Author’s response to reviews

Title: The role and prognostic value of apoptosis in colorectal carcinoma.

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Version: 2 Date: 27 July 2013

Author’s response to reviews: see over
July 10th 2013.
Dr Christopher Foote
Executive Editor
BioMed Central

Subject: Submission of revised manuscript.

Dear Editor in-Chief,

Please find enclosed the revised version of our manuscript, “The role and prognostic value of apoptosis in colorectal carcinoma”, previously submitted for publication as an original article in BMC Clinical Pathology.

The changes made in light of the reviewers' comments have been highlighted in yellow. Please also see below for a point-by-point response to the reviewers' comments.

We are pleased that the reviewers agree that the manuscript will be an article of interest in its field. We are very grateful to the reviewers for their helpful suggestions and we feel that the quality of the manuscript has been significantly improved as a result.

We look forward to seeing our manuscript in your journal.

Yours sincerely,

Julia Alcaide and Maximino Redondo on behalf of the authors.

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REVIEWER 1
Title: The role and prognostic value of apoptosis in colorectal carcinoma.
Version: 1 Date: 27 May 2013
Reviewer: Masaaki Tatsuka

Reviewer's report:
Apoptosis, a form of programmed cell-death defined morphologically, is a widely accepted biological process that is of a great interest to clinical and basic scientists who study cancer. Nowadays, there is a growing interest in the area of colorectal cancer and its management. Defects in the response to apoptosis are implicated not only in pathological aspects of colorectal cancer but also in its resistance to ionizing radiation and chemotherapeutic drugs. However, the authors in this paper demonstrated that apoptotic cells judged by TUNEL assay were frequent in samples from colorectal cancer patients with worse prognosis than good prognosis after excluding patients that had chemical treatment experiences or radiation therapy experiences. The paper is potentially interesting to know the biological aspects or physiological situation of aggressive colorectal cancers and might be valuable to establish a novel pathological diagnosis method with prognostic value.

To more better understand this paper's data, this reviewer recommends further studies as shown below.

Major concerns:
(1) This reviewer became concerned about which type of apoptosis is increased in aggressive colorectal cancers. For example, autophagy can be detected by immunostaining with anti-LC3B antibodies. Alternatively, mitotic catastrophe can be estimated by aberrant mitotic cells, which can be detected by immunostaining with anti-phosphorylated histone H3 antibodies. The authors’ observation is possibly the result of reflecting a high frequency appearance of dead cells in aggressively growing cancer cell populations. Such an accumulation of apoptotic cells may be due to mitotic defects caused by multiple centrosomes, disorganized mitotic spindles and cytokinesis errors.

The authors agree that several pathways of apoptosis can be activated in tumour development and progression. At the end of page 7 it is underscored that one important role of programmed cell death is the control of genetic damage. “This protective function explains why higher Alis may be found in tumours than in normal tissue, as these elevated Alis may indicate physiological attempts to eliminate the genetic alterations that are frequently found in neoplastic cells”, like DNA mutations or mitotic errors.

(2) This reviewer became concerned about which apoptotic signal is activated in aggressive colorectal cancers. For example, anti-caspase-9 antibodies are useful to detect the mitochondrial apoptosis signal. Anti-caspase-3 is also useful. Caspase-3 is likely to be implicated in cancer progression (Nature Medicine 17, 860–866, 2011). This reviewer wonders if it isn't time now to think of frequent caspase-3 activation states in aggressive colorectal cancers.
It has been observed that apoptotic cells can have an effect on the tumour microenvironment and the immune response in the associated stroma, leading to an activation of neoplastic progression (Noble et al, BJC 2013;108:2097-105). Therefore, a link between cell death and carcinogenesis exists. Caspase 3, a marker for apoptosis, has been proposed as this link by Huang et al in tumours treated with radiotherapy (Nature Medicine 2011;17:860-6), as reviewer remarks. The main limitations for using caspase 3 are that cleaved caspase 3 can be generated by other proteases and its prognostic significance has been studied with conflicting results (Leonardos et al, Cancer Lett 1999; 143:29-35; Noble et al, BJC 2013;108:2097-105; Jonges et al, Lab Invest 2001;81:681-8). Nevertheless, as a validated marker for determining apoptosis and a potential explanation for the connection between cell death and tumourigenesis, the authors have decided to add this reference to the final manuscript.

(3) This reviewer became concerned about which physiological situation is presented in aggressive colorectal cancers. For example, hypoxic conditions are thought to be important to progress colorectal cancers as niche, microenvironmental situation for cancer cells. Anti-HIF antibodies are available.

The authors agree with this comment. As you can read on page 8 of the manuscript, hypoxia is an important condition in the tumour development and a phenomenon that can connect also apoptosis and tumour progression. In fact, the authors are planning to include angiogenesis markers for future studies.

Minor concerns:

(1) Representative figures for judgement by TUNEL assay in each stage of colorectal cancers should be presented.

Figures have been attached.

(2) The authors should mention the relationship between the apoptotic cell rate and metastasis behavior.

On page 6 it can be read that “we found that the AIs were much greater in metastatic disease cases (stage IV) than in the localised stages of the disease (stages I, II and III), with $p=0.017$”. Besides, a research project with resected metastases from colorectal cancer is being carried out by the authors. We hope to have interesting results in the future.

**REVIEWER 2**

I think this paper is really proper for your journal. It is wonderful to analyze the apoptosis in colorectal carcinoma and the relation of long-term result.

Second, there are some problems in this article which are showed as follow:

(1) table 1 of the 103 patients’ characteristics is too simple, this lack of the tumor’s location, lymph condition, operation type, etc.
New items have been added to the table like tumour location. TNM stages have been separated individually, so now it is possible to see how many patients had positive lymph nodes in the primary tumour (stage III).

(2) where is the Cox’s proportional hazards survival analysis result? This may the article’s result unbelievable.

On page 6 and 7 the results of the analysis can be found:

Disease-free survival (DFS):
“A high apoptotic rate was significantly associated with an increased recurrence rate, and the independent relative risk (RR) was 2.03 (with a 95% confidence interval (CI) of 1.04-4.14). As expected, an advanced tumour stage was also significantly associated with a poor DFS (RR 2.48; the 95% CI was 1.10-5.59)”.

Overall survival (OS):
“In a multivariate analysis, we found that AI and tumour stage were independent prognostic factors for OS, with RR values of 2.18 (with a 95% CI of 1.08-4.37) and 2.41 (with a 95% CI of 1.20-4.85), respectively”.

(3)The two figures are not corrected.

Figure legends have been corrected to avoid misunderstanding when figures are not seen in color.

REVIEWER 3
Major concerns
• Prognostic significance of apoptotic index in colorectal carcinoma is controversial, as apoptotic index has been reported to be associated with both good and bad outcome in different studies. The authors should discuss this issue in more detail with reference to lack of uniformity in pre analytic processing variables especially cold ischemic times and fixation in formalin. Although the prognostic significance of increasing apoptotic rates and proliferation do lean towards poorer prognosis, it would be interesting to support this with additional data of additional markers of apoptosis like M30, cleaved caspase3 and maybe proliferative marker Ki-67 in the stepwise normal to adenoma to cancer sequence.

The authors agree with the comment about methodological and technical issues and their possible influence on conflicting results of previous studies. In fact, on page 8 it can be read: “Differences among the methods that are used for the detection of apoptosis could influence the data that are obtained in these studies. Nevertheless, regardless of the apoptotic detection method that is chosen, most authors have demonstrated an increase in the AIs that are observed during the course of the
progression from normal mucosa to adenoma to carcinoma [5-8,14].” The final manuscript has been amended to discuss this point with more detail.

Apoptosis can be effectively detected by other methods, although TUNEL is the most experienced in literature and is considered the reference standard (Koornstra et al, Histopathology 2004;44:9-17). As it has been described before, the prognostic significance of cleaved caspase 3 has been studied with varying results (Leonardos et al, Cancer Lett 1999;143:29-35; Noble et al, BJC 2013;108:2097–105; Jonges et al, Lab Invest 2001;81:681-8).

Regarding M30, it has been demonstrated a good correlation between in situ end labeling and M30 in paraffin-embedded tissue (Carr NJ, Arch Pathol Lab Med 2000;124:1768-72). This work showed an increase of apoptosis in carcinomas compared to adenomas with both methods. This finding was confirmed by Koornstra et al (Koornstra et al, Histopathology 2004;44:9-17), using the M30 antibody. They observed a correlation of apoptosis demonstrated by morphological criteria and M30 antibody. With both methods, apoptosis increased from normal mucosa to adenoma and carcinoma. Additionally, apoptosis was higher in advances stages compared to earlier stages. Apoptotic index was also higher in tumour than normal mucosa using both cleaved caspase-3 and M30 in the work of De Oliveira et al (De Oliveira et al, Oncol Rep 2009, 22:295-303), although they could not find a significant relationship with survival. In other studies, an association between elevated apoptotic index, measured by M30, and reduced survival was observed (Evans et al, BJC 2006; 94:1412-19; Ruppa et al, Cancer 2003;97:2404-11), in consistency with the results reported by the authors in this manuscript.

The proliferative marker Ki67 is being considered for future researches by the authors.

• In the Materials and Methods section the authors should specify the anatomical site and the timeframe of these 103 colorectal cancers. Were any rectal cancers included? If yes, why did they not receive neoadjuvant chemo/radiotherapy? Did the presence of some preexisting conditions prevent them from receiving treatment or were these patients excluded based on other factors? Another factor that the authors should discuss is the poor survival observed in distal tumors with lower apoptotic indices,(Sinicrope FA et al. Clinical Cancer Research Vol. 5, 1793–1804, July 1999.)

Site of primary tumours and the timeframe of the cases have been added to the text of revised manuscript. Rectal cancers were included, but no one received neoadjuvant chemo/radiotherapy previously to the surgery of the primary tumour, because it was not indicated according to the TNM stage of every case. The authors excluded those cases with treatment before the surgery, in order to avoid possible interactions between these treatments and apoptotic rates.

Indeed, a distinct correlation of apoptotic index with survival has been found in colon carcinomas according to their location. Nevertheless, not all the studies that have evaluated apoptosis in different parts of the colorectum have found the same results (Liu
et al, Gut 1999;45:45-50; Anti et al, Gut 2001;48:238-46). In this regard, Sinicrope et al (Sinicrope et al, Clin Cancer Res, 1999;5:1793-804) could not find differences (“No differences were found for Al, Mi, or Bcl-2 expression when stratified by anatomical site”) and when they analysed survival, neither primary tumour site nor apoptotic index were prognostic factors. In univariate analysis the only result that achieved statistical significance was a lower overall survival in distal colon carcinomas (all of them were lymph-node negative), with HR 0.39; p 0.05, without a difference in all colon tumours or in relapse free survival for distal tumours (HR 0.44; p 0.07). In multivariate analysis, low AI predicted poor survival for distal carcinomas. They could not find a correlation of apoptotic index with mitotic index or proliferative index. Furthermore, microsatellite status was not reported in this study. They concluded that their results needed to be validated prospectively and, to our knowledge, these findings have not been confirmed by other research.

- Terminal deoxyribonucleotidyl transferase mediated nick end labelling (TUNEL) and in-situ end labelling (ISEL) are the methods most often used to demonstrate and quantify apoptosis in histological tissue sections, and the interpretation and specificity of these techniques have been controversial. Another major issue is why TUNEL assay was not validated using the M30 antibody for immunohistochemistry that has proved useful in assessing apoptotic indices in colorectal carcinomas. As we have described before, TUNEL is the most experienced in literature and is considered the reference standard (Koornstra et al, Histopathology 2004;44:9-17) for determining apoptosis, and a good correlation with M30 expression has been demonstrated. For more details and references, see previous comment.

- Please elaborate if any steps were taken to avoid false positive staining in the TUNEL assay with regard to inhibiting endogenous endonucleases and endogenous alkaline phosphatase in the intestine. What is lacking is the need for supplementary data that would have complemented and added value to their findings as using a single method of detection fails to discriminate between different types of cell death. This is described on page 5 of the manuscript: “The pretreatment of sections with DNase served as a positive control for the enzymatic procedures; for a negative control, the same procedures were performed without the inclusion of the enzyme”.

- Apoptotic indices were determined in grouped stages (I&II and III&IV) and it is necessary to evaluate them in each stage individually and calculate OS and DFS in subgroups later. Similarly, AI should be correlated with age groups as it would be of interest to see if there was clustering of higher AI in the older age group (above 70yrs).
The available sample size is optimal to obtain information about the two main groups of colorectal cancer that we can differentiate: earlier stages, without metastases (stages I and II) and more advanced stages, with lymph node or distant metastases (stages III and IV). On page 6 of the manuscript, we have highlighted that “with respect to the tumour stage, we found that the AIs were much greater in metastatic disease cases (stage IV) than in the localised stages of the disease (stages I, II and III), with p=0.017. Apoptotic rates did not correlate with the gender, age or tumour grade of patients”.

Minor Essential Revisions

• The authors need to define the 20 “normal” colon tissues studied with regards to the following: Were they normal colonic tissue adjacent to cancer tissue or were they normal colonic tissues from non-cancerous specimens? If yes, what was the distance from the cancer? Apoptotic counts are higher in normal mucosa obtained from resection margins than in truly normal mucosa. The authors should comment on field change of inhibited apoptosis in mucosa adjacent to colorectal carcinomas.

Sections of normal colonic mucosa were obtained from surgical specimens, not adjacent but remote from carcinoma and considered as normal by pathologists. The text has been modified to include these specifications.

• The authors should provide photomicrographs that illustrate the differential AI across the colorectal carcinogenesis spectrum.

Figures have been attached.

Discretionary Revisions

An added suggestion would have been to try double labeling protocols for the TUNEL assay.

REVIEWER 4

1. As cited by the authors themselves, they showed that apoptosis activities were increased during the course of tumor progression from early to advanced stages of CRC. As such, the authors fail to explain why the manuscript is devoted to replicating this finding.

Our aim was to clarify the behaviour of apoptosis along the carcinogenesis sequence, as conflicting results were previously reported, as well as demonstrate a significant relationship between a high apoptotic index and poor survival in colorectal carcinoma. We finally have shown this relationship with both disease-free survival and overall survival. To our knowledge, this is the first study that reports these data.

2. The authors used TUNEL assay to detect apoptotic cells. They should show the images or higher power views to demonstrate the findings.

Figures have been attached.
3. Because there are many necrotic cells and irregular distribution of these apoptotic cells inside the tumors, they should provide the principles of which the areas of tumors for TUNEL assay they chose. This is the most crucial point in this article, and authors need to clarify this if AI becomes a CRC prognostic marker in the future.

The areas of tumours for the TUNEL assay were selected by expert pathologists, excluding necrotic areas. This sentence has been added to the final manuscript.

4. Authors should provide the overall survival data of patients, grouped by AI and compared to TNM stage. We are curious to know that whether TNM stage and AI value is the better prognostic marker in CRC.

On page 7 it could be read:

“With respect to the analysis of DFS by stages, we observed that a high AI was associated with a shorter survival in more advanced disease (stages III and IV) (p=0.004) (Figure 2). This association was not present in earlier stages.”

As well as:

“A low AI was associated with a more favourable OS (OS at 5 years: 74.6 ± 8.8 %), whereas a high AI was correlated with a poor outcome (OS at 5 years: 43.7 ± 11.9 %, with p=0.027). In a multivariate analysis, we found that AI and tumour stage were independent prognostic factors for OS, with RR values of 2.18 (with a 95% CI of 1.08-4.37) and 2.41 (with a 95% CI of 1.20-4.85), respectively”.