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ISSN 0722-5091

DOI 10.5414/NPP30047

FISH-based detection of 1p 19q codeletion in oligodendroglial tumors: procedures and protocols for neuropathological practice – a publication under the auspices of the Research Committee of the European Confederation of Neuropathological Societies (Euro-CNS)

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Key words

fluorescence *in situ* hybridization – oligodendroglial neoplasm – chromosomal arms 1p 19q

Abstract. The codeletion of chromosomal arms 1p 19q is a characteristic and early genetic event in oligodendroglial tumors, that is associated with a better prognosis and enhanced response to therapy. Over the last years, the increasing clinical demand to determine the 1p 19q status has led to the implementation of its testing in many neuropathology laboratories. Several different methods for 1p 19q testing are available: PCR-based loss of heterozygosity analysis, multiplex ligation-dependent probe amplification, array comparative genomic hybridization, and fluorescence *in situ* hybridization (FISH). Herein, we focus and critically discuss the latter method because a detailed description of procedures and protocols for FISH-based 1p 19q testing in practice is lacking. We present a practical approach to the FISH-based assessment of the 1p 19q status in oligodendroglial tumors, from commonly used locus-specific probes and technical protocols to the neuropathological interpretation of results. Thereby, we aim to facilitate the implementation of FISH-based 1p 19q testing for clinical purposes in standard neuropathology laboratories without special focus on brain tumor research.

Introduction

In gliomas, a number of biomarkers that provide diagnostic or prognostic/predictive

information are currently under debate, the most common being isocitrate dehydrogenase-1 (*IDH1*) mutation in low- and high-grade gliomas [1, 2], *BRAF* gene fusion in pilocytic astrocytomas [3], O⁶-methylguanine methyltransferase (*MGMT*) promoter methylation status in glioblastomas [4], and the codeletion of chromosomal arms 1p 19q in oligodendroglial tumors [5, 6, 7]. However, whether and how fast a biomarker translates from preclinical research to the routine diagnostic setting depends on both its clinical and analytical performance [8]. Among the various candidate biomarkers in gliomas, the 1p 19q codeletion in oligodendroglioma probably constitutes the best-characterized and most extensively investigated marker up to date. The 1p 19q codeletion is a characteristic and early molecular genetic event in oligodendroglial tumors, and 1p 19q codeleted tumors are associated with a better prognosis and enhanced response to therapy [5, 9, 10]. Over the last years, the increasing interest of medical oncologists in the 1p 19q status has led to the implementation of its testing in many neuropathology laboratories. However, despite the huge body of literature on this marker, no consensus guidelines or standard protocols for practical use exist.

Received
December 24, 2010;
accepted in revised form
January 11, 2010

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Table 1. FISH reagents.

Reagents
1M NaSCN Natriumthiocyanat (pH 6.0 – 8.5)
0.2N HCl
Pepsin buffer (0.9% NaCl, pH 2.0)
0.05% Pepsin-protease solution
3.7% Formaldehyde in 1 × PBS pH 7.2 (phosphate buffered saline)
Citrate buffer
2×SSC pH 7.0 (saline-sodium citrate buffer)
2×SSC/0.3% NP-40 wash solution
Aqua bidest
Fluorescence mounting medium (4',6-diamidino-2-phenylindole – DAPI)
Ethanol 70%/96%

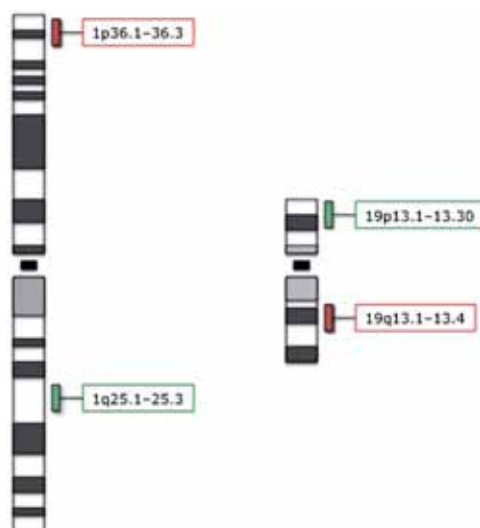


Figure 1. Locus-specific probes on chromosomes 1 and 19 (Vysis, Abbott Laboratories, Abbott Park, IL, USA). Chromosomal target regions are indicated in red, control regions in green.

Various methods for 1p 19q testing are available: PCR-based loss of heterozygosity (LOH) analysis, multiplex ligation-dependent probe amplification (MLPA), bacterial artificial chromosome (BAC)-array comparative genomic hybridization (aCGH), and fluorescence *in situ* hybridization (FISH) [11, 12, 13, 14]. The latter method has proven robust and cost-efficient, and straightforward to implement, but a detailed description of procedures and protocols for FISH-based 1p 19q testing in practice is lacking.

Herein, we present a detailed practical approach to FISH-based analysis of the 1p 19q status, including commonly used locus-spe-

cific probes, technical protocols and the interpretation of results, in order to facilitate implementation of FISH-based 1p 19q testing for clinical purposes in standard neuropathology laboratories without special focus in brain tumor research.

Procedures and protocols

Tumor tissue

1p 19q FISH can be performed on either RCL2- [15], or formalin-fixed paraffin embedded (FFPE), or fresh/frozen tumor tissue. It does not require additional blood samples of the patient.

Regions of interest

Tumor areas need to be pre-selected under the light microscope. Adequate areas should contain > 60 % tumor cell infiltration [16], and no necrosis or hemorrhage. Further processing of the block relies on the chosen approach: interphase nuclei may be isolated from punch preparations of the block [17], or whole-block slides are cut at a thickness of 4 – 5 microns [18]. FISH analysis is performed using a dual-color approach for chromosomes 1 and 19 separately. Target probes hybridize to subtelomeric 1p36 and 19q13.3 in combination with control probes on 1q and 19p, respectively (Figure 1). For evaluation, the signal ratio is assessed for 100 – 200 adjacent, non-overlapping interphase nuclei, and the results are expressed as percentage.

Probes

A number of companies provide locus-specific and fluorochrome-labeled DNA probes. Widely used, commercially available probes include those of Vysis® (Abbott Laboratories, Abbott Park, IL, USA) or Zytomed Systems® (Berlin, Germany). Among other suppliers are Qbiogene Inc® (Carlsbad, CA, USA), Cytocell Ltd® (Cambridge, UK), and Kreatech Diagnostics® (Amsterdam, The Netherlands). For some laboratories, in-house production of the probes may be an adequate alternative.

Table 2. MUV/MUI protocol for FISH on formalin-fixed paraffin-embedded sections.

Pre-treatment	
Step 1	Deparaffinize slides (4 – 5 microns thick) by incubating in xylene at 65 °C for 3 × 10 min, dehydrate 2 × 5 min in ethanol (100%) and air dry for 10 min
Step 2	Pretreat slides in 0.2 N HCl for 20 min at room temperature
Step 3	Wash slides in 2×SSC for 2 × 5 min at room temperature (shake slightly)
Step 4	Incubate slides in 1 M NaSCN or alternatively citrate buffer, both for 30 min at 80 °C (water bath)
Step 5	Wash slides in 2×SSC for 2 × 5 min at room temperature (shake slightly)
Step 6	Incubate slides in 0.05% protease-pepsin solution for 20 min at 37 °C (water bath)
Step 7	Rinse slides briefly in 2×SSC and wash for 2 × 5 min in 2×SSC at room temperature (shake slightly)
Step 8	Fix slides in 3.7% formaldehyde in 1 x PBS for 10 min at room temperature
Step 9	Wash slides in 2×SSC for 2 × 5 min at room temperature (shake slightly) and air dry for 10 min
Step 10	Dehydrate slides by a series of ethanol washes (70%, 90% and 100%) each for 2 min and air dry for 10 min
Co-denaturation and hybridization	
Step 1	Add probe mixture, cover by a coverslip and seal with rubber cement
Note: Handle slides in reduced light after probes have been applied	
Step 2	Co-denature slides on a hot plate at 78 °C for 8 min
Step 3	Hybridize slides over night in a preheated humidity chamber at 37 °C for at least 16 h
Washing procedure	
Step 1	Remove rubber cement and detach coverslip in 2×SSC carefully
Step 2	Wash slides in 2×SSC containing 0.3% NP-40 for 1 min at 37 °C
Step 3	Wash slides in 2×SSC containing 0.3% NP-40 for 1 min at 72 °C
Step 4	Wash slides in 2×SSC containing 0.3% NP-40 for 1 min at room temperature
Step 5	Wash slides briefly in distilled water, air dry for 10 min and cover with a fluorescence mounting medium containing DAPI

Reagents and protocols

Many companies provide ready-to-use FISH kits that contain all necessary reagents for pretreatment and washing procedures (e.g. the Histology FISH Accessory Kit K5599 by Dako, Glostrup, Denmark). However, for those laboratories, which expect only a limited number of investigations, preparation of fresh working solutions may be more suitable. A list of required reagents and working solutions is provided in Table 1.

Along with the FISH probes, manufacturers supply protocols and technical support. Exemplarily, the protocol of the Medical Universities of Vienna and Innsbruck (MUV/MUI protocol) for hybridization on paraffin-embedded sections is stated in Table 2. This protocol incorporates useful adaptations according to the institutional experiences. However, in addition to these standard protocols and procedures appropriate handling of the individual sample with slight variations based on practical experience is important. For instance, critical steps such as the right protease digestion time may differ from case to case, and require individual handling. For further reading on this issue a recent publication by Horbinski et al. [19] is recommended. Furthermore, standardization of slide denaturation and hybridization can be achieved by the use of automated systems (e.g. ThermoBrite system combined with the VP2000 Processor by Abbott Molecular Europe, Wiesbaden, Germany).

Equipment

Examination of slides requires a fluorescence microscope equipped with adequate filters (e.g. Vysis probes labeled with Spectrum Green and Spectrum Orange (Vysis Abbott Laboratories, Abbot Park, IL, USA) require Zeiss filter sets 49 (4',6-diamidino-2-phenylindole – DAPI), 15 (Cy3.5), 10 (FITC), 23 (red + green) (Carl Zeiss Microimaging, Jena, Germany).

Interpretation of FISH results

Signal ratios are assessed individually for chromosomes 1 and 19 and interpreted grossly according to the guidelines of the International Society of Paediatric Oncology (SIOP) Europe Neuroblastoma Pathology and Biology and Bone Marrow Group [16]. Whereas normal, diploid nuclei show a signal ratio of 2/2, a nucleus is considered to harbor a deletion, if the target signal is 0 or 1 in relation to normal or excess control signals (e.g. 2/0, 2/1, 3/0, 3/1, etc). Such deletions most likely correspond to a loss of heterozygosity (LOH) found by PCR. However, with increasing grade of malignancy genomic polyploidies

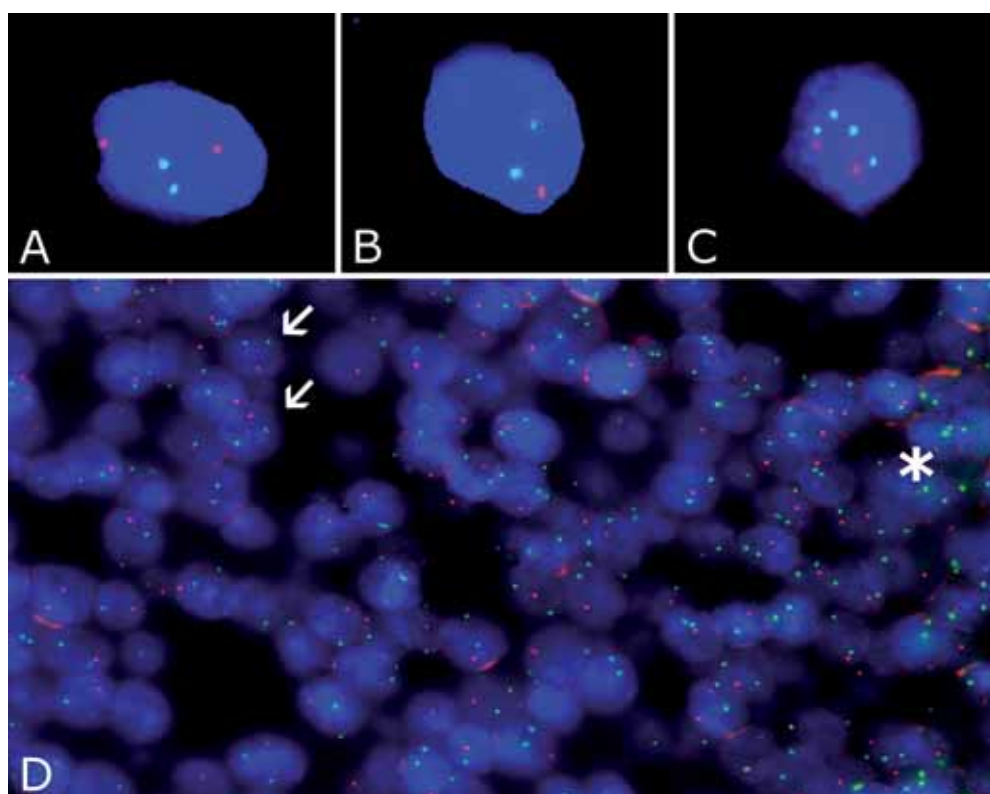


Figure 2. Representative FISH images (chromosome 1, analogous signals are obtained with probes for chromosome 19). Target signal = red, control signal = green, DAPI counterstained. A: Normal diploid signal ratio (2 controls/2 targets), magnification 63 \times . B: Deletion status (signal ratio 2 controls/1 target), magnification 63 \times . C: Imbalance with relative loss (4 control/2 target signals), magnification 63 \times . D: Fluorescence *in situ* hybridization of a 4 micron thick tissue section (magnification 40 \times) shows a deletion status (signal ratio 2 controls/1 target) of the majority of nuclei. For evaluation only non-overlapping nuclei (arrows) are analyzed, whereas clustered/overlapping nuclei (*) are not considered.

may be encountered. These chromosomal polysomies may be balanced (e.g. 3/3, 4/4, 5/5, etc) or imbalanced (e.g. 3/2, 4/2, 3/5, etc), indicating relative gains or losses. Whether an imbalance situation with relative loss of the target 1p or 19q corresponds to a hemizygous deletion in presence of reduplication cannot be resolved by FISH. Further clarification requires the use of ancillary tests such as PCR-based LOH [16]. For interpretation of FISH results, signal ratios for 100 – 200 adjacent, non-overlapping nuclei are evaluated. The number of nuclei exhibiting a balance, imbalance, or deletion are summed and expressed as percentages. If the number of “deleted” nuclei exceed a certain cut-off value (see next paragraph), the tumor is considered to show a “deletion” for the chromosome part targeted. In case of an imbalance situation with relative loss of the target (1p or 19q), the prevailing pattern (e.g. 4/2) and its prevalence (e.g. in 60% of the evaluated nuclei) are reported as such.

Cut-off definition

For proper interpretation of test results, clear definition of a cut-off for deletion or imbalance status is an essential prerequisite. Many neuropathology laboratories choose their cut-off arbitrarily. Cut-offs in use for the overall target-to-control signal ratios range from 15 – 70% [20, 21]. Instead of summary ratios, the SIOP examination guidelines recommend the evaluation of control-to-target signal ratios for individual nuclei and suggest a cut-off of 50% of nuclei that show a deletion [16]. An alternative approach is to determine a lab-specific cut-off on non-neoplastic, white matter oligodendrocytes (e.g. on temporal lobe specimens). Herein, the fraction of nuclei which deviate from the diploid 2/2 signal ratio (= deletion/imbalance/polysomy) is determined on a series of 5 – 10 cases. The cut-off value is then calculated by the mean plus 3 standard deviations [18, 22]. For example, the cut-off calculated by this method at

the Medical University of Vienna is 30% for 1p/1q and 19p/19q, respectively.

Documentation

As fluorescence signals bleach over time, adequate photo documentation is required in the routine clinical setting.

Discussion

Tumor biological background for combined 1p 19q assessment

Oligodendroglial tumors comprise pure oligodendrogliomas and mixed gliomas (oligoastrocytoma, glioblastoma with an oligodendroglial component) [23]. Together they account for approximately 35% of all gliomas [24]. More than 70% of oligodendrogliomas and roughly 40% of oligoastrocytomas display deletions of chromosomal arms 1p and 19q [25, 26]. The deletion typically involves both chromosomal arms, whereas solitary losses on either 1p or 19q are encountered only in a small fraction of tumors, in particular mixed oligoastrocytomas [25]. Although the characteristic codeletion is known since many years [27], the underlying mechanism that mediates the combined loss has been proposed a few years ago [28, 29]. A balanced whole-arm translocation between chromosomes 1 and 19 was described, which leads to the formation of two derivative chromosomes. Subsequent loss of the derivative chromosome composed of chromosomal arms 1p and 19q [der(1;19)(q10;p10)] results in the observed codeletion.

As the chromosomal status for 1p/1q most often corresponds to 19p/19q and *vice versa*, congruency of hybridization results on both chromosomal arms serves as internal validity control. Therefore, for quality reasons testing of both chromosomal loci is recommended in the routine clinical setting instead of 1p testing only [30, 31].

In contrast to the codeletion of whole chromosomal arms 1p and 19q, which is strongly associated with an oligodendroglial morphology, small interstitial deletions on 1p have been described in diffuse gliomas [14, 32, 33]. These partial 1p deletions were found

to be more prevalent in astrocytic as compared to oligodendroglial tumors and seem to be associated with a worse prognosis [32, 33]. For reliable detection of partial deletions, FISH as proposed in this paper is not useful, and more sophisticated methods such as aCGH need to be applied [14]. Such ancillary diagnostics are, however, beyond standard needs of contemporary clinical neuro-oncology.

Role of 1p 19q status as diagnostic marker

The relevance of the 1p 19q deletion as a potential diagnostic marker for oligodendrogliomas is controversially discussed [19, 34, 35, 36]. The issue of tumor typing on basis of the 1p 19q status has been raised [37, 38]. It has become clear, however, that the 1p 19q codeletion is closely but not absolutely associated with the oligodendroglial phenotype. There are a few morphologically typical oligodendrogliomas that do not show the characteristic codeletion. According to the current World Health Organization (WHO) 2007 consensus criteria, the diagnosis of an oligodendroglioma remains morphology-based irrespective of the 1p 19q deletion status [23]. Hence, 1p 19q losses constitute a prognostic molecular cytogenetic marker, but do not *per se* define a distinct tumor entity or variant.

Methodological considerations of FISH-based 1p 19q testing

FISH is an approved method for molecular cytogenetic testing on routinely available, FFPE tissues [39]. FISH analysis is independent from the age of the paraffin block, does not require reference tissues such as autologous blood samples, and the hybridization result is morphologically controlled. Thus, FISH constitutes a robust and straightforward to implement technique, suitable for the majority of neuropathology laboratories, including standard laboratories without diagnostic/research focus on brain tumors.

FISH analysis can be performed either on isolated nuclei or on tissue sections. In case of detecting a deletion such as 1p or 19q loss, the hybridization of isolated tumor cell nuclei has

been reported to be superior to conventional tissue sections due to the avoidance of truncated nuclei [40]. However, according to our experience, hybridization of tissue sections yields sufficiently reliable results as long as adequate thickness of the sections (4 – 5 microns) is warranted and a sufficient number of nuclei (100 – 200) are evaluated. The delicate pre-treatment and digestion procedures for FISH on isolated nuclei is laborious and time-consuming, requires a certain level of experience, and should be done only as second line investigation in the rare situation, in which FISH on sections does not allow proper evaluation of the test result because of nuclear overlap.

According to our experience, the neuropathological interpretation of the FISH results is straight-forward in most cases. A classic deletion with a signal ratio of 2/1 prompts an instant diagnosis. The evaluation of inconclusive, imbalance situations may require examination of a larger number of nuclei. In the diagnostic setting in practice, the exact cut-off value is not a major issue, as FISH usually yields clear-cut results far beyond the cut-off (defined as > 30% in our centers) in the case of a 1p 19q deletion status (e.g. > 80% of the nuclei showing a deletion). However, in case of an unclear FISH result additional hybridization of another section may be necessary.

According to our experience, FISH yields interpretable results in the vast majority of cases (> 90%). However, we came across single cases that stayed hybridization-refractory despite repetitive attempts with various adaptations of the protocol apparently due to poor DNA quality in the tissue specimen. Moreover, continued analytics on such specimens with an alternative method like a PCR-based LOH analysis, in our experience, will not likely yield an interpretable result either.

Limitations of FISH-based 1p 19q testing

FISH has some methodological limitations neuropathologists need to be aware of. First, commonly used and commercially available probes for 1p/1q and 19p/19q span a relatively large region (400 – 600 kb), and do not allow for detection of small interstitial deletions [14]. Secondly, imbalance situations with relative

losses of the targets 1p 19q (e.g. 4/2, 4/3, 5/3 ratios) could correspond to hemizygous deletions in the presence of reduplication. Such cases require further clarification by an alternate e.g. PCR-based method [16].

Analytical performance of FISH-based 1p 19q testing

FISH has been shown a reliable method for the analysis of the 1p 19q status in oligodendroglial tumors [17]. In addition, several independent studies found good concordance between FISH and PCR-based LOH or MLPA results [11, 13, 22, 41]. However, inter-observer and inter-laboratory variability have not yet been addressed in a systematic way, and common standards for 1p 19q FISH procedures across neuropathology laboratories are still lacking. Similarly, no accepted standards exist for PCR-based LOH or MLPA either [13]. There, the situation might be even more complicated as the interpretation of results strongly depends on the choice and quantity of probes/microsatellite markers along chromosomal arms 1p and 19q. Moreover, the number of microsatellite markers that are required to show a deletion seems unclear [13]. Thus, quality assurance remains an issue, and round-robin tests with regular participation of laboratories performing 1p 19q testing in the clinical setting need to be set up in future.

Clinical performance of FISH-based 1p 19q testing

The codeletion of chromosomal arms 1p 19q is considered a strong prognostic factor in oligodendroglial tumors, especially for the subset of anaplastic oligodendrogliomas, being associated with a longer progression-free and overall survival of the patients. Up to date, molecular genetic testing of the 1p 19q status is of particular relevance within the setting of clinical trials, where it serves as an important stratification factor (e.g. European Organization for Research and Treatment of Cancer (EORTC) trial 22033-26033), and has meanwhile led to the design of distinct clinical trials for 1p 19q intact and 1p 19q deleted tumors (EORTC 26053-22054 CATNON versus

EORTC 26081-NCCTG N0577 CODEL). In contrast to the role of the 1p 19q status as patient stratification factor or eligibility criterion in clinical trials, its significance in the routine clinical setting is that of a prognostic factor which needs to be weighed in conjunction with other prognostic factors (e.g. performance status, *IDH1* status, Ki67 index). So, knowledge of the 1p 19q status in practice is helpful for individual patient counseling, but does not *per se* define a common therapy/patient management standard [8, 37].

Considerations with regard to the usefulness of 1p 19q testing in particular situations

Testing of the 1p 19q status is useful in the case of oligodendroglioma and mixed oligoastrocytoma. In pure astrocytoma 1p 19q testing cannot be generally recommended, as a deletion status is quite rare. According to the authors' and others' experience repeated testing in case of tumor recurrence seems not useful, as the codeletion typically constitutes an early genetic event [19]. In pediatric and adolescent oligodendroglial neoplasms, 1p 19q testing is not so relevant due to the rarity of deletion in this age cohort.

Conclusions

FISH allows for fast and accurate detection of molecular cytogenetic alterations. It constitutes a straightforward assay for implementation in routine diagnostic assessment of the 1p 19q status in oligodendroglial tumors. This work is intended to serve as a manual for the implementation of FISH-based 1p 19q testing for clinical purposes in the standard neuropathology laboratory.

Acknowledgment

We thank Renate Vesely (Krankenanstalt Rudolfstiftung, Vienna) and Peter and Inge Ambros (St. Anna's Children's Cancer Research Institute, Vienna) for helpful comments and technical advice, and Gerda Ricken and Sabine Kaindl for excellent technical assistance.

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