Ancillary studies (Core and Non-core)

Immunohistochemistry for mismatch repair proteins and MLH1 promoter methylation

Immunohistochemistry (IHC) for mismatch repair (MMR) proteins is recommended in addition to analysis for MLH1 promoter methylation when there is immunohistochemical loss of MLH1 or PMS2 as a core reporting parameter.[1]

Endometrial cancer is one of the most common tumours in patients with Lynch syndrome (also known as hereditary non-polyposis colorectal cancer).[2,3] Around 3% of all endometrial carcinomas and approximately 10% of MMR deficient (MMRd)/microsatellite unstable endometrial carcinomas are causally related to germline mutations of one of the MMR genes MLH1, PMS2, MSH2 and MSH6 or a related gene, EPCAM.[4] 'Constitutive methylation' is also a rare cause of Lynch syndrome.[5]

Testing for MMR status/microsatellite instability (MSI) in endometrial carcinoma patients has been shown to be important for four key reasons:

1. Diagnostic, since MMRd/MSI is helpful to diagnose endometrioid carcinomas (as opposed to serous carcinoma or human papillomavirus (HPV)-associated cervical carcinoma);
2. It is part of the screening algorithm to identify potential patients with Lynch syndrome;[6]
3. Prognostic, as part of the TCGA surrogate molecular classification;[7] and
4. Therapeutically as a predictive biomarker for potential utility of immune checkpoint inhibitor therapy.[8]

Systematic clinical screening of personal and family history misses a significant proportion of women with Lynch syndrome, since up to 75% of patients do not fulfill the revised Bethesda Guidelines criteria.[9] International Society of Gynecological Pathologists (ISGyP) has recommended testing for MMR status/MSI in all endometrial carcinomas (preferably curettings or biopsy), irrespective of age.[1] This has also been recommended whenever resources are available by other societies/groups, such as the Manchester International Consensus Group.[10] The identification of Lynch syndrome in women with endometrial carcinoma can lead to the prevention of a second cancer in the patient and reduced incidence of cancers in family members through risk reducing strategies and heightened surveillance.

Microsatellite instability (MSI) can be detected by different methods, including polymerase chain reaction (PCR)-based approaches[9,11,12] and next generation sequencing (NGS).[13] NGS is in the process of being validated for this scenario. MSI can also be accurately predicted using IHC.

Immunohistochemistry (IHC) is cost effective and is implemented in most pathology departments. ISGyP guidelines recommend IHC as the best test for MMR deficiency and, indirectly, for MSI.[1] The IHC approach consists of an assessment of the expression of four DNA MMR proteins; MLH1, PMS2, MSH6, and MSH2. A simplified version includes only PMS2 and MSH6, with expanded analysis of MLH1 when PMS2 is lost, and of MSH2 when MSH6 is lost.[14] Carcinomas showing loss of MLH1 and PMS2 expression should be investigated for MLH1 promoter hypermethylation,[15] as its presence essentially excludes Lynch syndrome. Endometrial cancer patients whose tumours are MMRd, but not methylated at the MLH1 promoter, should undergo genetic counselling with consideration for germline testing.

Immunohistochemistry (IHC) may be not informative when the specimen has been subjected to poor pre-analytical conditions, such as inappropriate or delayed fixation. Furthermore, occasionally there are germline genetic abnormalities that do not result in abnormal expression of MMR proteins. In these cases, PCR-based techniques to assess MSI may be appropriate, particularly when the family history is highly suspicious for Lynch syndrome. MSI detected by PCR-based methods usually requires testing both normal and tumour tissue, although there is a recently described method that only requires tumour tissue.[16]

The cumulative incidences of colorectal, endometrial, ovarian, upper gastrointestinal, urinary and brain cancers in women aged 75 years with Lynch syndrome, depend on the specific mutation. The cumulative incidences have been reported as: germline *MLH1* mutation (46%,43% 10%, 21%, 8%, 1%);

germline *MSH2* mutation (43%, 57%, 17%, 10%, 25%, 5%); germline *MSH6* mutation (15%, 46%, 13%, 7%, 11%, 1%), respectively.[17] In contrast, PMS2 is mostly associated with a moderate increase in colon and endometrial cancer risk, with a cumulative incidence at age 80 years of 12% and 13%, respectively.[18]

The Cancer Genome Atlas (TCGA)-based molecular classification of endometrial carcinomas

Reporting of TCGA-based molecular classification of endometrial carcinomas is a non-core parameter. Diagnosis and classification of endometrial carcinoma has up until now largely been based on the microscopic appearance of the tumours.[19] The different histologic types have different molecular features, microscopic appearances, precursor lesions, and natural history, although in multivariate analyses,[20] International Federation of Gynaecology and Obstetrics (FIGO) stage and grade have more prognostic significance than histotype. Unfortunately, histological typing engenders problems with interobserver reproducibility and prognostication. While diagnosis is quite reproducible in low grade (FIGO grades 1 and 2) endometrioid carcinomas, which account for 70% of endometrial carcinomas, in typical serous and clear cell carcinomas, there is poor interobserver agreement in approximately 10% of tumours. This is particularly evident in a subset of endometrial carcinomas with high grade morphology[21-23] with microscopic and immunohistochemical features that are shared between high grade endometrioid and serous carcinomas.

The TCGA performed an integrated genomic, transcriptomic and proteomic characterisation of endometrial carcinoma.[24] Exome sequence analysis revealed four groups of tumours. Group 1 carcinomas (7% of endometrial carcinomas) have somatic inactivating hotspot mutations in the *POLE* exonuclease domain and a very high mutational burden (ultramutated). FIGO grade 3 endometrioid carcinomas are highly represented in group 1, some of which resemble serous carcinomas. Irrespective of grade, group 1 tumours have an excellent prognosis, although this is not confirmed in all of the recent literature.[24-27] Group 2 and Group 3 show similar progression-free survival rates that are intermediate between groups 1 and 4. With additional research, it is becoming apparent that groups 2 and 3 are heterogeneous, each having genomically-defined subgroups of tumours, some of which are prognostically favourable and others that are unfavourable.[24,28-30] Group 2 (28% of tumours) include endometrioid carcinomas with MSI (hypermutated), frequently with MLH1 promoter hypermethylation and high mutation rates. Group 3 tumours (39% of endometrial carcinomas) include endometrioid carcinoma with low copy number alterations, and low mutational burden, while lacking *POLE* mutations and MSI-high (MSI-H). Group 3 tumours have also been referred to as 'no specific molecular profile (NSMP)'. Finally, Group 4 (serous-like or copy-number high; 26% of carcinomas) show a low mutation rate, nearly universal (95%) *TP53* mutations, and a highly unfavourable prognosis. Most of these tumours are serous carcinomas, but up to 25% of endometrioid (mostly high grade) and clear cell carcinomas, along with carcinosarcomas, can be found in this group.

In an attempt to bring the TCGA molecular-based classification into clinical practice, different groups have proposed a surrogate (simplified) algorithm precluding comprehensive tumour profiling.[7,29,30] The algorithm includes three immunohistochemical markers (p53, MSH6 and PMS2) and one molecular test (mutation analysis of *POLE*). Several studies have demonstrated the prognostic value of this TCGA-surrogate approach, and ISGyP have recommended this scheme.[1,28,31]

According to this simplified algorithm, tumours with pathogenic *POLE* mutations correspond to ultramutated tumours. MSH6 or PMS2 abnormal expression defines tumours in the hypermutated group. Abnormal expression of p53 (mutated pattern), characterises the high copy number group. Finally, NSMP is defined by the absence of *POLE* mutation, and a normal expression pattern for MSH6, PMS2 and p53.[7,30]

The TCGA surrogate approach has been shown to be particularly helpful in the group of high grade endometrioid carcinomas, including cases in the grey zone between endometrioid and serous carcinomas. High grade endometrioid carcinoma had been regarded as an aggressive tumour type with some similarities to serous carcinoma. However, application of the TCGA surrogate shows that there is a group of high grade endometrioid carcinomas with an improved prognosis (tumours with pathogenic

POLE mutations), and a group with a very poor prognosis (p53-abnormal tumours). MSI-H and NSMP grade 3 endometrioid carcinomas have an intermediate prognosis.[32] Application of this algorithm for clear cell carcinoma,[33] undifferentiated carcinoma,[34] neuroendocrine carcinoma,[35] and carcinosarcoma[36] is possible, but this is currently considered investigational as these tumours were not included in the original TCGA paper.[24] The vast majority of low grade endometrioid carcinomas are NSMP or MSI, with *POLE*-mutated, or *TP53*-abnormal tumours accounting for less than 10%. Moreover, the vast majority (95%) of serous carcinoma are *TP53* abnormal.

There is still discussion about whether to apply the molecular classifier to all endometrial carcinomas or just in diagnostically challenging high grade tumours. An important factor in the decision to base therapy selection on genomic subgrouping, includes that most evidence is still retrospective. Prospective studies are awaited and ongoing (e.g., Post Operative Radiation Therapy in Endometrial Carcinoma-2 (PORTEC) 4a). The availability of resources, particularly for *POLE* mutation analysis, are not always accessible. However, perhaps the most important argument against generalised introduction of the molecular classifier is that studies so far have not shown that risk stratification using TCGA molecular data is superior to the European Society for Medical Oncology (ESMO) classification, which relies on clinicopathological data.[7] Also, most evidence in support of the TCGA classification is based on two large but retrospective cohorts.[7,30] There are two additional complexities to *POLE* testing: distinguishing between pathogenic and non-pathogenic mutations,[37] and coexistence of ultramutation (i.e., pathogenic *POLE* mutation) with secondary mutations in *TP53* and/or one or more of the DNA MMR genes.[38] These 'multiple classifier' cases are currently thought to retain the favourable prognosis of *POLE* mutated tumours, regardless of the MMR or p53 status but this is still an evolving field.

Other markers

Immunohistochemistry (IHC) may be helpful for diagnosis. With a differential diagnosis involving endometrioid and serous carcinomas, loss of expression of DNA MMR proteins, PTEN and/or ARID1A expression would favour endometrioid carcinoma, whereas both serous and endometrioid carcinomas can show aberrant p53 staining and p16 overexpression (both more common in serous carcinoma).[39] Napsin A, HNF1-beta and AMACR (together with negative estrogen receptor (ER))[40,41] may be helpful in diagnosing clear cell carcinoma. A combination of cytokeratin staining, EMA, PAX8 and E-cadherin may also be useful in distinguishing between undifferentiated carcinomas and high grade endometrioid carcinomas since the former generally shows markedly reduced staining with these markers compared to the latter. Neuroendocrine markers can help in recognition of neuroendocrine tumours,[42] and GATA3, TTF1, CD10 and calretinin may help in recognising mesonephric-like carcinoma.[43,44] Finally, a panel including p16, ER, progesterone receptor (PR), and high risk *HPV* in situ hybridisation may be useful in ruling out an HPV-associated endocervical adenocarcinoma.[45]

There are also immunohistochemical markers of prognostic and predictive value. HER2 protein overexpression and/or *HER2* gene amplification is encountered in approximately 25-30% of endometrial serous carcinomas,[46-48] and 14% of endometrial carcinosarcomas.[49] Intratumoural heterogeneity of HER2 expression and gene amplification are common in these tumours and should be taken into consideration when evaluating their HER2 status.[46,50] HER2 positivity in endometrial serous carcinomas is associated with worse progression free and overall survival,[51] but they can be therapeutically targeted by adding trastuzumab to the standard chemotherapy regimen.[52,53] It has been recently shown that *HER2* amplification is characteristic of p53-abnormal endometrial carcinomas as defined in the molecular classification, and is not restricted to the serous carcinoma category.[54] Although currently no official endometrial cancer-specific pathology HER2 scoring guidelines exist, a new set of criteria have been recently proposed based on the successful clinical trial experience.[55]

L1CAM expression has been touted as a marker of aggressive behaviour amongst the NSMP carcinomas and is associated with non-endometrioid histology, distant metastasis and poor survival.[56-58] Mutations in *CTNNB1* (but not necessarily nuclear expression of beta-catenin with IHC) are considered by some to be associated with diminished survival in low grade endometrioid carcinomas, but this is not universally accepted.[30,59,60]

Estrogen receptor (ER) expression has been associated with tumour behaviour and survival in endometrioid tumours.[61,62] ER/PR may assist with tumour classification and may be important to some clinicians for treatment planning, although there is some controversy on whether the expression status of the initial hysterectomy specimen reflects the status of the progressive disease at a later stage. A recent systematic review confirmed improved response rates to endocrine therapy in tumours with positive ER and PR, especially when determined in the metastatic tissue.[63]

WT1 expression may be helpful to distinguish between a primary endometrial serous carcinoma and a tubo-ovarian high grade serous carcinoma since the latter is more likely to be positive. However, up to 30-40% of endometrial serous carcinomas may exhibit some degree of WT1 positivity.[64]

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