Histone H3 mutations and H3 K27 trimethylation (me3) (Non-core)

Reason/Evidentiary Support

Any standard sequencing method can be used to detect the H3 K27M mutation, including pyrosequencing, Taq Man PCR, droplet-digital PCR, Sanger sequencing, and NGS. A similar array of sequencing methods can be used for H3 G34 mutations, however due to the GC rich nature of this region, targeted methods can be more difficult to set up. For detection of both mutations using targeted methods (and alignment of non-targeted methods), consideration needs to be given to the high degree of homology among the H3 genes (human H3 variants include H3.3, H3.1, H3.2, CENP-A, H3t, H3.X and H3.Y) and the number of genes encoding each protein (H3.3 is encoded by two genes, *H3F3A* and *H3F3B*, while H3.1 and H3.2 are each encoded by multiple genes found within gene clusters). The exact gene being tested and the method used should be provided in the report.

Histone H3 K27M Mutation (Sequencing) and Expression (Immunohistochemistry)

Recurrent mutations in *H3F3A* (H3.3) and *HIST1H3B/C/I* (H3.1) with lysine 27 substituted for methionine (H3 K27M) are characteristic of paediatric high-grade astrocytomas with a predilection for a midline location; less commonly, these mutations are found in adult midline diffuse gliomas.[1-3] These tumours have a poor prognosis. The H3.3 K27M mutation is found in approximately 70% of diffuse intrinsic pontine gliomas and H3.1 K27M in a further 15%. Furthermore, in the paediatric age group, H3.3 K27M is also found in approximately 50% of high-grade diffuse gliomas involving the thalamus and spinal cord. H3 K27M mutations also occur in a broader range of patient ages, morphologies, and locations; the median age to date is the third decade for spinal cord and thalamic tumours with patients as old as 65 years being reported with the alteration. Other locations include third ventricle, hypothalamus, pineal region and cerebellum.[4] H3 K27M mutation can also be found in diffuse astrocytomas without classic high-grade features that generally behave more aggressively than their wild type counterparts. In occasional cases, the mutation has been found in other tumour types, including ganglioglioma,[5] pilocytic astrocytoma[6] and ependymoma.[7] Testing for this alteration should be considered, at a minimum, in all midline diffuse gliomas in patients under the age of 30. These alterations can be identified by sequencing or a mutation-specific antibody. Detection of the mutation by either immunohistochemistry or sequencing is required for the diagnosis of *Diffuse midline glioma, H3 K27M mutant*. Lack of H3 K27-me3 is not a specific marker of H3 K27M status.

Immunohistochemistry with an antibody against the N-terminus of the mutant protein is highly sensitive and specific for detection of the H3K27M protein from either H3.3 or H3.1.[8,9] In practice, the antibody can produce a fair amount of background cytoplasmic staining in non-tumour cells and only diffuse strong nuclear staining in most (or all) tumour cells should be considered positive. Further, poorly fixed tissue or tissue from post-mortem or older blocks may be false negative. If equivocal, a sequencing-based method (see below) should be considered as the standard of care.

Histone H3 G34 Mutations (Sequencing) and Expression (Immunohistochemistry)

Recurrent mutations in *H3F3A* (H3.3) with glycine 34 substituted for arginine (H3 G34R) or infrequently valine (H3 G34V) are found most commonly in hemispheric high-grade gliomas of the adolescent and young adult population (median age 15 years; range 9-51 years).[10] The H3G34R mutation is found in ~15-20% of hemispheric high-grade glioma cases in the pediatric age group.[11] Outcome is slightly better than in H3K27M-mutant tumours in a midline location, with a median survival of approximately 18 months. Testing for this alteration should be considered, at a minimum, in hemispheric, IDH-wildtype, high-grade gliomas in patients under the age of 30, particularly if ATRX

is lost and p53 is diffusely immunopositive. These alterations can be identified by sequencing or a mutation-specific (H3 G34R) antibody.

Immunohistochemistry with an antibody against the mutant protein is specific for detection of the H3G34R protein.[12] In practice, the antibody works well on FFPE tissue with specific nuclear staining but does not stain every tumour cell; as a result, sensitivity may prove to be an issue as more experience is gained with the antibody. If immunohistochemical results are equivocal or if suspicion for mutation is high, a sequencing-based method should be considered as the standard of care.

Histone H3 K27me3 Expression (Immunohistochemistry)

The presence of the H3 K27M mutant protein is associated with a fairly widespread (and thus detectable on Western blot or immunohistochemistry) loss of the repressive trimethyl (me3) mark on lysine 27 (K27me3). Tumour cells harbouring the H3 K27M mutation (either H3.1 or H3.3 K27M) will typically show loss of nuclear expression of this protein on immunohistochemistry with retention of staining in entrapped non-neoplastic cells, e.g., endothelial cells (similar to the pattern seen with ATRX or INI1). However, it should be noted, that while loss of H3K27me3 is sensitive for detection of H3 K27M mutant tumours, it is not specific. Other tumours, notably some posterior fossa ependymomas,[13] will also show loss of H3 K27me3; in ependymomas this lack of immunoreactivity aligns with the posterior fossa group A (PFA) tumours.[13,14] Similarly, in some H3-wildtype cases, partial loss may be seen. Thus, while helpful for confirmation when combined with an H3 K27M stain, loss of H3 K27me3 staining by itself should be considered a non-specific surrogate marker for identifying H3 K27M-mutant diffuse midline gliomas.

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