Histol Histopathol (2011) 26: 1207-1213

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Many facets of chromosome 3p cytogenetic findings in clear cell renal carcinoma: the need for agreement in assessment FISH analysis to avoid diagnostic errors

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Summary. Abnormalities of the locus chromosome 3p and the entire chromosome 3 are involved in the cancerogenesis of clear cell renal carcinoma and may be detected by interphase fluorescence in situ hybridization (interphase FISH). We observed a variable detection rate of chromosome 3p/3 abnormalities in different series of clear cell renal carcinoma. Therefore, we focused on problematic issues when performing analysis on routinely available formalin-fixed and paraffin embedded tissue. A group of studies encountered a single approach to chromosome 3p detection, by using probe/s to map different codes of the short arm 3p without a control of the entire chromosome 3. Deletion of chromosome 3p and monosomy of chromosome 3 ranged from 38% to 100% in clear cell renal carcinoma. Cut-off values for the threshold were chosen randomly or obtained by calculation of the mean value plus 1 or 2 or 3 standard deviations. Loss of chromosome 3p was assessed either as the percentage of single signals on the total number of nuclei, or applying a double approach with corrections of control chromosome 3. Moreover, cut off values were sometimes arbitrarily corrected with the findings from normal adjacent renal parenchyma.

A consensus of experts in the field is needed in order to define the best methodological approach and the appropriate threshold in assessment 3p deletion when interphase FISH is performed in clear cell renal carcinoma. This harbours relevant diagnostic and therapeutic implications, at light also of targeted therapies recently available to clear cell renal carcinoma.

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Key words: Clear cell renal carcinoma, Chromosome 3p, Chromosome 3, Fluorescence in situ hybridization (FISH), Interphase

Introduction

Clear cell renal cell carcinoma accounts for the majority, around 75%, of renal cell malignancy (Eble et al., 2004). Abnormalities of the short arm of chromosome 3 (3p) and the entire chromosome 3 (CEP3) are involved in the early stages of the cancerogenesis of clear cell renal cell carcinoma (Brunelli et al., 2008). The most recent classifications of renal cell carcinoma issued by the World Health Organization (WHO) and the Armed Forces Institute of Pathology (AFIP) defined the same subtypes of renal cell neoplasms and both highlighted, at a cytogenetic level, abnormalities of chromosome 3p/3 as the hallmark of clear cell renal cell carcinoma (Eble et al., 2004). Loss of heterozygosity in loci 3p14, 3p21.3 and 3p25 has been demonstrated with high frequency in both sporadic and hereditary forms of clear cell renal cell carcinoma, while the von Hippel–Lindau (*VHL*) gene at 3p25 is the one mostly involved in oncogenesis (Kovacs, 1994). At the molecular level, *VHL* gene inactivation can occur in several different ways including genomic mutations (50%-80%), deletions (60%-of 80%), or abnormal DNA methylations (20%-25%) (Zbar, 1995). Therefore, the *VHL* gene represents the key point in developing the neoplastic phenotype (Brauch et al., 2000) from normal renal epithelium. Figure 1 briefly summarizes morpho-molecular common findings in clear cell renal cell carcinoma.

Cytogenetically, loss of 3p can occur either by deletion (terminal or interstitial) or by unbalanced translocation between the 3p region and another chromosome (Gnarra et al., 1994; Herman et al., 1994). It has also been suggested that the loss of heterozygosity of 3p is not confined to the clear cell RCC but is rather a 'universal' phenomenon in RCC, with different underlying mechanisms involved in the different subtypes of RCC (Velickovic et al., 2001).

Interphase fluorescence *in situ* hybridization

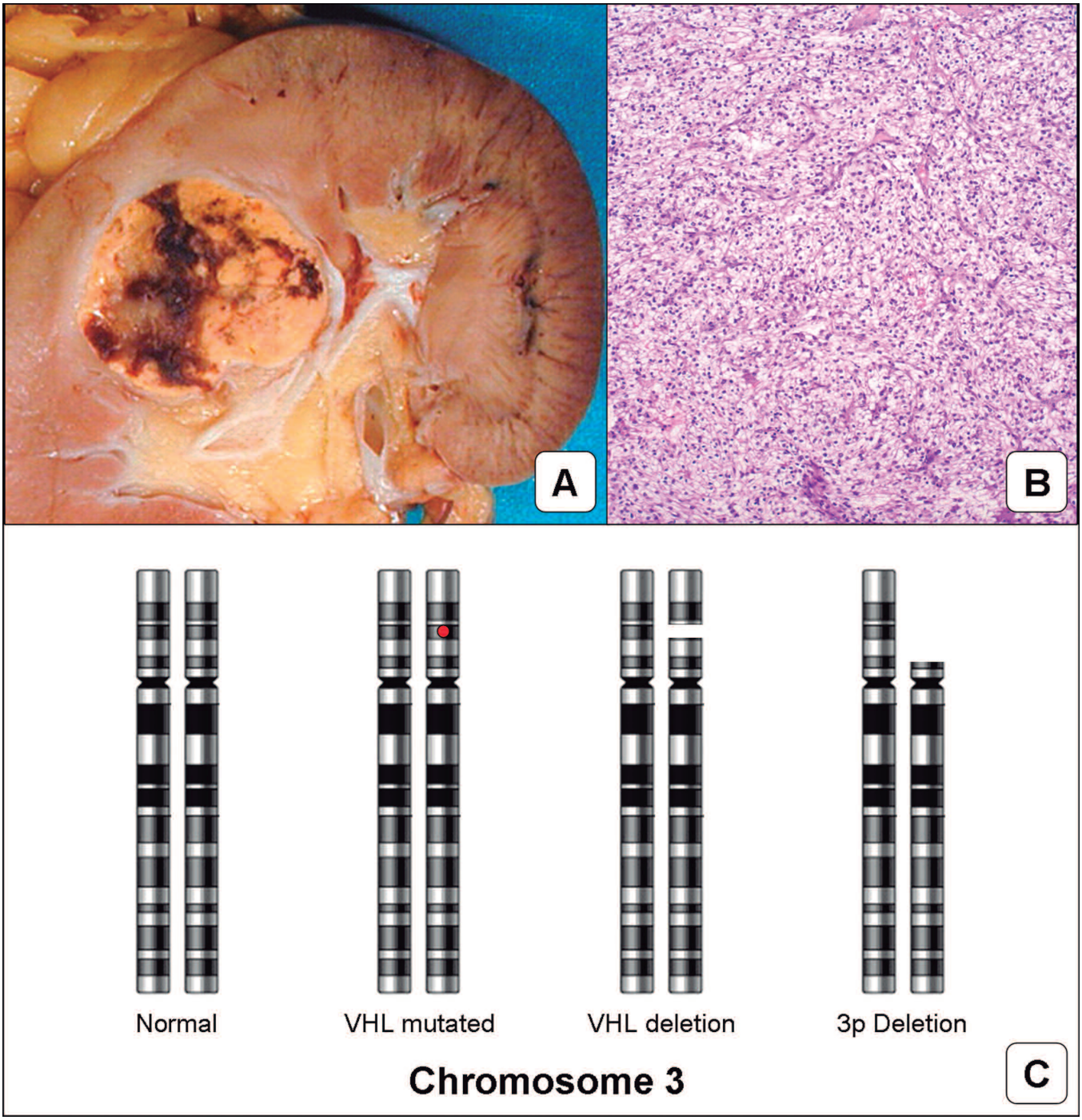

Fig. 1. Common features of a clear cell renal cell carcinoma. A: macroscopic yellowish solid nodule. B: solid arrangement of neoplastic cells with clear cell cytoplasms intermixed in a vascular network. C: Genetic alteration involving *VHL*.

(interphase FISH) has been recently emphasized as a useful molecular tool in problematic cases of renal cell neoplasms. The precise differential diagnosis among RCC histotypes has both diagnostic and prognostic implications for patients, including variable follow-up management (Jones et al., 2005; Brunelli et al., 2008) or suitability and response to targeted therapies.

In certain scenarios, fluorescence *in situ* hybridization (FISH) analysis offers one of the most sensitive, specific, and reliable strategies for identifying acquired chromosomal changes associated with renal cell carcinoma. With the growth in the understanding of the importance of cytogenetic abnormalities associated with these diseases and the availability of commercial FISH probes, this area of clinical laboratory testing is rapidly expanding.

At a therapeutical level, the development of new targeted agents in advanced RCC has increased the need for molecular testing when assessing the appropriate subtype of renal cell carcinoma, such as the clear cell. Since December 2005, 3 targeted agents have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced RCC: sorafenib, sunitinib and temsirolimus. Sorafenib and sunitinib are synthetic, orally active agents shown to directly inhibit vascular endothelial growth factor receptors -2 and -3 (VEGFR-2, VEGFR-3) and platelet-derived growth factor receptor beta (PDGFR-beta), while temsirolimus is an mTOR inhibitor.

We observed a consistently variable incidence of chromosome 3p/3 abnormalities in different series of clear cell renal cell carcinoma by interphase FISH (Jones et al., 2005). Therefore, we focused on problematic issues when performing analysis on routinely available formalin-fixed and paraffin-embedded tissues to explain, in part, the spectrum of these abnormalities. Looking at the ISCN 2009, unanswered questions remain with regard to reporting the heterogeneous molecular findings with potential clinical impact.

Here we describe the multifaceted chromosome 3p and 3 abnormalities in clear cell renal cell carcinoma detected by interphase FISH analysis, and discuss how to overcome the problematic issues in the appropriate assessment. The correct subtyping of renal cell neoplasms provided by FISH analysis may have tremendous impact for prognosis, clinical management and therapeutic decision making of RCC patients.

Spectrum of chromosome 3p/3 abnormalities in clear cell renal cell carcinoma

Brief findings are reported in Table 1

A subset of studies utilized a single methodological approach to chromosome 3p analysis, by using probe/s to map different codes of the short arm 3p without a control of the entire chromosome 3.

Sanjmyatav et al. used probes mapping the 3p24pter and 3p13p14 regions on interphase nuclei of tumor specimens prepared from 50 μm frozen tissue sections and fixed on slides (Sanjmyatav et al., 2005). They reported in 6/7 (86%) clear cell renal cell carcinoma at least a single loss for the locus specific probes mapping 3p14 or 3p24, with double simultaneous losses in 3/7 cases and gain of the 3p24 in one case.

Siebert et al. performed FISH analysis by using seven specific probes for chromosome 3 on isolated nuclei from 26 uncultured sporadic RCC (Siebert et al., 1998). Alterations of chromosome 3 have been identified in 19 (73%) RCC cases. They observed monosomy and/or 3p-deletions in 15 of 19 (79%) non-papillary/clear cell RCC but not in other morphologic su-groups. The mean percentage of cells containing loss of 3p in a specimen was 45%. Deletion mapping indicated that large deletions affecting different regions in 3p were predominant. Chromosomal region 3p24 was repeatedly involved in all RCC with a deletion in 3p. Based on these findings the authors concluded that interphase FISH for the detection of loss in 3p provided a sensitive and feasible method for the genetic classification of kidney tumors and the description of recurrently deleted regions in 3p (Siebert et al., 1998).

Receveur et al. observed that 11/11 clear cell renal cell carcinoma displayed loss of at least one BAC or PAC mapping for a locus specific on chromosome 3p (Receveur et al., 2005).

Other studies employed a double methodological FISH approach, using two probes, respectively mapping the centromere of the chromosome 3 (CEP) and a locus specific probe (LSI) for the telomeric or the subtelomeric part of chromosome 3p.

Yamaguchi et al. detected deletions of 3p25 in 38/50

Table 1. Interphase FISH on chromosome 3 in clear cell renal cell carcinoma.

Author	Year	Pts	Material	Loss 3p	Monosomy chr 3	Polysomy chr 3
Sanjmyatav	2005	25	frozen	6/7 (90%)	1/7 (15%)	-
Siebert	1998	22	frozen	38%	27%	-
Barocas	2006	20	biopsy	16/20	43	-
Moch	1998	42	frozen	24/35 (69%)	7 (20%)	11 (31%)
Receveur	2005	11	short term culture	11/11 (100%)	-	-
Gronwald	1997	25	paraffin	60%	-	-
Yamaguchi	2003	50	isolated nuclei (frozen)	38/50 (76%)	13/50 (26%)	10/50 (20%)
Nagao	2005	50	isolated nuclei (frozen)	40/50 (80%)	-	-

(76%), monosomy of chromosome 3 in 13/50 (26%) and gain of chromosome 3 (polysomy) in 10/50 (20%) cases of clear cell renal carcinoma. In this study both a centromeric chromosome 3 and locus specific 3p probes have been utilized (Yamaguchi et al., 2003).

Nagao et al. examined 50 RCCs by dual-color FISH with DNA probes for the centromere 3 and for the locus 3p25.1, approximately p25.3 (Nagao et al., 2005). They found in 40/50 (80%) loss of LSI 3p.

Moch et al. studied 53 clear cell RCCs (Moch et al., 1998) using a dual-color FISHwith probes for the *VHL* gene and the chromosome 3 centromere. Deletion was detected in 69% of clear cell RCCs. A considerable genetic heterogeneity of *VHL* deletions was seen in clear cell RCCs, including *VHL*-deleted subclones with different chromosome 3 counts within individual tumors, as well as clones with and without *VHL* deletions (Moch et al., 1998).

Barocas et al. found loss of 3p in 9/16 (56%) clear cell RCCs in biopsy specimens (Barocas et al., 2006, 2007).

Gobbo et al. studied some renal cell neoplasms with papillary architecture but showing a large number of cells with clear cytoplasm. They detected 3 cases with loss of chromosme 3p and classified these tumours as clear cell renal cell carcinomas (Gobbo et al., 2008).

Spectrum of cut-offs proposed for the detection of chromosome 3p/3 abnormalities in clear cell renal cell carcinoma

Barocas et al. considered that locus 3p or chromosome 3 was lost if 10% of the cells counted had only one signal for that locus or chromosome and cells containing four signals (tetraploid) were deemed normal (Barocas et al., 2007).

Samjanatov et al. calculated the cut-off value for each probe and for each fluorescent dye. Control experiments were done in 25 normal kidney tissues using the two interphase FISH test sets (Sanjmyatav et al., 2005). Cut-off values for all probes were defined as the mean percentage of normal diploid cells in the 25 normal tissues minus 2SE, with diploid signals per probe ranging from 87 to 98% nuclei.

Yamaguchi et al. stated the numerical aberration as monosomy (cells with one centromeric signal) or polysomy (more than three centromeric signals) when this was observed in more than 10% of the total counted cells on each slide. They chose this cut-off in analogy with the approach for chromosome 8p (c-myc) in prostate adenocarcinoma (Yamaguchi et al., 2003). Decreased or increased fraction was defined as the fraction with decreased or increased numbers of cosmidic compared with centromeric signals. Nuclei with one centromeric and one cosmidic signal were regarded as monosomy, and were included in the decreased fraction. It was deemed to be a chromosomal deletion or gain when decreased or increased fractions exceeded the mean value of the five normal kidney tissues studied minus 2 standard deviations. Finally the che cut-off value for the decreased fractions of cCI3-865 was set to 40.9%.

Moch et al. defined monosomy as the presence of >20% of monosomic cells (cells with one centromere signal) (Moch et al., 1998) The definitions used for loci deletions were similar to those of previous studies (Sauter et al., 1994; Wagner et al., 1995). As regards deletion, the percentage of deleted cells was calculated for each hybridization as the number of cells containing either one centromere signal or fewer cosmidic/Pl than centromeric signals over the total number of cells. A tumor was deemed to harbour deletion if the percentage of deleted cells was >40%.

Siebert et al. indicated deletions and duplications as selective loss or gain of one or more YAC-specific signals with respect to the centromeric signals (Siebert et al., 1998). Interphase cells carrying a deletion in 3p contained a lower number of fluorescent YAC-specific signals compared to centromere D3Z1- specific signals. In these cases, the amount of D3Z1- specific signals indicated the copy number of chromosome 3. Normal tissues were used as controls and the level of 6.4% for YAC 7566 was defined as the diagnostic cut-off for deletions for all the investigated YACs.

Nagao et al. defined the presence of chromosomal deletion when the percentage of cells with fewer signals than the control (decreased fraction) exceeded 41% for cCI3-865 (Nagao et al., 2005). Cut-off was calculated in 6 normal kidney specimens as the mean value minus 2 S.D. for chromosome 3 and the mean minus 3 S.D. for chromosome 5. These cut-off values were set at twice the maximum percentage of the signals in the normal kidney tissue (Sauter et al., 1995). The authors chose this in analogy to the cut-off used for c-myc in bladder cancer (Sauter et al., 1995).

Receveur et al. simply observed the percentage of nuclei exhibiting “monosomic” or “polysomic” signals with a range from 5 to 100% (Receveur et al., 2005).

Wada et al. used the criteria previously proposed by Hopman and colleagues (1988, 1989, 1991, 1994). Five normal renal specimens were used as controls. In these samples, the mean minus 2 SDs of the frequency of each signal was considered as the cut-off level from background. In normal renal tissue from five different controls, the mean ± SD percentages of nuclei with 1 signal and >3 signals were 12.9±8.5% and 3.5±1.6% respectively (Wada et al., 1998).

Gobbo et al. developed a FISH ratio in order to overcome the problem of finding the deletion in euploid versus poliploid nuclei (Gobbo et al., 2008a, b). In brief, for each slide, 100 to 150 nuclei from tumor tissue were scored for FISH at x 1000 magnification. Non-neoplastic kidney tissue was used as control. Definitions of loss of chromosomes 3p and chromosome 3 were based on the Gaussian model and related to the non-neoplastic controls. Any tumor with a signal score beyond the cut-off value was considered to harbour a gain or loss for the specific chromosome. The cut-off for each probe was set

at the mean value of the controls plus 3 standard deviations. 3p deletion was statistically analyzed in analogy with a previous study on chromosome 1p and 19q deletions in oligodendrogliomas.

Detection of chromosomal loss in euploid versus polyploid nuclei

Most clear cell renal cell carcinomas show only simple chromosomal changes, such as loss of chromosme 3p in euploid (46XX chromosomes) neoplastic nuclei (Kardas et al., 2005). However, a subgroup of RCC, usually with a more aggressive behaviour, displays a more complex cytogenetic pattern, indicating that further accumulation of chromosome changes could play a role in tumour progression. Abnormalities in the cell division machinery have also been described. Structural chromosomal changes at two or more ploidy levels at cell division have been observed, including copy number alterations, chromosomal breakage-fusion-bridge events and multipolar configurations and supernumerary centrosomes (Gisselsson and Hoglund, 2005). All the aforementioned chromosomal rearrangements finally contribute to the evolution of complex karyotypes and genetic intra-tumour heterogeneity in a subset of RCC (Gisselsson, 2003).

In the context of these neoplasms the detection of loss of part of a chromosome such as loss of 3p may be difficult to detect at the analysis of interphase nuclei; when the DNA content harbours normal ploidy (i.e. 46XX, -3p) a single fluorescent signal with the probe specific for the locus 3p is expected (Figure 2). Differenlty, the loss of chromosome 3p in polyploid nuclei may be obscured by the overall supernumerary ploidy (78<x>92, XX, -3p) (Figure 2). In this case, the nuclei used for the score may not show the real presence of the structural 3p loss. We developed a simple method for the detection of chromosomal loss of 3p in polploid nuclei in an attempt to overcome the bias of the interphase analysis.

Normal renal parenchyma

Few studies have reported on FISH analysis in normal renal parenchyma so far, probably due to the methodological variability of the procedures. This important issue should always be taken into account since this is the baseline from which standardized FISH results are extrapolated. Percentages of single signals, greater than 26, 27, 22, 26, and 30% have been reported for chromosomes 1, 2, 6, 10, and 17 respectively using a non-confocal microscope (Kernek et al., 2004). In normal epithelial cells of the renal tubules, nuclei with three or four centromeric signals were occasionally seen but never exceeded the 12% of the total (Brunelli et al., 2003a,b; Cossu-Rocca et al., 2006)

Tibiletti et al. reported a spectrum of single signals ranging from 1,7 to 15,5% of nuclei (Tibiletti, 2007).

Conclusions

The FISH methods widely used in clinical laboratory studies involve hybridization of a fluorochrome-labeled DNA probe to an *in situ* chromosomal target. FISH can be applied to a variety of specimen types, to date also on formalin-fixed and paraffin embedded tissue. Metaphase preparations from cultured cells that are routinely used for cytogenetic analysis are considered the "gold standard" because chromosome morphology and position of the signals can be visualized directly. However, a major advantage of FISH is that it can also be performed on nondividing interphase cells. Interphase nucleus assessment from uncultured preparations allows

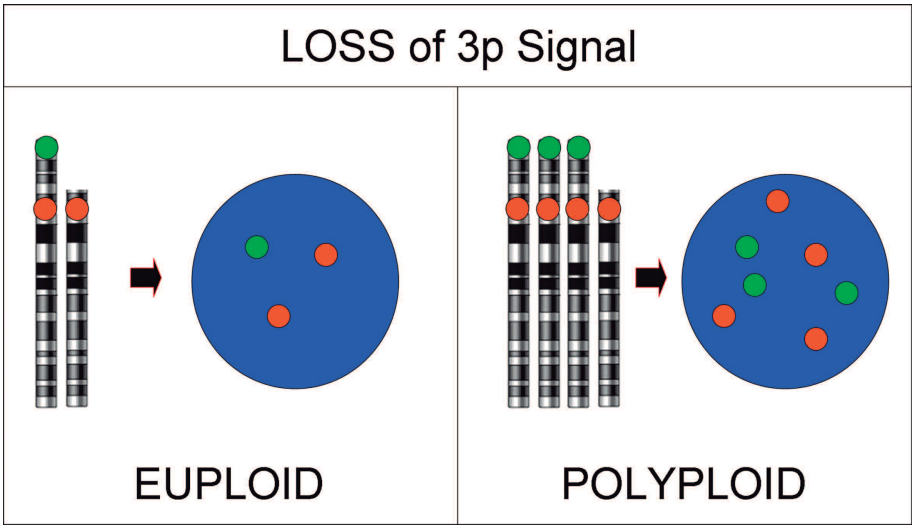

Fig. 2. Error in reporting interphase FISH analysis on chromosome 3p. Case with a normal ploidy, i.e. 46XX, -3p, is expected to show a single fluorescent signal with the probe specific for the locus 3p region. Differently, the loss of chromosome 3p in polyploid nuclei may be obscured by the overall supernumerary ploidy (78<x>92, XX, -3p); in this case, the nuclei used for the score may not show the real presence of the structural 3p loss.

for a rapid screening for specific chromosome rearrangements or numerical abnormalities associated with renal cell neoplasia.

Interphase FISH on tissue sections from formalin-fixed paraffin-embedded tissue needs particular care. i) the sectioning of the nuclei may cause underestimation of trisomies and overestimation of monosomies when focusing on detection of chromosomal losses, such as loss of chromosome 3p or chromosome 3, if the size of the nuclei is not evaluated correctly. ii) Efficient hybridization can influence the cut-off levels. FISH analyses with more than 10% of cells without the fluorescent spots usually are not acceptable for scoring chromosomal abnormalities because they lead to overestimation of monosomies and underestimation of trisomies. iii) Deletion of chromosome 3p and monosomy of chromosome 3 range from 38% to 100% in clear cell renal cell carcinoma in different studies. Loss of chromosome 3p can be assessed by using a single approach (percentage of cells with single signals over total nuclei) or a a double approach with correction for control chromosome 3 (percentage of nuclei harbouring 3p signals < control chromosome 3). iv) The cut off values have been arbitrarily corrected with the findings in the normal adjacent renal parenchyma.

Cut-off levels for both monosomy and trisomy change depending on the type of probe and the type of target nuclei. For this reason it is not possible to use the same threshold level for the detection of different chromosomal abnormalities. Based on the data of the present review we can conclude that: each probe (LSI and CEP) needs an appropriate cut-off level based on the count in the normal tissue of different subjects. The cut-off levels may be evaluated differently for specific types of nuclei. The omission of the cut-off point for specific types of nuclei introduces a bias in the evaluation of chromosomal abnormalities, particularly under- and overestimation of monosomies.

FISH is a readily available, relatively simple, highly accurate method of detecting losses, gains and translocations of genetic material. Although interphase FISH is a versatile and highly sensitive technique for detecting chromosomal abnormalities, the lack of accurate controls can result in the misdiagnosis of some abnormalities. Looking at the ISCN 2009, unanswered questions remain in regard to reporting the heterogeneity of FISH findings from molecular analysis in clear cell renal cell carcinomas.

Regardless of the method used for the assessment chromosome 3p loss, other biological reasons may in part explain the heterogeneous spectrum of findings. Intratumoral heterogeneity is a major reason. A considerable genetic heterogeneity of *VHL* deletions was observed in clear cell RCCs, including *VHL*-deleted subpopulations with different chromosome 3 counts within individual tumors, as well as populations with and without *VHL* deletions.

A system for FISH nomenclature, the International System for Human Cytogenetic Nomenclature (2009), including both metaphase and interphase analysis, has been developed. Although the system may seem confusing to those not working directly with chromosomes, correct nomenclature designations are important to convey the precise nature of a result. However, as the number of critical loci involved in neoplastic chromosome rearrangements or numeric abnormalities continues to expand, the diversity of FISH probes and unique probe sets will undoubtedly improve. FISH has become an important means both for definition of the initial chromosome changes in a disease process, as well as a reliable means for the ongoing monitoring of response to therapy and disease remission.

We believe that a consensus of experts in the field is needed to define the appropriate methodology and identify the threshold when interphase FISH is performed. A better definition of the criteria for the assessment of chromosome 3p deletion may help the pathologists identify this subtype with potentially relevant diagnostic and therapeutic implications.

Acknowledgements. Fondazione Unicredit, Cassa di Risparmio di Verona, Vicenza, Belluno, Ancona; Diagnostica Molecolare in Oncologia; Ministero Istruzione, Università e Ricerca (MiUR); Banco di Sardegna, ARC.

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Accepted April 6, 2011