Peer Review File

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REVIEWER A:

General comment .- *This is a case report of an endoluminal tumor of a 72-year-old patient with intraluminal metastasis to the anal fistula, as indicated by FISH and immunophenotyping.*

The paper is well written and clearly understood. This paper describes metastasis of the primary rectal tumor as disseminating through the lumen to the anal fistula.
The approach the authors used to distinguish between a related tumor clone and an unrelated tumor with FISH (and immunochemistry) is simple, effective and conclusive, and in this case ruled out a more aggressive anal adenocarcinoma.

Answer to the general comment.- We thank the reviewer for his/her positive comments about the manuscript and the work contained in it.

Comment 1.- *The authors concluded that since the FISH assay showed the same chromosomal nature in the two tumors, they were likely derived from the same clone. However, the KRAS mutation was only seen in the rectal tumor and not in the anal fistula tumor. They provided two mechanisms to explain this: 1) divergent evolution and 2) sampling issues. So had the cytogenetic abnormalities as determined by FISH been different, it could also mean they had a divergent evolution consequent to the metastasis and not necessarily were two different and unrelated tumors. To this, perhaps a more exhaustive panel of FISH probes in addition to those for chromosomes 7 and 8 could be added as a more comprehensive assay to exclude divergent evolution. Perhaps the authors could add a sentence for more clarity.*

Answer to comment 1.- In order to determine that it is the same tumor clone and exclude a divergent evolution, we have added to chromosomes 7 and 8 probes directed at chromosomes 13q and 18q, frequently altered in this disease. In addition, a new sentence has been added in the case description and discussion section of the revised manuscript, following the suggestion of the reviewer. Now, the figure 2 (figure 3 in the revised manuscript) show the complete panel of FISH probes studied from tumor cells from both tumors, including normal mucosa.

Comment 2.- *Minor points: line 116 "chromosomal" rather than "chromosomic"*

Answer to comment 2.- We thank the reviewer for pointing out this mistake that has now been corrected.

REVIEWER B

Comment 1.- *Dr. Abad and colleagues describe a study dedicated to endoluminal tumor implant of a colorectal cancer in an anal fistula analyzed by FISH. The manuscript is intended to be not only a simple case report, but a review of similar cases, as well. This is a fairly good report of an unusual case. However, there is real need to support more the main technical conclusions of the manuscript by an extension of the discussion dedicated to FISH or, more precisely, interphase FISH.*
Authors try to demonstrate that interphase FISH is a truly valuable technique in given circumstances. It is true, but explanations of FISH applicability are not convincing. One may recommend to expand discussion about the applicability of interphase FISH (spectrum of interphase FISH methods and the application, ability to analyze immense cell population using visualization, single cell genome visualization, immunoFISH, parameters of interphase FISH which are unique to this technological platform etc.).
Moreover, a brief comparison or highlighting of uniqueness of possibilities offered by interphase FISH is required (e.g. several phrases dedicated to). To succeed, one can recommend to look through and cite thereof a number of reviews dedicated to single cell genome analysis using interphase FISH. These are doi: 10.3390/genes10050379, doi: 10.1186/1755-8166-3-1, doi: 10.1002/bies.201400218, doi: 10.1007/978-3-540-70581-9, doi: 10.2174/1389202128025104 39.

Answer to comment 1.- We fully agree with this suggestion of the reviewer. Following his/her indication a more detailed and improved description of the interphase FISH techniques (iFISH) has been added in the text of the discussion section of the new revised manuscript, in which the applicability of this technique is specifically highlighted. The references have also been updated.

Comment 2.- *Unfortunately, the review of similar cases is not significant enough to entitle the manuscript using a phrase part "with review of the literature". This is a simple case report (well-reported case report).*

Answer to comment 2.- Following the comment of the reviewer, "Review of literature" has been removed from the title.

Comment 3.- *Finally, some presentational aspects should be considered by authors. English is to improve (some word combinations are genetically incorrect – e.g. "chromosomic aberrations" etc.). One sentence paragraphs are presentationally incorrect.*

Answer to comment 3.- The language of the manuscript has been carefully revised by a native English Professor, following the recommendation of the reviewer.

REVIEWER C:

Comment 1.- *This is a case study of an elderly patient with an adenocarcinoma that metastasized from sporadic colorectal cancer into an anal fistula. Another rectal tumor was identified as well. This case in unusual since sCRCs usually spread to the liver, lung and peritoneum, while here a more distant location was observed. The authors wanted to determine whether there is genetic connection between the primary tumor and the distant tumors.*
To do so they performed the traditional immunohistochemistry stainings and PCR amplifications, but also applied DNA FISH to the samples. The probes used were directed to specific genomic regions commonly amplified in sCRC. The FISH analysis showed that both tumors had loss of chromosome 8 but no alteration of chromosome 7, which is rather different than the alterations typically found in sCRCs.
In addition, a KRAS G12D mutation was detected in the rectal tumor by PCR but not in the primary tumor. The authors suggest how the discrepancy between the two findings might be explained.

The authors do not really explain in the end why FISH should be used – do they mean to say that one should always look at tumors with DNA FISH? Also, is this comparison done for clinical use or just for understanding the genetic origin of the tumor?

Answer to comment 1.- The information requested by the reviewer has been included in the discussion section of the revised manuscript, which the usefulness of the FISH techniques for the identification of tumor cell clones according to the chromosomal alterations, as well as its clinical implications are now discussed.

Comment 2.- *What about showing the actual data, the PCR data at least, and maybe some of the IHC.*

Answer to comment 2.- Following the comment of the reviewer, information has been included in the case presentation section of the revised manuscript about PCR data obtained for the mutation *KRAS*. In addition, a new figure showing the immunohistochemical profile of patient tumors has been added (Figure 2).

Comment 3.- *Figure 2 bottom (chr 8) – there is no control of a regular diploid pattern of chr 8 in other cells to compare to. I assume the authors mean to say that normally one would see 6 FISH dots but only 3 are seen in the tumor cells. Also, isn't it is strange that in some cases the 3 dots are not close by as would be expected for a chromosome territory, rather, one of the dots (unclear which color) is quite far from the other two – is the chromosome linear?*

Answer to comment 3.- A control diploid patterns of all chromosomal regions analyzed of the normal mucosal tissue of the patient studied have been added to figure 2 (now figure 3), following the recommendation of the reviewer. In addition, the image of the

cells of the primary tumor hybridized with chromosome 8 probes has been replaced by a new capture.

Comment 4.- *Wouldn't SKY karyotyping give the same results about chromosome loss/presence/truncation and much more (and info for all chromosomes)?*

Answer to comment 4.- Following the comment of the reviewer, in the discussion section we have added the advantages of FISH *vs*. other molecular techniques, including SKY karyotyping.

Comment 5.- *The English could use some brushing up*

Answer to comment 5.- The language of the manuscript has been carefully revised by a native English Professor.