

# Assessment of Apoptosis by M30 Immunoreactivity and the Relationship with the MSI status and the Clinicopathological Characteristics of Colorectal Carcinomas

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**Background :** The monoclonal antibody M30 recognizes a neoepitope of cytokeratin 18 that's produced during the process of apoptosis, and it is reactive in formalin-fixed, paraffin-embedded tissue. The detailed nature of apoptosis in colorectal cancer is unclear, especially in regard to the MSI status and the clinicopathologic factors. The purpose of this study was to elucidate the apoptosis assessed by M30 immunoreactivity in colorectal cancer and its relationship with the MSI status and the various clinicopathologic factors of colorectal cancers. **Methods :** 101 colorectal cancers were classified according to levels of MSI as 12 MSI-H, 4 MSI-L and 85 MSS. Apoptosis was quantified by immunohistochemistry with using M30 CytoDEATH antibody. **Results :** The apoptotic index assessed by M30 was significantly increased in the MSI-H and MSI-L colorectal cancer compared to that in the MSS colorectal cancer. Right sided colon cancer showed a significant higher apoptotic index than did the left sided colon cancer. There was also a tendency for decreased apoptosis in metastatic colorectal cancers (Duke's stage D). There was somewhat of an increase of apoptosis in colorectal cancers with mucinous carcinoma and medullary carcinoma, and also in the colorectal cancers with an increased TIL count, but this was not statistically significant. **Conclusion :** M30 immunoreactivity is a valuable method to detect apoptosis in formalin-fixed, paraffin-embedded tissue and it might explain that MSI-H colorectal cancer shows better clinical behavior than MSS colorectal cancer in regard to the increased apoptosis.

**Key Words :** M30; Apoptosis; Colorectal cancer; Microsatellite instability

Apoptosis is programmed cell death, and it plays an important role in many physiologic and pathologic processes.<sup>1</sup> An important function of apoptosis is the elimination of damaged cells. For example, the cells with genetic damage caused by exposure to carcinogens may be deleted by undergoing apoptosis, and this prevents their replication and the accumulation of clones of abnormal cells. There is increasing evidence to support the hypothesis that failure of apoptosis may be an important factor in the evolution of colorectal cancer and also its poor response to chemotherapy and radiation.<sup>2</sup>

Apoptotic cells can be recognized by careful light-microscopic examination of the hematoxylin and eosin (H & E) stained sections. The morphologic criteria of apoptotic cells, as defined by Kerr *et al.*,<sup>3</sup> include the formation of apoptotic bodies with nucle-

ar condensation or nuclear fragmentation, and there is cytoplasmic shrinkage and the loss of contact with the surrounding cells. Because of the brief duration of these morphologic changes, apoptosis has been difficult to identify on H & E stained slides. Therefore, the techniques most widely used to investigate apoptotic cell death in human colorectal mucosa are terminal deoxyribonucleotidyl transferase mediated nick end labeling (TUNEL) and in situ end labeling (ISEL).<sup>4</sup> However, there are some limitations for the above-mentioned methods to apply them for evaluating apoptosis in the gastrointestinal tract. Both techniques mark necrotic and autolytic cells in addition to apoptotic cells.<sup>5</sup>

Several antibodies have recently been introduced that specifically identify apoptotic cells in different human cell types.<sup>6,7</sup> One of these antibodies is the murine monoclonal antibody M30,

which reacts with a caspase-cleaved product of cytokeratin 18. M30 immunoreactivity is confined to the cytoplasm of apoptotic epithelial cells and it is expressed during early apoptosis.<sup>8</sup> A clear advantage of M30 over TUNEL and ISEL is that M30 is not expressed in necrotic cells.<sup>9</sup> Due to this lack of expression in necrotic cells, M30 is a more desirable method to detect apoptosis than is the TUNEL or ISEL method.

In recent years, it has become apparent that there are three genetically distinct subgroups of sporadic colorectal cancer (CRC), as based on the level of DNA microsatellite instability (MSI).<sup>10-12</sup> The majority of sporadic CRCs (70-80%) show allelic losses with no MSI and so they are defined as microsatellite stable (MSS). The other two subgroups are defined as MSI-low (MSI-L) and MSI-high (MSI-H), and each comprises 10-15% of the sporadic CRCs, and they both develop small insertion and deletion mutations in repetitive DNA.<sup>10-12</sup> MSI-H cancers are predominantly right sided, they are more likely to be the mucinous type and to have a signet ring cell component or to have a solid growth pattern (medullary carcinoma), and to show increased numbers of tumor-infiltrating lymphocytes (TILs).<sup>13,14</sup> Some studies have suggested that colorectal carcinomas with MSI-H tend to have an improved survival rate<sup>15,16</sup> and they may respond to adjuvant chemotherapy differently than do the MSS tumors.<sup>17,18</sup>

It has been shown that apoptosis can be induced by the over-expression of the mismatch repair genes hMSH2 or hMLH1,<sup>19</sup> suggesting that tumors with the MSI phenotype lose their ability to undergo efficient apoptosis.<sup>20</sup> However, it was described by two studies that apoptotic cell death was actually more frequent in colorectal tumors with MSI than in those colorectal tumors without MSI.<sup>13,21</sup>

Therefore, the purpose of this study was to determine the correlation between apoptosis and the MSI status, and to analyze the relationship between apoptosis and the clinicopathological characteristics.

## MATERIALS AND METHODS

### The patients, tissue samples and the clinical characteristics

101 cases of colorectal carcinomas were obtained from 101 patients who were treated at Pusan National University Hospital from 2003 to 2004. None of the patients had received chemotherapy or radiotherapy prior to tumor resection. All of tumors were tested for their MSI status by polymerase chain reaction (PCR) amplification of the microsatellite repeats. The mean age

of patients was  $61.2 \pm 10.2$  years (age range: 39-85), and the male to female ratio was 1.8:1.

The cecum, ascending colon, and transverse colon were regarded as the right sided colon, while the descending colon, sigmoid colon and rectum were referred to as the left sided colon. For the statistical analysis, according to metastatic status, the tumors at modified Duke's stages A, B and C were compared with the modified Duke stage D tumors. For the depth of invasion, the tumors that extended to the subserosa were compared with those with serosal exposure.

All the H & E stained sections were reviewed. TILs were defined as the lymphocytes that were infiltrating carcinomatous glands in nests or sheets, and this was exclusive of the inflammatory cells that were associated with stromal tissue or luminal debris. The lymphocyte counts were recorded as the total TILs/10 high power fields (HPF). Other histologic features were defined as 1) mucinous carcinoma with the mucinous component representing >50% of the tumor 2) medullary carcinoma with medullary features in >50% of the tumor; medullary features were defined as small to medium sized cells with variable amounts of eosinophilic cytoplasm that were growing in solid sheets, and they were often associated with abundant lymphocytes in the stroma 3) tumor differentiation (well or moderately differentiated vs poorly differentiated). The tumors were regarded to be poorly differentiated when >50% of the lesion showed poor gland development with formation of irregular clusters or sheets.<sup>14</sup>

### Immunohistochemistry (M30)

Four  $\mu\text{m}$  thick sections from the paraffin-embedded formalin fixed blocks were deparaffinized and hydrated through a graded series of alcohol solutions. After inhibition of endogenous peroxidase activity by immersion in a 3%  $\text{H}_2\text{O}_2$ /methanol solution, antigen retrieval was carried out with 10 mmol/L citrate buffer (pH 6.0) in a microwave oven for 10 min at  $120^\circ\text{C}$ . The sections were then incubated with the primary antibody M30 CytoDE-ATH (Boehringer Mannheim, Mannheim, Germany). After a thorough washing with phosphate-buffered saline (PBS), they were next incubated with biotinylated secondary antibody, and then with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). Finally, the immune complexes were visualized by incubation with 0.01%  $\text{H}_2\text{O}_2$  and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin.

### Evaluation of the staining results with M30 antibody

Evaluation of M30 staining was performed using light microscopy. Quantitative analysis was performed randomly in the carcinoma. M30 positivity was identified as brown cytoplasmic staining. In all the cases, at least 1000 epithelial cells were counted and the M30 positive cells were expressed as a percentage of the total number of counted cells (the apoptotic index).

### Molecular detection of microsatellite instability

The DNA of cancerous tissue and of the corresponding normal colorectal mucosa was obtained from the formalin-fixed, paraffin-embedded blocks. The DNA was extracted by proteinase K digestion and a phenol-chloroform procedure. The extracted DNA was amplified by PCR with fluorescent dye labeled primers for five microsatellite loci as 2 mononucleotide markers (BAT25 and BAT26) and 3 dinucleotide markers (D5S346, D2S123 and

D17S250) as per the NCI criteria. The DNA was detected by a temperature-controlled DNA Sequencer (PRISM 377, Perkin-Elmer Corp., Foster City, CA), and fragment analyses were carried out with Genscan software (Perkin-Elmer). The MSI status was determined by size variation and the occurrence of additional bands in the PCR product from the tumor DNA and this was not observed from the DNA of the normal tissue from the same patients. MSI-H was defined as instability in at least two of the five microsatellite loci; MSI-L was defined as a shift in only one locus; and MSS was defined as when none of the loci were shifted, according to the NCI criteria.

### Statistical analysis

The mean M30 counts were compared across the MSI status and the different clinicohistological characteristics with using Student's t-test. Correlation between apoptosis and the TILs was assessed using the Pearson test. p values <0.05 were considered significant.

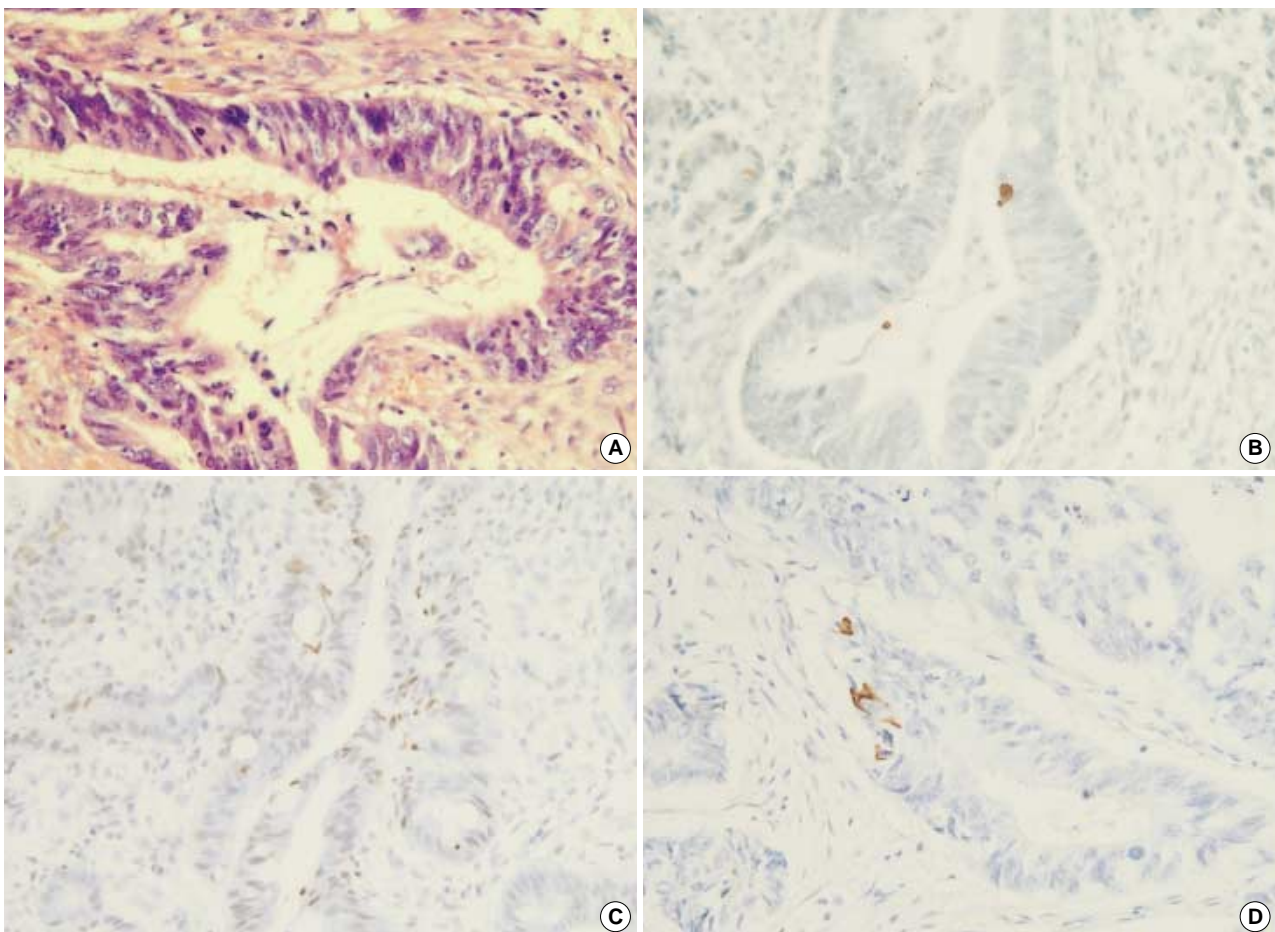


Fig. 1. Apoptotic cells which have nuclear condensation and cytoplasmic shrinkage are detected by M30 (A: H&E stain, B: M30 CytoDEATH,  $\times 400$ ). MSI-H cancer (C) shows higher apoptotic index than MSS cancer (D) (C, D: M30 CytoDEATH,  $\times 400$ ).

## RESULTS

### Apoptosis assessed by M30 in the adjacent normal mucosa around the tumor and in colorectal cancer

In the adjacent normal mucosa around the tumor, apoptotic cells that were assessed by M30 were observed in the mucosal surface and also in the crypts in a few cases. In the carcinoma, apoptotic cells were randomly distributed throughout the tumor. M30 was expressed in the apoptotic cells, but not in the necrotic cells and debris. Even though there was no morphological evidence of apoptosis on the routine H&E staining, apoptotic cells could be identified by M30 immunostaining (Fig. 1). There was no difference between the different grades of carcinomas.

### Relationship between the apoptotic index as assessed by the M30 and the MSI status

12 (11.9%) of the 101 cases were classified as MSI-H. 4 (4.0%) cases showed instability at one locus (MSI-L), and 85 (84.2%) cases showed no instability at any of the five loci (MSS). M30 positive cells were randomly distributed throughout the tumor. MSI-H and MSI-L colorectal carcinomas showed a two fold higher apoptotic index than did the MSS colorectal carcinoma and this was statistically significant ( $p < 0.05$ ) (Fig. 1, 2).

### Apoptosis assessed by M30 in relation to the clinicopathological characteristics

The clinicopathological data from the 101 colorectal cancers are summarized in Table 1. Right-sided colon cancer showed a

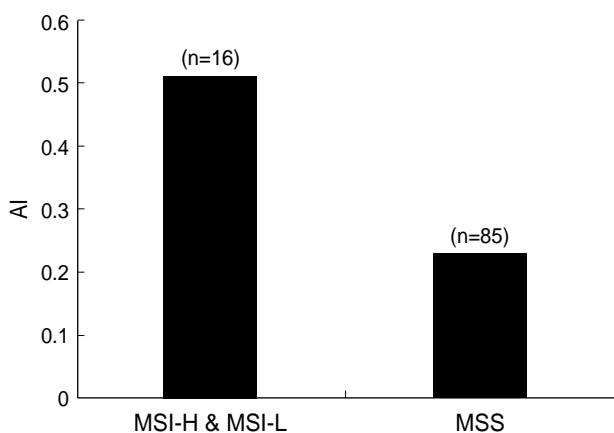


Fig. 2. MSI-H and MSI-L colorectal carcinomas show a two fold higher apoptotic index than the MSS colorectal carcinoma and this is statistically significant ( $p < 0.05$ ). AI, apoptotic index.

higher apoptotic index than did the left-sided colon cancer ( $p = 0.011$ ). There was also a tendency for decreased apoptosis in metastatic colorectal cancer (Duke's stage D) ( $p = 0.055$ ). There was somewhat of an increase of apoptosis in the colorectal cancers with mucinous features and the medullary carcinoma, and also for the colorectal cancer with an increased TIL count, but this finding was not statistically significant. The mean tumor size was 5.2 cm (range: 0.6-14.8). The apoptotic index was higher in the larger sized tumors than that in the small tumors, but this was not significant. There was no significant relationship of the apoptotic index with other clinicopathologic factors such as depth of invasion, lymphovascular tumor emboli, perineural invasion and lymph node metastasis.

## DISCUSSION

Failure of apoptosis may be an important factor in the evolution of colorectal cancer. In a systematic review,<sup>4</sup> the methods

Table 1. Relationship between apoptosis and the clinicopathologic features

Clinicopathologic features	Number of cases	AI	p value
Size			
<5 cm	61	0.26 ± 0.10	0.465
≥ 5 cm	40	0.31 ± 0.95	
Mucinous carcinoma	13	0.34 ± 0.10	0.489
Non mucinous carcinoma	88	0.27 ± 0.03	
Medullary carcinoma	7	0.28 ± 0.11	0.652
Non medullary carcinoma	94	0.22 ± 0.03	
Differentiation			
Poorly	12	0.34 ± 0.09	0.485
Well to moderate	89	0.27 ± 0.03	
Site			
Right	31	0.40 ± 0.06	0.011
Left	70	0.22 ± 0.03	
Depth			
Less than serosa	78	0.30 ± 0.03	0.159
Serosal exposure	23	0.19 ± 0.05	
Duke's stage			
A, B, C	85	0.30 ± 0.03	0.055
D	16	0.13 ± 0.04	
Nodal status			
N0	46	0.28 ± 0.05	0.875
N1-2	55	0.27 ± 0.04	
Lymphovascular emboli			
Present	48	0.27 ± 0.04	0.858
Absent	53	0.28 ± 0.04	
Perineural invasion			
Present	40	0.29 ± 0.06	0.636
Absent	61	0.26 ± 0.03	

AI, apoptotic index.

that were most widely used to identify apoptotic cells in paraffin sections were TUNEL and ISEL. Both methods detect the DNA strand breaks by adding nucleotides, including deoxyuridine triphosphate labeled with biotin, to the ends of the broken DNA. The only essential difference between the two methods is that TUNEL uses the enzyme terminal deoxyribonucleotidyl transferase, whereas ISEL uses DNA polymerase I or its Klenow fragment.<sup>9,22</sup> Both methods have their own disadvantages. Most importantly, they mark necrotic and autolytic cells in addition to apoptotic cells, and they require pre-treatment steps that need careful optimization.<sup>5,22</sup> There is also evidence that the physical act of cutting tissue to make sections produces TUNEL activity.<sup>4</sup> This creates serious reservations concerning the applicability of the TUNEL method for evaluating apoptotic cell death in the gastrointestinal tract.<sup>4</sup> The advantage of the new method that uses monoclonal antibody M30 over the other two methods is that M30 is exclusively expressed in apoptotic epithelial cells.<sup>23</sup> M30 immunoreactivity has been shown to be positive in epithelial cells that have apoptotic characteristics such as chromatin condensation, nuclear fragmentation and detachment of cytoplasm, whereas the cells having normal morphology and the end-stage apoptotic cells were M30 negative.<sup>8</sup> In most cases, the apoptotic counts were slightly higher in the case of employing morphological cell criteria compared with the M30 immunostaining. This suggests that there are false-negative staining results with the M30 method. First, it must be noted that the various types of cells show the morphological features of apoptosis, including apoptotic epithelial cells, apoptotic lymphocytes and apoptotic mesenchymal cells, whereas M30 positivity is limited exclusively to epithelial cells. Second, M30 immunoreactivity eventually disappears in end-stage apoptotic cells.<sup>8</sup> In a previous study, the M30 immunoreactivity and ISEL positivity were strongly correlated. Since M30 immunohistochemistry is technically simpler and easier to interpret than ISEL, it has the potential to become the method of choice for demonstrating apoptosis in colorectal epithelial cells.<sup>9</sup>

Two previous studies reported that apoptotic cell death was more frequent in MSI colorectal cancer than in MSS colorectal cancer.<sup>13,14</sup> In our current study, MSI-H and MSI-L colorectal cancers ( $0.51 \pm 0.09$ ) expressed a higher apoptotic index than did the MSS colorectal cancers ( $0.23 \pm 0.03$ ) ( $p=0.002$ ). The MSI-H colorectal cancers were predominantly right sided and poorly differentiated, they were the mucinous and medullary types, they had a signet ring cell component and they showed increased TIL.<sup>13,14</sup> We assumed that colorectal cancer with a high apoptotic index might have the clinicopathological characteris-

tics of MSI-H and MSI-L colorectal cancers. In the present study, the apoptotic index was higher in the right sided colon cancer ( $0.40 \pm 0.06$ ) than that in the left sided colon cancer ( $0.22 \pm 0.03$ ) ( $p=0.011$ ). There was somewhat increased apoptosis in the colorectal cancers with mucinous features, medullary features and an increased TIL count, but this was not statistically significant. There were no significance between apoptosis and the other characteristics such as depth of invasion, lymphovascular tumor emboli, perineural invasion and lymph node metastasis.

Although the use of the MSI-L category is widespread, there is some debate as to whether a discrete MSI-L group truly exists. One study<sup>13</sup> showed that MSI-L cancers had an intermediate apoptotic index between MSI-H cancer and MSS cancer. However, MSI-L cancer is not a distinctive category. One study<sup>24</sup> showed that MSI-H tumors were predominantly poorly differentiated and they arose more often in the proximal colon, whereas MSI-L and MSS tumors were moderately differentiated and they originated at the same frequency in the proximal and distal colon. That study also showed that there was no significant difference between the MSI-L and MSS groups in terms of age, gender, the clinical stage, differentiation or the anatomical location. MSI-H cancers form a well defined group with distinct clinicopathologic features that are characterized by an overall better long-term prognosis. Yet the MSI-L cancers have been shown to differ in their clinicopathologic features or in their molecular features from the MSI-H cancers and MSS cancers because MSI-L cancer has different biological behaviors. Some studies have suggested that the *K-ras* mutation,<sup>12</sup> methylation of the O<sup>6</sup>-methylguanine DNA methyltransferase (MGMT) promoter,<sup>25</sup> and loss of the MGMT gene expression<sup>24</sup> are all associated with the MSI-L phenotype. There were only 4 MSI-L cancers in our study. Because the number of MSI-L cancers was limited, we could not obtain significant results about the clinicopathologic characteristics.

Two reports<sup>26,27</sup> have shown that the apoptotic indices were higher in tumors that were more highly differentiated and had not invaded or metastasized than in those tumors that were poorly differentiated and invasive or metastasizing. Takano *et al.*<sup>28</sup> also found higher apoptotic indices in tumors without lymph node metastasis or distant metastases in comparison to the tumors that had metastasized, but they found no correlation with the degree of tumor differentiation. In the present study, the apoptotic index was relatively decreased in cancers with metastasis (Duke's stage D) ( $p=0.055$ ). On the other hand, Hawkins *et al.*<sup>29</sup> demonstrated that Dukes A carcinomas had lower apoptotic indices than the Dukes B-D carcinomas. Metastatic dissemination may depend upon the resistance of metastatic cells to apoptosis.<sup>30</sup>

In several murine and human cancer cell lines, a more aggressive metastatic phenotype was associated with increased resistance to apoptosis.

In conclusion, M30 immunohistochemistry was a useful method to identify the apoptotic epithelial cells. There was more apoptosis in the MSI cancer than in the MSS colorectal cancers, and apoptosis was predominant in the right side colon. More study is needed to clarify the relationship between distant metastasis and apoptosis.

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