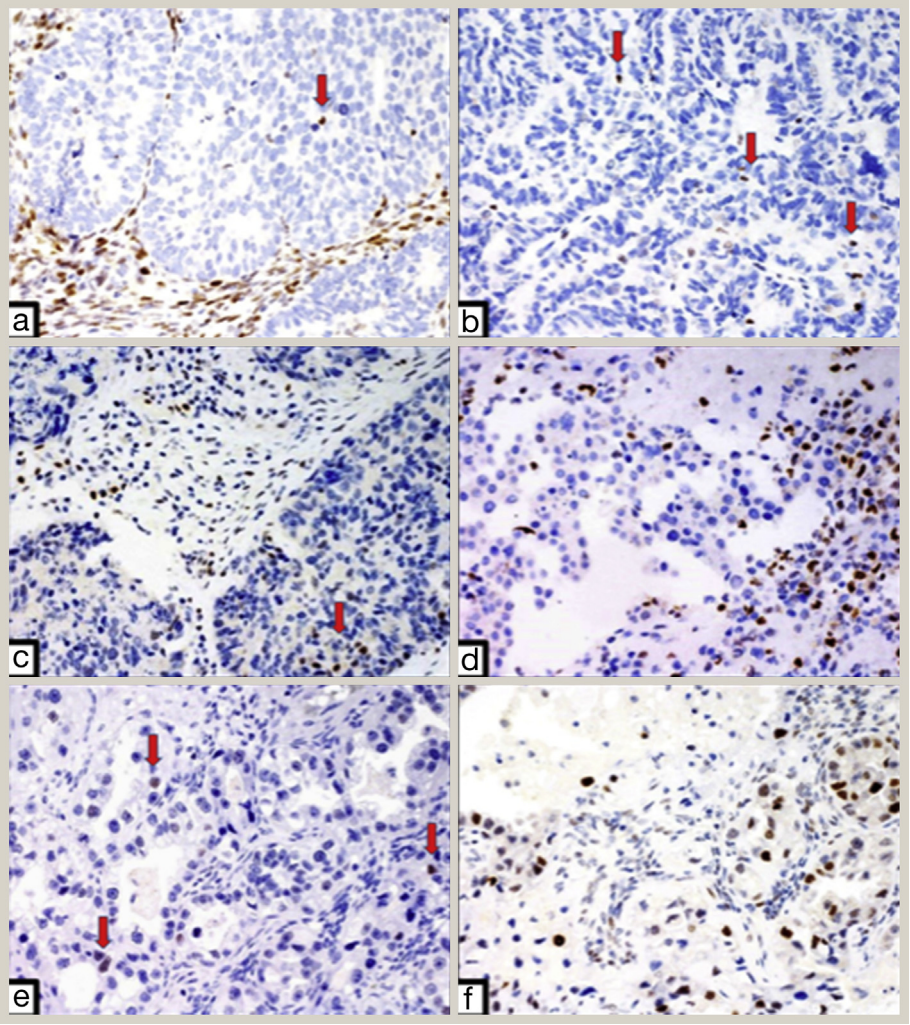

Figure 7 Null pattern of p53 expression. p53-null pattern in high-grade serous carcinoma demonstrates complete absence of staining in tumour cell nuclei with clearly positive internal control, including stromal and inflammatory cells (arrow) (**a**), but sometimes there may be scant internal control (arrows) requiring careful examination to confirm the null pattern (**b**). Another case showing rare p53-positive tumour cells (arrow) in an otherwise p53-null pattern (**c**); this may be non-specific and the tumour could be classified as having a null pattern. p53-null pattern in cell blocks can be difficult to evaluate as the presence of numerous inflammatory cells can give the impression of a wild-type pattern at low power, but on higher magnification tumour cells can be appreciated to be completely negative (**d**). Wild-type pattern may sometimes be difficult to distinguish from null pattern, as reflected by a case of clear cell carcinoma stained on two platforms, one of which resembling null pattern except for occasional weakly stained tumour cell nuclei (arrows) reflecting wild-type expression (**e**), while the other platform shows stronger and more extesnive staining with variable intensity (**f**).

Typically the staining intensity is at least of moderate intensity but may be weaker; the best way to recognize this is a low power impression of increased staining within the tumour in contrast to the background, which on closer examination is seen to be predominantly due to cytoplasmic expression.

Recommended terminology for reporting: in daily practice, it is recommended that normal p53 expression pattern be described as "wild-type" or "normal", whereas mutation-type patterns may be collectively reported as "mutation-type", "mutant", "aberrant" or "abnormal", preferably specifying the exact pattern observed (overexpression, null or cytoplasmic). Cases with heterogeneous pattern should have a combination of the patterns listed. To avoid confusion, reporting p53 expression simply as positive or negative is strongly discouraged.

Problems and pitfalls in p53 interpretation in ovarian carcinoma

Since many of the p53 expression patterns can exhibit substantial variability in the extent and intensity of staining, pathologists should be aware of the technical and diagnostic issues that may potentially lead to incorrect designation and misdiagnosis.

- **Problems with fixation may result in misclassification of p53 patterns.** Apart from the inherent tumour biology, suboptimal fixation is a major factor determining the staining observed. Antigen degradation may lead to difficulties differentiating overexpression from "high" wild-type pattern, or "low" wild-type from null pattern. Attention should be paid to looking for an unequivocally

positive internal control in the area being assessed, while excluding those areas without valid internal controls from the interpretation.

- **Optimization of immunohistochemistry performance is necessary for quality assurance.** The use of different antibody clones and staining protocols may give discrepant results of p53 expression, due to staining that is either too strong or too weak. External controls including both "high expressor" and "low expressor" positive controls with negative control are recommended.[22] HGSC with overexpression is commonly used as a "high expressor" positive control, while colon/appendix may serve as both "low expressor" positive and negative controls[22]; tonsillar tissue is particularly recommended as an on-slide control as the full range of expression can be seen: strong in the basal epithelial layers and germinal centres of lymphoid tissue, and weak in the mature epithelial and lymphoid components.
- **Wild-type p53 expression is seen in a small subset of tubo-ovarian HGSC.** As discussed, certain *TP53* mutations may produce wild-type p53 expression.[25] Pathologists should be cautious but not completely discouraged from making a diagnosis of HGSC when it is p53 wild-type but the clinical context, histological picture and other immunohistochemical results are otherwise typical.
- **Interpretation of cytoplasmic expression can be problematic.** The cytoplasmic pattern is relatively recently described, infrequent and can be subtle in some cases. Cytoplasmic expression is generally accompanied by weak nuclear staining. Awareness of this pattern and correlation with morphology can help to prevent misinterpretation as wild type staining, especially when weak.
- **A very faint nuclear blush can occur in 'null' pattern p53 expression, and should not be interpreted as wild-type staining.** HGSC with a null pattern of mutant p53 can show occasional weakly staining nuclei; this should not be interpreted as wild type staining which, in any analogous, rapidly proliferating tumour, would demonstrate clearcut but variable expression.

p53 IHC in endometrial carcinoma

Endometrial carcinoma (EC) classification has traditionally been based on morphology and a broad clinical division has been applied, separating tumours into types 1 (endometrioid) and 2 (serous and other non-endometrioid) with more indolent as opposed to more aggressive behaviour respectively. This simplistic approach has been problematic as a significant proportion of cases do not fall within these clear groups morphologically or prognostically, with the potential that these cases are either over- or under-treated. The Cancer Genome Atlas (TCGA) has defined four clinically distinct molecular groups of EC based on tumour mutational burden and numbers of somatic copy number alterations (SCNA)[27]; going forward this needs to be implemented into clinical practice as the molecular subtype has far-reaching implications for patient management[28]:

i. an ultramutated group characterized by an exceptionally high mutation rate (232 x 10^{-6} mutations/MB), higher than that seen in any other human cancers: this ultramutated state results from pathogenic hotspot mutations in the proof-reading, exonuclease domain of the enzyme DNA Polymerase epsilon (*POLE*mut). These tumours show high-grade morphology with scattered cells with bizarre nuclei, and large numbers of tumour-infiltrating lymphocytes. Significantly, although these are liable to fall morphologically into a 'high-risk' category, these tumours have an exceptionally good prognosis, and are potential candidates for treatment de-escalation.
ii. a hypermutated group still showing high mutation rates but approximately 10-fold less than *POLE*mut tumours (18 x 10^{-6} mutations/MB): this hypermutated state results from a defect in the function of the mismatch repair proteins MLH1, PMS2, MSH2 or MSH6. The mismatch repair defect (MMRd) may be hereditary in a minority of cases (Lynch Syndrome), or sporadic, as discussed below. These tumours are also often morphologically high-grade and show a high propensity for lymphovascular space invasion. These respond well to radiotherapy but do not respond well to chemotherapy, and are considered candidates for immune checkpoint inhibitor therapy.
iii. a group that is MMR proficient and shows low mutation frequency (2.9 x 10^{-6} mutations/MB) as well as relatively low numbers of SCNA: this largest group of tumours, also known as copy number-low (CN-L) or no special molecular profile (NSMP), accounts for classical endometrioid carcinomas, and shows stage-dependent prognosis.
iv. a group with low mutation frequency (2.3 x 10^{-6} mutations/MB) but extensive SCNA, consisting largely of classical high grade uterine serous carcinoma: these are characterized by *TP53* mutations and described as copy number-high (CN—H), 'serous-like' or p53 abnormal (p53abn). This group of EC shows significant improvement in survival with platinum/taxane-based chemotherapy, in addition to potential benefit from trastuzumab and PARP inhibitors in selected cases.

The interpretation of p53 in EC is affected by the underlying molecular events. About 3% of EC show more than one molecular classifying feature, and the majority of these are the occurrence of *TP53* mutations in tumours that are either *POLE*mut or MMRd.[29] In these situations, the *TP53* mutations are secondary or passenger events that do not confer the same poor prognosis as is typical of serous-like or CN—H tumours. The molecular classification of EC should therefore follow an algorithm and p53 IHC is an accurate surrogate with *TP53* mutation status in *POLE* wild-type and MMR proficient tumours.[30]

Problems and pitfalls in p53 interpretation in endometrial carcinoma

- **Subclonal/heterogeneous patterns of p53 IHC can be seen in POLEmut or MMRd EC**. The presence of more than one pattern of p53 expression in a tumour may be described as the heterogeneous pattern, most commonly represented by coexistence of wild-type and overexpression patterns. Excluding patchy staining related to suboptimal fixation, true heterogeneous pattern is rarely encountered in tubo-ovarian HGSC as *TP53* mutation is an early event in these tumours[25] (27,840,695). In endometrioid carcinomas of endometrium or ovary, however, the

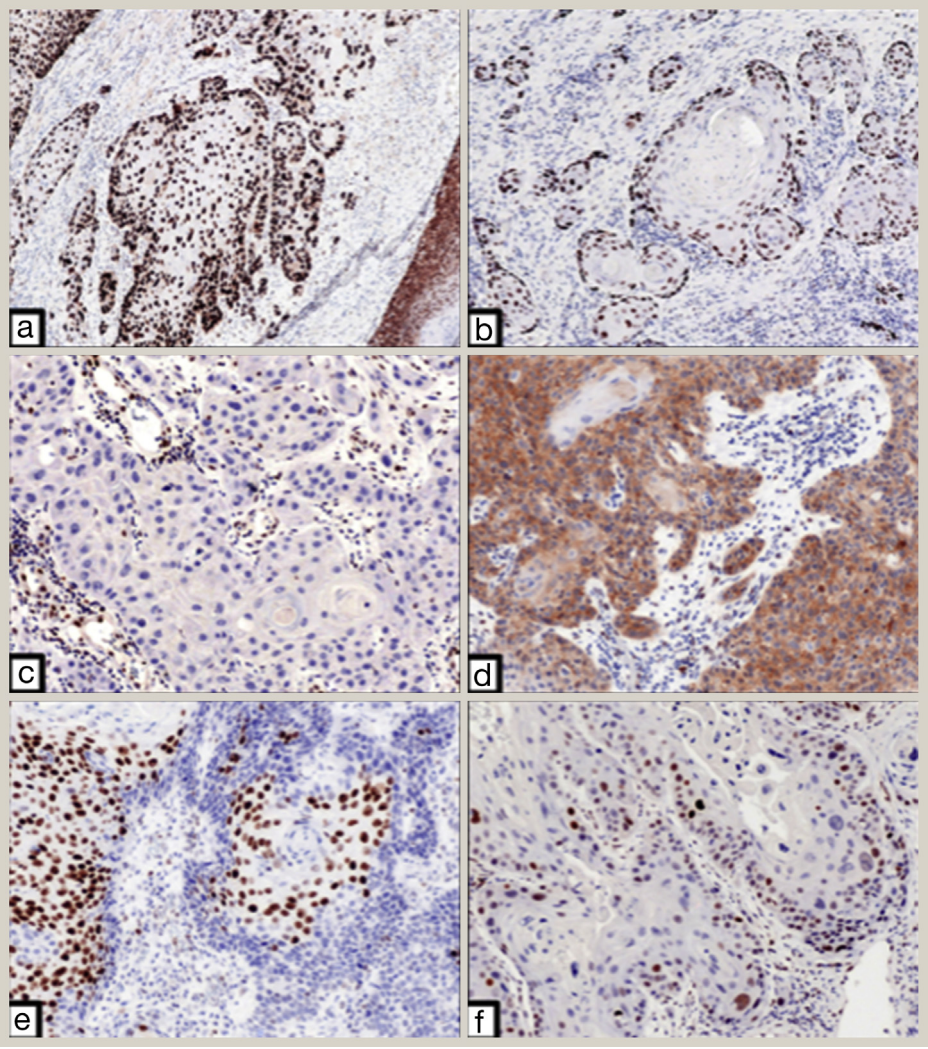

Figure 8 p53 expression in vulval squamous cell carcinoma. There are four patterns of mutation-type p53 expression in HPV-independent vulval carcinomas. Diffuse overexpression shows strong nuclear staining in the basal epithelium, as well as parabasal or even all the epithelial cells (**a**). Basal overexpression is characterized by strong nuclear staining in almost all basal epithelial cells with no or minimal parabasal extension (**b**); this pattern may be easily mistaken as wild-type due to the low percentage of positively stained tumour cells. Null pattern features completely absent nuclear staining with definite positive internal control (**c**). Cytoplasmic pattern shows definite cytoplasmic staining with variable nuclear expression (**d**). In HPV-associated carcinomas, mid-epithelial staining of strong intensity can occur while the basal layer is negative (**e**); this should not be mistaken as aberrant p53 overexpression. Wild-type p53 expression may occur in a subset of HPV-independent vulval carcinomas (**f**).

presence of heterogeneous p53 expression may be explained by the acquisition of *TP53* mutation in a tumour subclone as part of tumour progression, most commonly signifying underlying *POLE* mutation or mismatch repair deficiency.[30]

- **Mutation-type p53 is not specific to serous-like CN−H EC.** As noted already mutation-type p53 IHC expression can be seen in *POLE*mut or MMRd tumours. The application of p53 IHC should therefore ideally form part of an algorithm.[31]
- **Mutation-type p53 expression is not specific to serous histotype in endometrial carcinomas. I**n the differential diagnosis of high-grade endometrial carcinomas, it should be noted that mutation-type p53 may be found in some cases of high-grade endometrioid carcinoma and clear cell carcinoma.[32] Although it has been questioned whether it is necessary to distinguish between p53 mutant endometrial carcinomas of different histotypes as they all belong to the high risk category,[33] currently the morphology and a panel of markers still form the basis of histotype designation.
- **Specific diagnostic considerations apply to adenocarcinomas in the cervix.** When applying p53 to the diagnosis of an adenocarcinoma involving the cervix, it should be noted that a high-grade adenocarcinoma which is p53 mutant and p16 diffuse positive most likely represents serous adenocarcinoma of endometrial or tubo-ovarian origin, instead of a cervical primary (please note the comment above relating to HPV-associated endocervical adenocarcinoma with p53 expression resembling 'null' pattern). A subset of cervical gastric-type adenocarcinomas are p53 mutant but their morphological features should offer clues to the diagnosis.[12]

p53 IHC in vulval carcinoma

It is currently recognized that vulval squamous cell carcinoma (VSCC) and its precursors follow three distinct pathogenetic pathways with different prognostic implications[17]: HPV-associated, HPV-independent p53 abnormal and HPV-independent p53 wild-type. While it has been shown that p16 is a good surrogate for HPV-associated VSCC,[4] assessing the performance of p53 IHC for subclassifying HPV-independent lesions has been problematic. Interpretation of p53 IHC patterns indicative of an underlying *TP53* mutation is more complex in stratified squamous than in glandular epithelia. This is because maturation of epithelial cells, as occurs in stratified squamous epithelium, allows even abnormal p53 protein to be degraded in the upper epithelial layers.[34] Previously p53 IHC has been interpreted with variable cut-offs indicating abnormal staining, resulting in confusing results in the literature. Comparison of p53 IHC patterns with *TP53* sequencing results shows that in HPV-independent *TP53* mutant VSCC and its precursor lesions, four mutation-type patterns can be reproducibly recognized with sensitivity and specificity comparable to that in HGSC (Figure 8)[35]:

i. parabasal/diffuse: strong basal staining with definite parabasal/diffuse nuclear staining. This is the most common pattern seen in *TP53* mutated VSCC.
ii. basal overexpression: strong nuclear staining in all (or almost) all basal epithelial cells and no/minor parabasal extension.
iii. completely absent nuclear staining: as in other cancers this should be accompanied by definite positive internal control
iv. cytoplasmic staining: definite cytoplasmic staining with variable nuclear expression and a normal internal control

Problems and pitfalls in p53 interpretation in vulval squamous cell carcinoma

- **p53 interpretation in vulval squamous lesions should be interpreted in the context of HPV status.** p53 staining in HPV-related usual-type vulval intraepithelial neoplasia and squamous cell carcinomas is absent in the basal layer (basal sparing) but there can be conspicuous positive nuclear staining in the middle layers of the squamous epithelium; strong mid-epithelial staining with basal sparing should not be mistaken as aberrant overexpression indicative of a *TP53* mutation. Careful scrutinization of the basal layer should be performed. This can be difficult in poorly differentiated tumors comprising of small infiltrative nests, where the basal layer is attenuated. In some cases of HPV-associated VSCC, p53 staining spares the basal layer as well as parabasal layers, mimicking the appearance of a null-type pattern.[58] Some cases of usual-type vulvar intraepithelial neoplasia can show superimposed lichen simplex chronicus, mimicking the appearance of dVIN.[36] In this situation, the recognition of a HPV-related p53 pattern and strong p16 staining, will help pathologists correctly categorize the lesion as usual-type vulvar intraepithelial neoplasia.
- **Interpretation of p53 in HPV-independent dVIN and associated VSCC can benefit from comparison with background normal skin.** Most HPV-independent dVIN and VSCC are characterized by aberrant p53 expression, mostly commonly seen as parabasal/diffuse overexpression. As mentioned, p53 overexpression in squamous epithelium commonly exhibit diffuse strong nuclear staining limited to the basal and parabasal layers,[37] although some cases may display full thickness positivity.[38] There is often a sharp demarcation of the abnormal p53 pattern, in comparison with the background skin or hair follicles, which can be a helpful feature. The p53 basal overexpression pattern is amongst the rarest patterns, and is prone to misinterpretation. Poor fixation and can result in confusion with strong wild-type staining. The recognition of null pattern in squamous lesions also requires cautious comparison with the adjacent non-neoplastic squamous epithelium, as the distinction from a weak wild-type pattern can be challenging.[38]
- **A recently characterized group of HPV-independent squamous premalignant lesions are p53 wild-type.** While dVIN is well recognized as an HPV-independent premalignant lesion with aberrant p53 expression, a separate group of p53 wild-type squamous premalignant lesions have gained attention in recent years, represented by two probably overlapping entities known as vulvar acanthosis with altered differentiation (VAAD)[39] and differentiated exophytic vulvar intraepithelial lesion (DEVIL)[40] respectively. These lesions usually display a verruciform appearance and the basal nuclear atypia is minimal.[41] With the wild-type p53 expression, the distinction from non-neoplastic mimics can be very difficult especially in small biopsies. Correlation with the clinical appearance and evaluation of multiple sizable biopsies may help arrive at the diagnosis.

Mismatch repair protein immunohistochemistry in endometrial cancer

Biology of mismatch repair

Before a cell divides it needs to make an exact replica of its entire DNA content. High-fidelity DNA replication is essential for preservation of the genome and there are accordingly a few highly conserved biological processes that govern the accuracy of DNA replication.[41] These are, firstly, accurate selection of the correct DNA base at each position and secondly, a proof-reading process that checks that a complementary nucleotide base has been inserted at each position corresponding to that on the original DNA template that is being replicated; both of these functions are carried out by DNA polymerases (ε and δ). Two types of *mismatches* may occur despite these checks: base—base mismatches, e.g. C inserted opposite to A instead of T, and insertion-deletion (indel) errors at repetitive sequences. The latter occur in segments of repetitive bases within the DNA strand known as short tandem repeats or microsatellites, and are particularly resistant to detection by the proof-reading function of DNA polymerases. The mismatch repair system is a post-replication mechanism that acts closely with the DNA polymerases, serving to detect and correct all mismatches to prevent their being passed on to the daughter cells.[41,42] The mismatch repair proteins, MLH1, PMS2, MSH2 and MSH6, function together in two heterodimers, MLH1-PMS2 and MSH2-MSH6, serving to recognize mismatches and target them

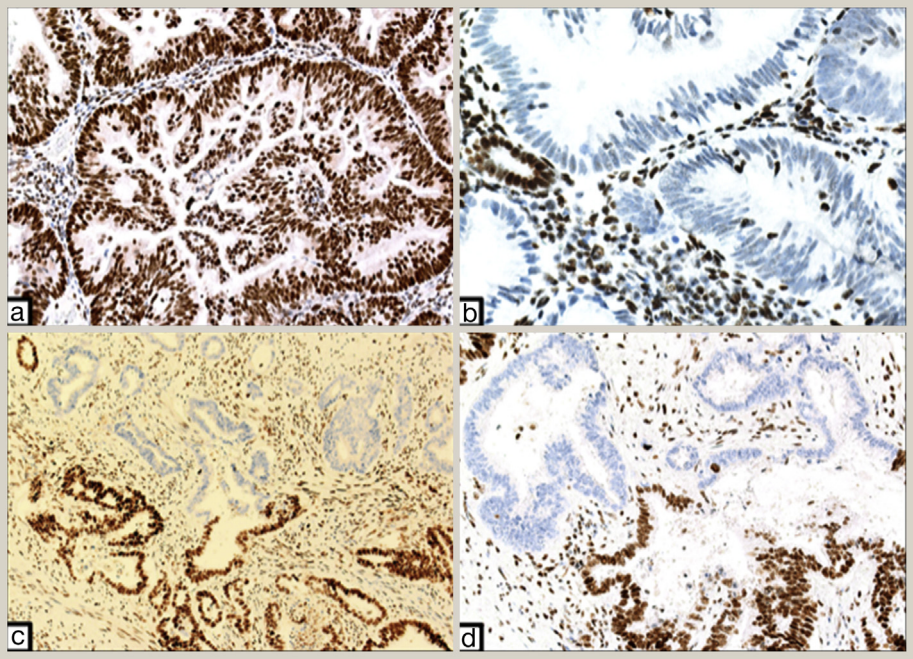

Figure 9 Normal, deficient and heterogeneous/subclonal patterns of mismatch repair IHC. Normal expression of MSH6 in an endometrioid carcinoma shows strong diffuse nuclear staining in tumour cell nuclei (**a**), while loss of PMS2 in the same case demonstrates negative staining in tumour cell nuclei with preserved expression in endometrial stroma, residual normal glands and stromal/intratumoral lymphocytes (**b**). Heterogeneous/subclonal staining for MSH6 in another case of endometrioid carcinoma features diffuse strong nuclear staining in some tumour glands (bottom) coexisting with loss of nuclear staining in others (top), in the presence of preserved staining in stromal cells (**c**). Higher magnification of the same case demonstrates heterogeneous staining within the same tumour gland (**d**).

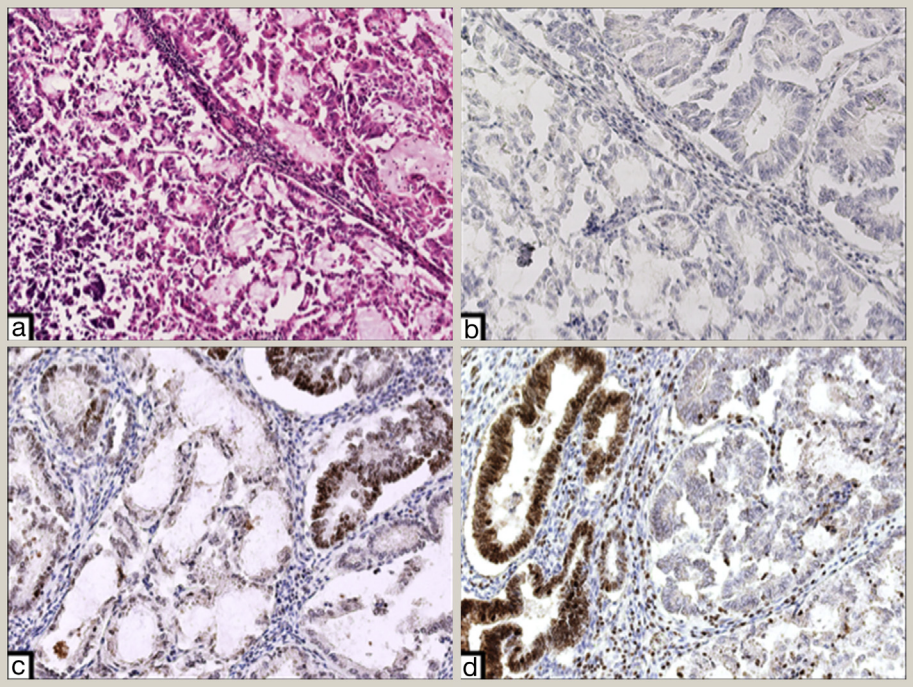

Figure 10 Fixation problems in mismatch repair IHC. In a suboptimally fixed endometrioid carcinoma showing marked artifacts (**a**), the MSH6 staining is not interpretable in an area where both the tumour cells and the internal control (stromal and inflammatory cells) are negative (**b**). In another better fixed area of the same slide, there is positive nuclear staining in some tumour cells (**c**). MLH1 staining of the same case shows negative tumour cell nuclei (right) with valid internal positive control including normal endometrial glands (left), stromal and inflammatory cells (**d**).

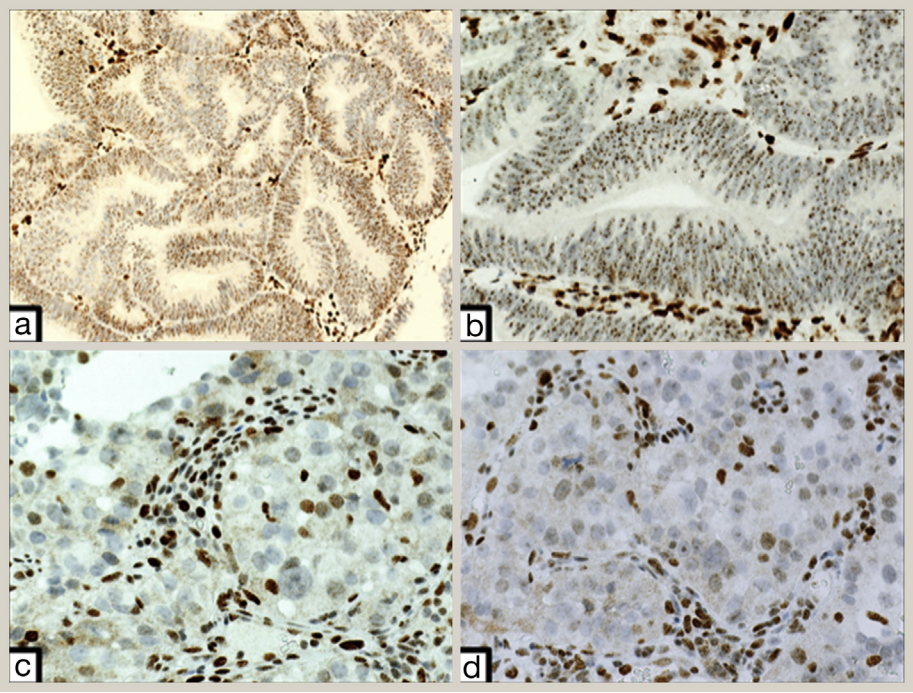

Figure 11 Caveats in interpretation of mismatch repair IHC. Loss of MLH1 in an endometrioid carcinoma may show an uncommon form of artefactual staining with a punctate/dot-like pattern in the tumour cell nuclei (**a, b**); this should not be mistaken as intact nuclear staining. Loss of MSH6 in a clear cell carcinoma of the endometrium may manifest as weak, focal nuclear positivity in the tumour cells, coupled with staining of overall stronger intensity in the internal control (stromal cells and intratumoral lymphocytes) (**c, d**); this should be reported as abnormal (deficient) pattern instead of intact expression.

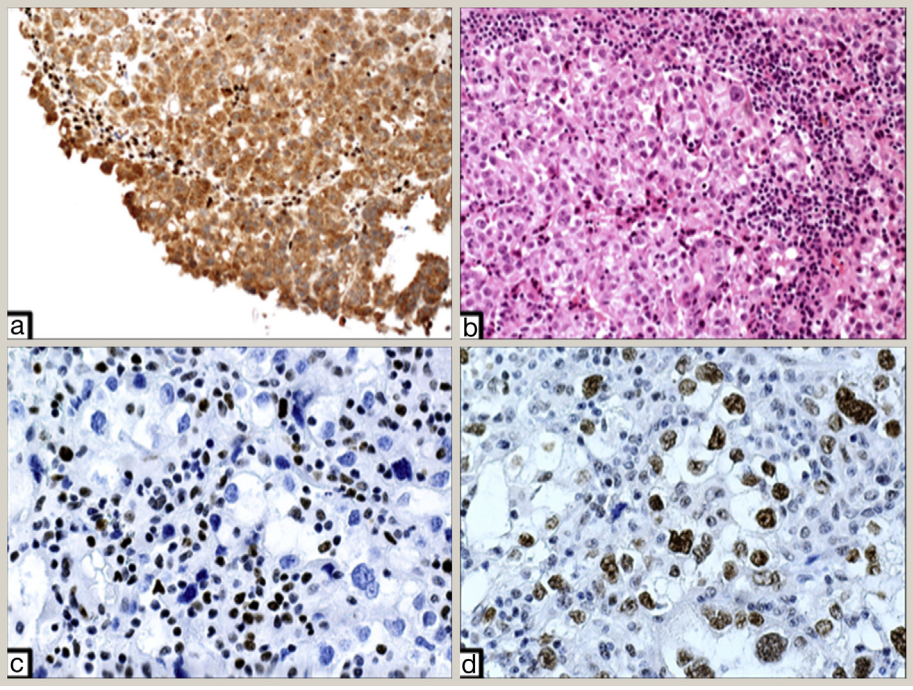

Figure 12 Interpretive issues in mismatch repair IHC. Artifactual cytoplasmic staining for PMS2 as observed here should not be mistaken as intact expression; the protein is lost in this case (**a**). In a case of endometrial clear cell carcinoma with prominent intratumoral lymphocytic infiltrate (**b**), the loss of MSH6 expression may be obscured by substantial number of lymphocytes, which may be misinterpreted as positive staining in tumour cells, especially at low power magnification or when stromal cells or several lymphocytes overlap giving the impression of a bigger positive nucleus (**c**). The preserved staining for MLH1 in the tumour cells of the same case is included for comparison (**d**).

Recommended terminology for reporting mismatch repair protein immunohistochemistry (MMR IHC) +/- *MLH1* promoter methylation results[a,b,c,d]

MMR result	Recommended report
Normal, MLH1, PMS2, MSH2 and MSH6 tested	**MMR IHC Normal:** The tumour cells show normal nuclear staining for MLH1, PMS2, MSH2 and MSH6. Conclusion: There is no immunohistochemical evidence of a mismatch repair deficiency*.
Normal, only MSH6 and PMS2 tested	**MMR IHC Normal:** The tumour cells show normal nuclear staining for PMS2 and MSH6. Conclusion: There is no immunohistochemical evidence of a mismatch repair deficiency*.
Abnormal, MSH6 loss	**MMR IHC Abnormal, MSH6 loss:** The tumour cells show loss of expression of the mismatch repair protein MSH6 (with normal nuclear staining for MLH1, MSH2 and PMS2). Conclusion: This mismatch repair deficiency is associated with Lynch and related syndromes. This patient should be referred to Clinical Genetics services.
Abnormal, PMS2 loss	**MMR IHC Abnormal, PMS2 loss:** The tumour cells show loss of expression of the mismatch repair protein PMS2 (with normal nuclear staining for MLH1, MSH2 and MSH6). Conclusion: This mismatch repair deficiency is associated with Lynch and related syndromes. This patient should be referred to Clinical Genetics services.
Abnormal, MSH2 and MSH6 loss	**MMR IHC Abnormal, MSH2 loss:** The tumour cells show loss of expression of the mismatch repair proteins MSH2 and MSH6 (with normal nuclear staining for MLH1 and PMS2). Conclusion: This mismatch repair deficiency is associated with Lynch and related syndromes. This patient should be referred to Clinical Genetics services.
Abnormal, MLH1 and PMS2 loss, *MLH1* promoter hypermethylation absent	**MMR abnormality, MLH1 loss and *MLH1* Promoter hypermethylation absent:** The tumour cells show loss of expression of the mismatch repair proteins MLH1 and PMS2 (with normal nuclear staining for MSH2 and MSH6). *MLH1* promoter hypermethylation is not present. Conclusion: While this mismatch repair deficiency could be sporadic, it is probable that this mismatch repair deficiency is due to Lynch or related syndromes. This patient should be referred to Clinical Genetics services.
Abnormal, MLH1 and PMS2 loss, *MLH1* promoter hypermethylation present	**MMR abnormality, MLH1 loss and *MLH1* Promoter Hypermethylation present:** The tumour cells show loss of expression of

Table 3 (*continued*)

MMR result	Recommended report
	the mismatch repair proteins MLH1 and PMS2 (with normal nuclear staining for MSH2 and MSH6). The *MLH1* promoter shows hypermethylation is present in the tumour. Conclusion: This combination indicates that this mismatch repair deficiency is almost certainly sporadic rather than due to Lynch Syndrome. This patient does not require referral to Clinical Genetics services*.
Abnormal, MLH1 and PMS2 loss, *MLH1* promoter hypermethylation not tested	**MMR abnormality, MLH1 loss and *MLH1* Promoter hypermethylation not tested:** The tumour cells show loss of expression of the mismatch repair proteins MLH1 and PMS2 (with normal nuclear staining for MSH2 and MSH6). *MLH1* promoter hypermethylation has not been tested. Conclusion: This pattern is likely to be sporadic, although it is possible that this mismatch repair deficiency is due to Lynch or related syndromes. Testing for *MLH1* Promoter hypermethylation is recommended OR this patient may be referred to Clinical Genetics services.
Abnormal, MLH1 and PMS2 loss, *MLH1* promoter hypermethylation pending	**MMR abnormality, MLH1 loss and *MLH1* Promoter Hypermethylation testing results pending:** The tumour cells show loss of expression of the mismatch repair proteins MLH1 and PMS2 (with normal nuclear staining for MSH2 and MSH6). *MLH1* promoter hypermethylation testing in the tumour has been requested. Conclusion: This pattern of mismatch repair deficiency may be either sporadic or due to Lynch or related syndromes — the result of testing for *MLH1* promoter hypermethylation will provide further information. A supplementary report will be issued when these results become available.

*Referral to Clinical Genetics services should be considered despite this result in the presence of a strong family/clinical history.

[a] For referral laboratories only reporting mismatch repair status the report should include: Specimen type, Site of sample, Diagnosis, Overall cellularity (biopsy samples only): High/average/low, Percentage neoplastic nuclei in test area for DNA extraction.

[b] Good fixation is important for obtaining reliable and reproducible patterns of MMR expression by IHC and can be evaluated by assessing MMR expression by internal control cells. Pre-operative biopsies are often better fixed than hysterectomy specimens and may be considered as a better sample for MMR IHC testing, depending on availability. MMR IHC should be reported only in the presence of positive internal control cells, such as stromal cells or lymphoid cells, that are immediately adjacent to the tumour cells under analysis; it must be stated if there is no internal control for comparison.

[c] Rare abnormalities of mismatch repair protein expression are not included in this table and these may be reported as free text where present; examples include weak/patchy/cytoplasmic/punctate or dot-like nuclear patterns of abnormal MMR expression, subclonal/heterogeneous patterns of MMR staining abnormality, and loss of expression of different combinations of MMR proteins (other than the expected MLH1 & PMS2 — or — MSH2 & MSH6 combinations).

[d] The molecular mechanism for the strong association of *BRAF* mutation with CRC harbouring somatic *MLH1* hypermethylation is incompletely understood but appears to be tissue/tumour-specific; unlike algorithms in use for CRC, BRAF immunohistochemistry or sequencing cannot be used as a proxy for somatic *MLH1* hypermethylation in gynecological cancers, as oncogenic *BRAF* mutations occur so rarely in these.

Table 3

for excision, accurate resynthesis and ligation. In the presence of a defective mismatch repair system therefore, the cell becomes prone to acquiring large numbers of mutations and the development of cancer. Although all mismatches accumulate in the presence of a mismatch repair defect (MMRd), microsatellites are particularly susceptible to errors, resulting in a state of microsatellite instability,[43] or the widespread occurrence of indel errors in microsatellites, which are present throughout the genome in coding and non-coding DNA. MSI is thus the result of MMRd, and the two terms are often used interchangeably.

About 25–30% of EC are characterized by defective MMR.[27] This occurs sporadically within the target tissue, in this case the endometrium, in the majority of cases, most commonly as a result of methylation of the *MLH1* promoter region and resultant epigenetic silencing of *MLH1*. About 10% of MMRd is inherited as part of Lynch Syndrome (LS); this is an inherited cancer susceptibility syndrome in which the patient inherits one defective allele from a parent and loss of function of the second allele occurs in the target tissue, most commonly colorectal or endometrial, resulting in cancer.[44]

Detection of MMRd can be carried out through immunohistochemistry for the MMR proteins or through MSI testing.[45] The two methods have comparable sensitivity are show approximately 96% concordance, with IHC having the advantages of being cheaper, easily accessible to pathologists, amenable to IHC external quality assurance schemes, allowing correlation with morphology and enabling identification of the defective protein, thereby guiding downstream testing. The discussion of MSI testing is outside the remit of this review which will now focus on MMR IHC testing.

MMR IHC in endometrial (and ovarian) carcinoma

The indications of MMR IHC in EC (and endometrioid and clear cell ovarian carcinomas) are all of the following:

i. screening for LS: it is estimated that one in 250–300 individuals are affected by LS and that >95% of these cases are unaware of their cancer-susceptibility risk. EC is often the first or sentinel cancer in a pedigree, and therefore provides an opportunity for detection of LS in a family.[46] An EC patient with LS may go on to develop other cancers and EC precedes subsequent cancers such as colorectal carcinoma by approximately a decade.[47] The diagnosis of LS in a family allows surveillance and preventative measures that significantly reduce the mortality from subsequent LS-related cancers.[48]

ii. molecular diagnosis of EC: the TCGA classification requires MMR IHC or MSI testing of all cases for identification of the hypermutated MMRd/MSI category of EC,[31] with important management implications; such tumours are unlikely to respond to conservative treatment with progesterone, show a high likelihood of lymphovascular space invasion justifying a sentinel or other nodal procedure, and chemotherapy results in no significant survival benefit in these tumours, which on the other hand, respond well to radiotherapy.

iii. predictive testing for MMRd: MMRd tumours of all sites are eligible for targeted treatment with immune checkpoint inhibitors.[49]

Interpretation guidance and terminology

MMR protein expression in normal tissues is seen as nuclear staining of variable intensity.[50] In cancers, generally characterized by high proliferation rates relative to normal tissue, there is typically strong nuclear staining. Although the intensity may be variable, this is generally higher than that seen in the background stroma, normal glands or inflammatory cells that serve as an internal control (Figure 9). In the presence of a mismatch repair defect, there is loss of expression of one or more of the MMR proteins. As mentioned previously, the MMR proteins occur as heterodimers with MLH1 pairing with PMS2 and MSH2 with MSH6. While MLH1 and MSH2 can stabilize in the cell by forming heterodimers with other proteins, PMS2 and MSH6 can only exist stably in the cell in the presence of MLH1 and MSH2 respectively. This has two important consequences. The first is that MMRd results in four typical MMR IHC patterns:

1. loss of both MLH1 and PMS2; this occurs in MLH1 deficiency
2. loss of both MSH2 and MSH6; this occurs in MSH2 deficiency
3. isolated loss of MSH6; this occurs in MSH6 deficiency
4. isolated loss of PMS2; this occurs in PMS2 deficiency

The second consequence is that testing for just two proteins, PMS2 and MSH6, can be used to screen for MMRd with equivalent accuracy to testing for all four proteins,[45,51] provided there is due regard to the pitfalls listed below.

MMR IHC forms part of a testing algorithm, particularly with regard to LS screening. It is vital that standard reporting terminology is used with appropriate emphasis and recommendations for further testing. The implications of MMR IHC results vary for each pattern seen, and this is reflected in the standard recommended terminology summarized in Table 3.[46,52]

Problems and pitfalls in MMR interpretation

- **MMR IHC is fixation-sensitive.** Poor fixation is a common problem in pathological reporting of EC. It is vital that well-fixed areas are examined when reporting MMR IHC, to avoid erroneous interpretation of one or more stains as loss of expression (Figure 10). For this reason MMR IHC should be carried out on biopsies, with the added advantage that this vital information is available at the time of diagnosis of EC.[50]
- **Very weak or very focal expression may be seen in the presence of MMRd.** Very weak or very focal MMR expression can occur in the presence of a defective MMR protein (Figure 11) and similarly weak or focal MSH6 expression may be seen in the presence of MSH2 mutation.[53] As already stated the expression of MMR proteins is generally strong and diffuse relative to the internal control and *any deviation* from this, including heterogeneous/subclonal expression, or very weak/focal expression, should be noted and reported either as defective or equivocal. Repeating the staining on a different section or a biopsy rather than the hysterectomy specimen can solve some of these issues.
- **Subclonal expression may occur in a minority of cases.** Subclonal expression of MLH1 ± PMS2 can occur when *MLH1* promoter methylation is acquired during tumour progression; this pattern therefore generally reflects a sporadic rather than acquired MMRd. An acquired or secondary subclonal MSH6 loss is also described and

occurs secondary to any mechanism of MMRd due to the occurrence of a mutation in a microsatellite within the MSH6 molecule.

- **A low proportion of MLH1 loss cases can show punctate nuclear expression that may be erroneously interpreted as retained/normal expression.** This pattern has been described in previous publications and should be reported as loss of expression.[54–56] To the best of our knowledge this does not occur with any other protein.
- **Cytoplasmic/membranous staining does not constitute normal expression and should be reported as abnormal.** The MMR proteins are localized to the nucleus. In some cases, possibly related to technical reasons, there is relatively conspicuous cytoplasmic or membranous staining in the absence of nuclear staining (Figure 12); such cases should be reported as abnormal.[50]
- **In addition to the typical patterns listed above, a range of other patterns/problems may occur, such as loss of three or more proteins, discordance between MMR IHC and MSI or between MMR IHC and genetic testing.**[50,57] A superadded somatic defect, usually *MLH1* promoter methylation, can be seen in any MMRd, resulting in unusual MMR IHC patterns. Mutations that result in a functionally defective but antigenically preserved protein can give rise to MMRd with normal IHC. It is important to note that MMR IHC loss with absence of *MLH1* promoter methylation does not equate to LS, and only about half of the cases will be proven to have an inherited defect; this is reflected in the recommended terminology.

Conclusions

Immunohistochemistry for p16, p53 and MMR proteins have a central place in classification and management of gynaecological neoplasms and pathologists need to be aware of current guidance on usage, interpretation, pitfalls and terminology for each of these markers. ◆

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