

# Apoptosis and Proliferation in Paired Primary Colorectal Adenocarcinomas and Their Liver Metastases

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The proliferation potentials and the level of apoptosis were compared in paired primary colorectal adenocarcinomas and their liver metastases within each individual. From a total of 22 patients 44 specimens of paired primary and metastatic tumors were obtained for analysis. The levels of spontaneous apoptosis (a spontaneous apoptosis index, SAI: % apoptotic nuclei among a total of 1000 nuclei) and of proliferation (KI-67 index: % positively stained cells for KI-67 among a total of 1000 cells) were analyzed between primary and metastatic tumors. Survival rates and its relationship with the clinical parameters were also analyzed. The overall survival rate at 5 years was 16.9% with the median survival time of 45 months. T-stage ( $p=0.005$ ) and time to liver metastasis (synchronous versus metachronous,  $p=0.03$ ) showed statistical significance in relation to survival. The mean SAI of primary tumors was  $1.35 \pm 0.25$ , which was not statistically different from the  $1.58 \pm 0.18$  of metastatic tumors ( $p=0.33$ ). The mean KI-67 indices in primary and metastatic tumors were  $23.9 \pm 3.4$  and  $16.4 \pm 2.5$ , respectively, and this difference was statistically significant ( $p=0.016$ ). Subset analysis showed significant difference in the KI-67 index in the synchronous group but not in the metachronous group. No significant difference was shown in the relative ratios of apoptosis to proliferation between the primary tumor and the metastasis within each individual. The results in this study may partly explain the indolent behavior of liver metastasis from colorectal cancer and provides a rationale for the active treatment of metastatic tumors as well as of primary disease.

**Key Words:** Colorectal adenocarcinoma, liver metastasis, proliferation, apoptosis

## INTRODUCTION

The past decades have witnessed remarkable improvements in the treatment results of colorectal cancer by successful multimodality treatments with surgical resection, chemotherapy, and radiotherapy. However, treatment failure still remains to be a problem to be overcome.

Liver metastasis is commonly seen in colorectal cancers, and is reported to be 25 - 30% at the time of diagnosis and 40 - 50% after radical resection.<sup>1,2</sup> While the presence of distant metastases represents a dismal prognosis in many other types of cancer, liver metastasis from colorectal cancer does not preclude curative treatment. Five-year survival following the resection of isolated colorectal cancer liver metastases has been reported to be as high as 38%.<sup>3</sup> Several non-surgical experimental therapeutics have been developed for those who are not amenable to surgical resection and applied in clinic.<sup>4-9</sup> However, therapeutic success still remains to be improved.

Understanding tumor characteristics in terms of biological parameters can provide more insight in developing a treatment strategy.<sup>10-12</sup> Various biological parameters have been investigated in colorectal cancers in comparison to its metastases.<sup>13-15</sup> Of those, the potentials of proliferation and apoptosis have been shown to be important, hence novel regulating molecules are under investigation as therapeutic targets.<sup>13,16,17</sup>

In this study, we investigated relative prolifera-

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tion potentials and levels of apoptosis in paired primary colorectal adenocarcinomas and their liver metastases within each individual.

## MATERIALS AND METHODS

### Materials

From 1994 to 2000, a total of 49 colorectal cancer patients with liver metastasis registered at our institute. Of these 22 patients whose tissue samples were available from archives were included in the study. Their liver metastases were found either at the time of initial diagnosis of the primary disease (synchronous) in 13 patients or during the follow-up period after treatment for primary disease (metachronous) in 9 patients. As summarized in Table 1, the patients had initial T stages of T3 except 2 patients with T4, and the N stages were N0 in 8, N1 in 4, and N2 in 10 patients. The number of metastatic nodules in the liver was 1 in 7, 2 in 4, 3 in 3, and more than 4 in 8 patients. Forty-four specimens of paired primary and metastatic tumors were obtained from the archives for analysis.

### Assessment of apoptosis

The hematoxylin and eosin-stained slides of

histologic paraffin sections were reviewed for spontaneous apoptosis by 2 investigators, with no knowledge of patient's clinical history. Apoptotic cells were scored in coded slides at 400x magnification. The morphological features used to identify apoptosis in these tissue sections have been previously described and illustrated.<sup>18</sup> Ten fields of nonnecrotic areas were randomly selected across each slide and in each field the spontaneous apoptosis index (SAI: the percentage of apoptotic nuclei among a total of 1000 nuclei) was calculated.

### Assessment of proliferation

Four  $\mu\text{m}$ -thick sections of paraffin-embedded tumor tissues were immunohistochemically stained with streptavidin-biotin peroxidase (Universal LSAB kit, DAKO, Carpinteria, CA, USA). The paraffin sections were deparaffinized in xylene, rehydrated by treating them with sequential concentrated alcohols, and rinsed in two changes of PBS for 15 min. To enhance the staining, slides were subjected to microwave antigen retrieval (800 W,  $2 \times 5$  min) in 0.01 M sodium citrate buffer (pH 6.0) and then endogenous hydrogen peroxidase activity was blocked by 3%  $\text{H}_2\text{O}_2$  for 10 min. To minimize nonspecific binding, the tissues were reacted with a protein-blocking agent (DAKO kit) for 20 min. Specimens were incubated with primary antibody (MIB-1, DAKO) at room temperature (RT) for 1 h, washed 3 times with PBS, incubated with biotinylated anti-rabbit and anti-mouse immunoglobulin G for 30 min, and washed 3 times in PBS. Slides were then treated with streptavidin buffer diluent for 20 min at RT and washed 3 times in PBS. The peroxidase reaction was visualized by treating with 3-amino-9-ethyl-carbazole in N, N-dimethyl formamide (AEC) for 3-10 min and then were counterstained with Mayer's hematoxylin and mounted. Proliferation status was expressed as the KI-67 labeling index, the percentage of positively stained cells among a total of 1000 cells.

### Statistical analysis

The statistical significance of difference was analyzed using the paired t-test for the level of

**Table 1.** Summary of Patients' Characteristics

Characteristics	No. of patients (%)
Age (years)	32-71 (median; 59)
Male:female	17: 5
Stages: T Stage	T3: 20 (90.9) T4: 2 (9.1)
N Stage	N0: 8 (36.4) N1: 4 (18.2) N2: 10 (45.4)
Onset of liver metastasis	Synchronous: 13 (59) Metachronous: 9 (41)
Time to liver metastasis	6-45 months (median 14 months)
No. of metastatic nodules	1: 7 (31.8) 2: 4 (18.2) 3: 3 (13.6) more than 4: 8 (36.4)

apoptosis or proliferation in paired primary and metastatic tumors within each individual. The relative ratio of apoptosis to proliferation was calculated for the primary tumor and the metastasis within each individual and their difference was also analyzed using paired t-test.

Survival rates were calculated using Kaplan-Meier method. Any possible relationship was analyzed using log-rank test between survival results and the parameters involving stage, time to liver metastasis, SAI and KI-67.

## RESULTS

### Clinical results

In the metachronous group, the time interval to liver metastasis ranged from 6 to 45 months with a median time of 14 months. The overall survival rate at 5 years was 16.9% and the median survival time was 45 months. The median survival times in the synchronous and in the metachronous group were 28 months and 54 months, respectively, ( $p < 0.03$ ). Survival result was also analyzed for any possible relationship with the parameters involving stage, time to liver metastasis, SAI and KI-67. Only T-stage ( $p = 0.005$ ) and time to liver metastasis (synchronous versus metachronous,  $p = 0.03$ ) showed statistical significance.

### Spontaneous apoptosis index (Fig. 1 and 2)

SAI was assessed in a total of 44 specimens. The mean SAI of metastatic tumors was  $1.58 \pm 0.18$ , which was not significantly different from the  $1.35 \pm 0.25$  of the primary tumors ( $p = 0.33$ ). The level of SAI showed an increase in 13 and a decrease in 9 metastatic tumors compared to their primary counterparts.

### KI-67 labeling index (Fig. 3 and 4)

KI-67 index was assessed in a total of 44 specimens. The mean KI-67 indices in primary and metastatic tumors were  $23.9 \pm 3.4$  and  $16.4 \pm 2.5$ , respectively and this difference was statistically significant ( $p = 0.016$ ). The KI-67 index showed a

decrease in 15 of 22 metastatic tumors compared to their primary counterparts.

### Analysis of SAI and KI-67 with respect to time to metastasis

SAI and the KI-67 index were analyzed within subgroups; i.e., the synchronous or metachronous groups. In the synchronous group, the mean KI-67 indices of the primary and the metastatic tumors were  $24.8 \pm 6.7$  and  $15.8 \pm 4.3$ , respectively, and this difference was statistically significant ( $p = 0.03$ ), while no similar significant difference was found for the SAI. In the metachronous group, neither the SAI nor the KI-67 index showed any significant difference between the primary and metastatic tumors. The relative ratios of apoptosis to proliferation were also analyzed. No significant difference was shown between the primary tumor and the metastasis within each individual.

### Relationship between clinical parameters and the SAI or the KI-67 index

Presence of a relationship was examined between the SAI or the KI-67 index and clinicopathologic factors including size and stage of the primary tumor, lymph node status, and number of metastatic tumor. No significant correlation was found.

## DISCUSSION

It has been well established that colorectal carcinogenesis is due to a deregulation of cell proliferation, which is related to the accumulation of various genetic alterations.<sup>19</sup> More recently, cell loss by apoptosis has received attention as an important component of tumor growth.<sup>20,21</sup> Apoptosis is inhibited during the development of colorectal cancer and its frequency is inversely related to the invasiveness and metastatic activity of colorectal cancers.<sup>16</sup>

We previously studied the deregulation of cell proliferation and cell loss in paired primary and recurrent colorectal adenocarcinomas and showed significantly decreased apoptosis in recurrent tumors compared to their primary counterparts.<sup>22</sup> The underlying mechanisms were also found to

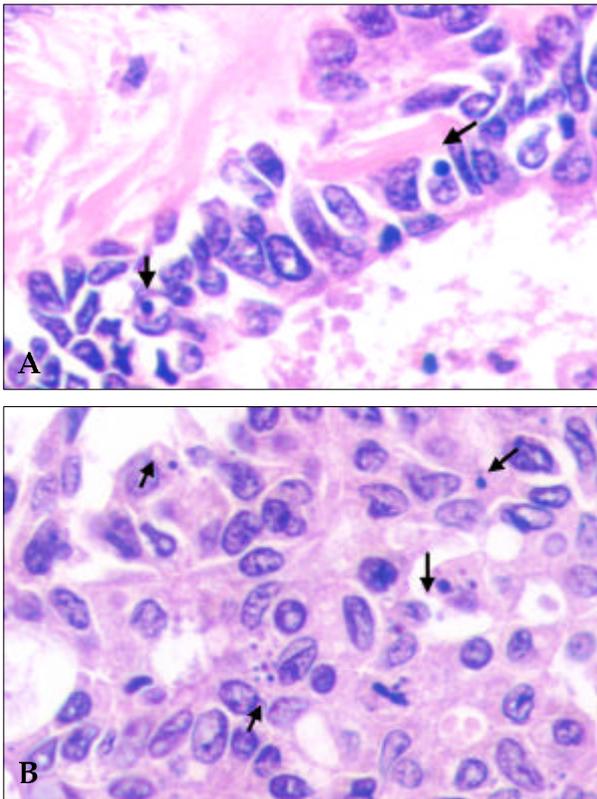


Fig. 1. Spontaneous apoptosis (arrows) in a hematoxylin and eosin-stained tissue section of a primary tumor (A) and liver metastasis (B).

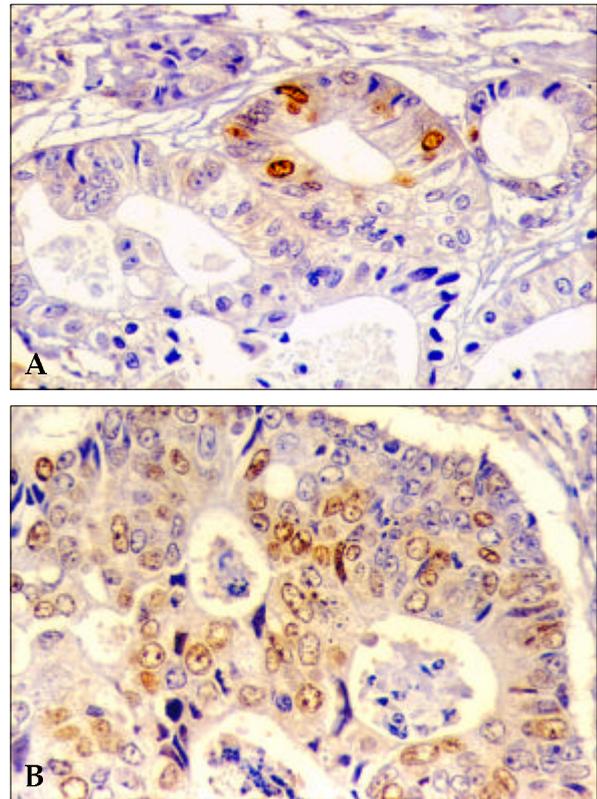


Fig. 3. KI-67-stained cells (brown color), indicating a proliferative status, are shown in a tissue section of primary tumor (A) and in a liver metastasis (B).

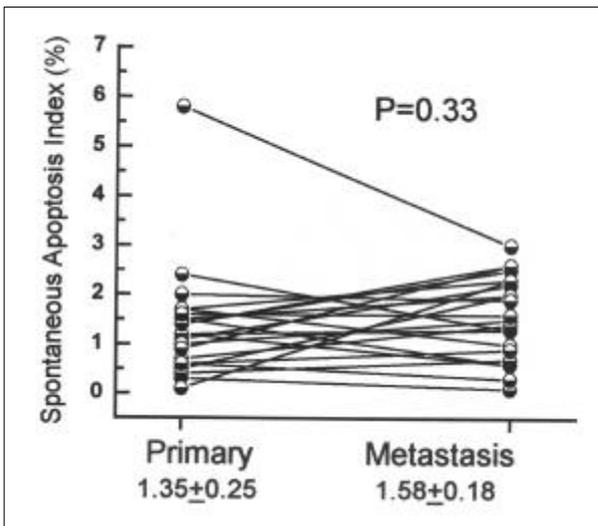


Fig. 2. A plot showing changes in the level of spontaneous apoptosis (SAI) in paired primary colorectal cancer and in its liver metastasis. SAIs in the primary and in the liver metastasis were  $1.35 \pm 0.25$  and  $1.58 \pm 0.18$  ( $p=0.338$ ).

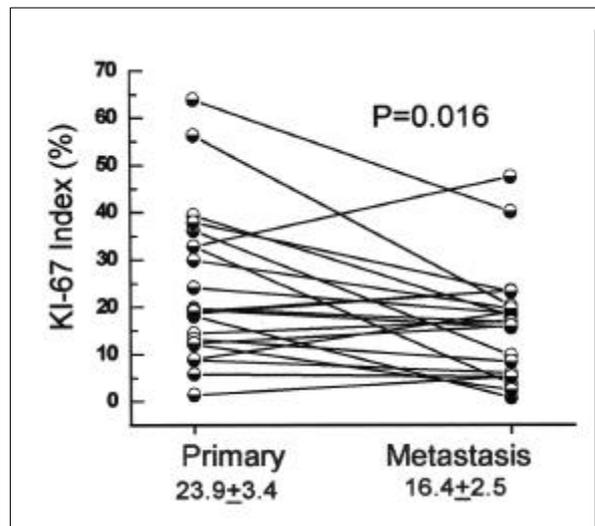


Fig. 4. A plot showing the change in the level of KI-67 index in paired primary colorectal cancer and its liver metastasis. KI-67 indexes in the primary and the liver metastasis were  $23.9 \pm 3.4$  and  $16.4 \pm 2.5$  ( $p=0.016$ ).

involve the increased expression of p53 and the alterations in the levels of cell cycle regulators. In the present study the levels of apoptosis were rather similar in the primary and metastatic tumors. However, the proliferation status was found to be significantly decreased in the metastatic tumors, suggesting that intrinsic biologic characters of metastatic and primary tumors differ.

Several studies have compared the biological behaviors of metastatic liver tumors with those of their primary counterparts. Tatebe et al.<sup>13</sup> showed increased proliferative potential and apoptosis in liver metastasis compared to the corresponding primary tumor. Termuhlen et al.<sup>23</sup> also reported increased apoptosis in liver metastasis. Agui et al.<sup>15</sup> reported the opposite result, i.e., decreased proliferation and increased apoptosis in liver metastasis.

The decreased proliferation potential of the metastasis in the present study concurs with the findings of Agui et al.<sup>15</sup> The combination of reduced proliferation and a similar level of apoptosis in metastasis might be interpreted to reflect somewhat slow-growing nature of metastatic liver tumors. In fact, the mean doubling time of liver metastasis from colorectal cancer has been reported to be no more than 155 days, which implies a relatively slow growth rate.<sup>24</sup> To summarize, published data do not agree in terms of differential proliferation potentials of primary colorectal cancer and its liver metastasis, while increased apoptosis in liver metastasis is a commonly shared finding. One of the reasons why results do not agree, seems to be due to the limited number of samples analyzed, which is in the range of 15 to 37.

Heterogeneity within liver metastases in terms of its biological characteristics might be another reason. For example, it is questionable whether synchronous metastasis is biologically equivalent to metachronous metastasis. Foster et al.<sup>25</sup> reported poorer survival in the synchronous group than that in the metachronous group (12.5% vs. 31%). In contrast, Butler et al.<sup>26</sup> reported a lack of survival difference between the synchronous and metachronous groups. Although the point is still argued, clinically it appears that synchronous liver metastases tend to be associated with poorer clinical results than metachronous metastasis. In

the present study, subset analysis showed that a significant difference between the in KI-67 indices of primary and metastatic tumors in the synchronous but not in the metachronous group. Therefore, this difference in terms of the time to liver metastasis needs to be considered in analyses of this kind.

Currently, experimental therapeutic approaches have achieved some success in the management of liver metastasis from colorectal cancer. A common characteristic shared by these approaches is that those are local not systemic treatments. The results in this study may explain in part the indolent behavior of liver metastasis from colorectal cancer and provide a rationale for the active treatment of liver metastasis using local modalities. In the case of synchronous metastasis in particular, the results of the present study suggest that active treatment should be carried out on both liver metastasis and the primary tumor. In fact, combined treatment by surgery for both the primary and the liver metastasis plus postoperative pelvic radiotherapy was effective in terms of not only local control but also in survival in a group of rectal cancer patients with synchronous liver metastasis.<sup>27</sup>

While further study is warranted upon a larger number of samples, including a detailed analysis of regulating factors, we hope that the results of this study may be useful for developing a new therapeutic strategy for the treatment of colorectal cancer with liver metastasis.

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