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CLINICAL STUDY

The clinical significance of apoptosis and M30 expression in colonic cancer progression

Stylianos Kykalos[1], Dimitrios Dimitroulis[2], Evangelia Ntikoudi[2], and Anastasios Karayiannakis[1]

[1]2nd Department of Surgery, Democritus University of Thrace, Alexandroupolis and [2]2nd Department of Propedeutic Surgery, Medical School of Athens University, Laiko General Hospital, Athens, Greece

Abstract

Background/aim: The aim of this study is to identify the significance of M30, an early apoptosis indicator, in colorectal cancer and its liver metastasis. *Patients and methods*: The expression of M30 was immunohistochemically estimated at colonic and liver metastatic tissues of 66 patients. The results were correlated to clinical and pathological features of the tumors. *Results*: High expression of M30 was observed in 15.5% of cases. No metastatic tissue showed expression of M30, while stage D tumors (metastasis included) showed a statistic significant lower expression of M30, when compared to earlier tumor stages. *Conclusion*: Low expression of M30 implies the development of resistance mechanisms against apoptosis, facilitating the progression of colon cancer.

Keywords

Cleaved CK18, colon cancer, liver metastasis

History

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Introduction

This report is presented as a continuous study of a previous analyzed sample of 68 patients suffering from colorectal cancer (1). The aim is to evaluate the significance of the tissue expression of M30 (cleaved CK-18), an indicator of apoptosis, in the several stages of colorectal cancer. Liver metastatic tissue has been examined as well. A further correlation with the expression of Fas apoptotic protein will be also attempted. The cleavage of cytokeratin 18 by caspases occurs early in the cascade of apoptosis, prior to morphological nuclear disruption. This degradation generates a neo-epitope in epithelial cells that is recognized by the M30 monoclonal antibody, allowing the early detection of apoptosis.

Patients and methods

The patients (35 men, 33 women) were offered an operation for colorectal cancer, without neoadjuvant chemo- or radiotherapy, during the period from 2008 to 2010. At the second phase of this cohort study, the histological specimens of two initially included patients were proven marginal with insufficient tissue and therefore not amenable for further examination. These two patients were therefore excluded from further analysis.

Patient's cohort was separated into two groups with regard to the presence of liver metastasis or not, practically D and non-D Stage. Of 27 patients with stage D disease, in 6 cases, there was only colon specimen; in 13, only the liver metastasis; and in the remaining 8 cases, colon as well as liver metastasis (synhron or metachron) was available. In the non-D group, 14 patients had stage A disease, while 9 and 16 patients had disease of stage B and C, respectively.

Paraffin wax-embedded surgically resected thin tumor sections (3–4 μ) were deparaffinized in xylene and rehydrated before analysis. M30 antibody of Cell Signaling Technology with an incubation period of 40 min and a Benchmark XT and Ventana automatic immunohistochemistry machine were used.

The evaluation of the results was made using an optic microscope Nikon eclipse 50i with adapted camera Nikon Digital sight DS-S1 (Nikon Corporation, Japan) with capacity of 100-fold magnification. The immunoreaction was considered to be high if more than 10% of the cells were stained.

The results were evaluated with χ^2 test and where necessary with Fisher's exact test. The significant levels are bilateral and the statistical significance was set at 0.05. The analysis of all data was made with the program SPSS 17.0 (SPSS Inc., Chicago, IL).

Results

The features of all 74 examined tissue samples (from 66 examined patients) are presented in Table 1. Of the examined specimens, 47.3% were of stage D disease, with liver metastases consisting the 60% of cases.

The incidence of low and high expression of M30 protein has been related to the following parameters: tumor stage,

Address for correspondence: Stylianos Kykalos, Tilou str. 12, 15344 Athens, Greece. Tel: +30 210 6390861, +30 6974992828. E-mail: kykalos@gmail.com

Table 1. Tissue sample features.

	N	%
DUKES		
A	14	18.9
B	9	12.2
C	16	21.6
D	35	47.3
Tissue origin of stage D		
Bowel	14	40
Liver metastases	21	60
Tumor – T		
T2	20	37.7
T3	32	60.4
T4	1	1.9
Nodes – N		
No	27	50.9
Yes	26	49.1
Metastasis		
No	53	71.6
Yes	21	28.4
DIFFER		
Well = 0	13	17.6
Moderate = 1	45	60.8
Poor = 2	16	21.6
ASTLER-COLLER		
B1	14	18.9
B2	9	12.2
C1	3	4.1
C2	13	17.6
D	35	47.3

Table 2. M30 expression according to stage.

	Low M30 expression		High M30 expression		
	N	%	*N*	%	*p* - χ^2 test
Astler-Coller					
B1 - C2	28	75.7	9	**24.3**	**0.032**
D	32	94.1	2	**5.9**	
Nodes					
No	19	76	6	24	0.679
Yes	21	80.8	5	19.2	
Metastasis					
No	40	78.4	11	**21.6**	**0.027***
Yes	20	100	0	**0**	
Differentiation					
0	9	69.2	4	30.8	0.100*
1–2	51	87.9	7	12.1	
Death					
No	4	100.0	0	0.0	>0.999*
Yes	25	83.3	5	16.7	

*Fisher's exact test
Bold values indicate statistically significant values ($p < 0.05$)

differentiation, metastatic tissue, lymph node involvement, Fas expression and survival. Finally and after performing the immunohistochemistry, three further specimens could not be evaluated at optic microscope, due to staining failure. In more details, two sections of primary colonic tissue and one section of liver metastasis proved to be non-evaluable. The final results of the 71 remnant samples are presented in Table 2.

The patterns of low and high M30 expression at colonic tissue are depicted in Figures 1 and 2. The characteristic low M30 staining at liver metastasis is shown in Figure 3.

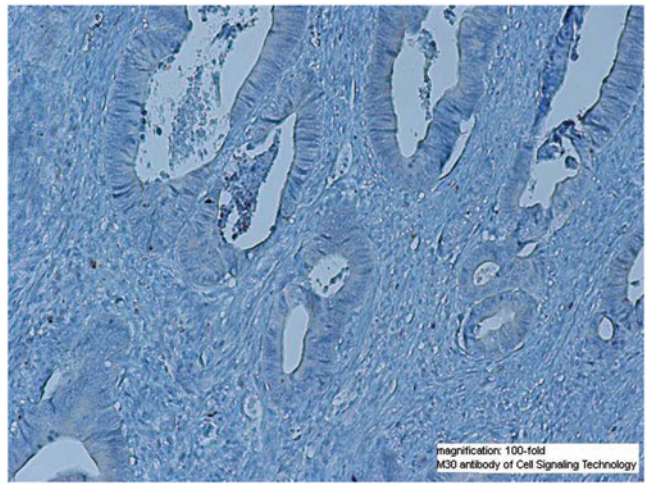

Figure 1. Low M30 expression – colonic tissue.

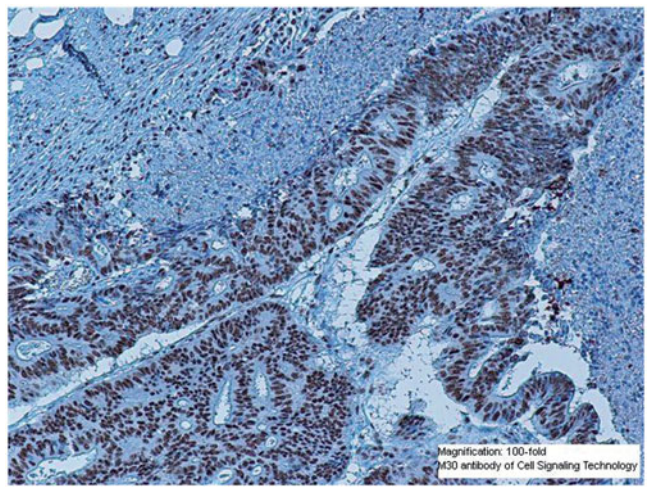

Figure 2. High M30expression – colonic tissue.

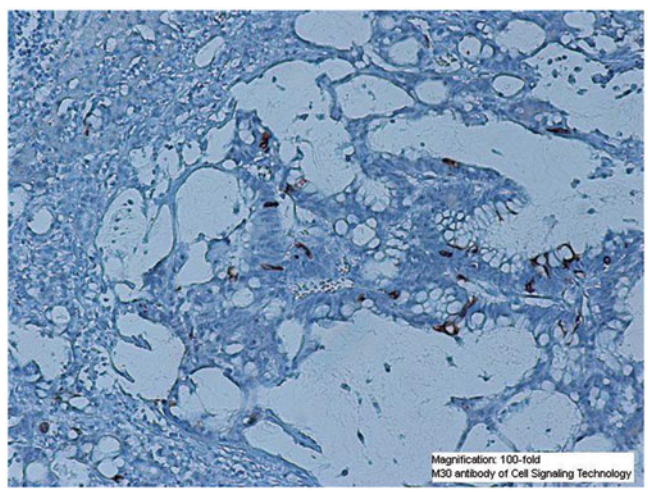

Figure 3. Low M30expression – liver metastasis.

High tissue expression of M30 (M30-T) was observed at 15.5% of cases. Cancer cells of stage B1 to C2 (non-D) disease showed higher expression of M30, in comparison to stage D tumors ($p = 0.032$) (Table 2; Figure 4). On the other

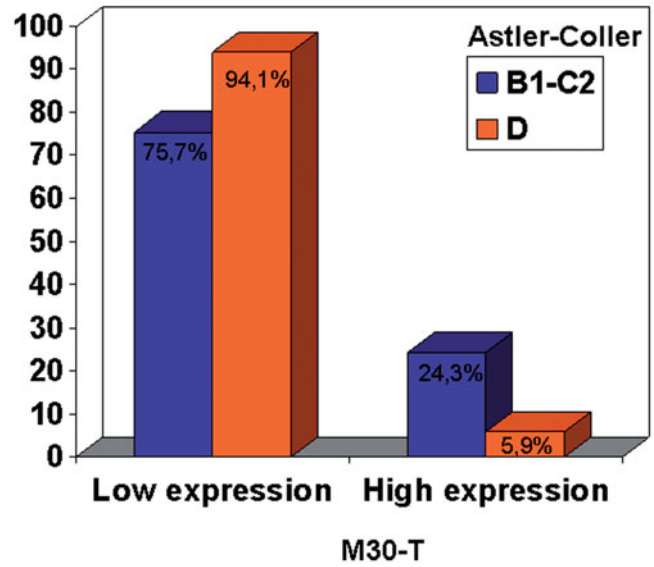

Figure 4. M30 expression according to tumor stage.

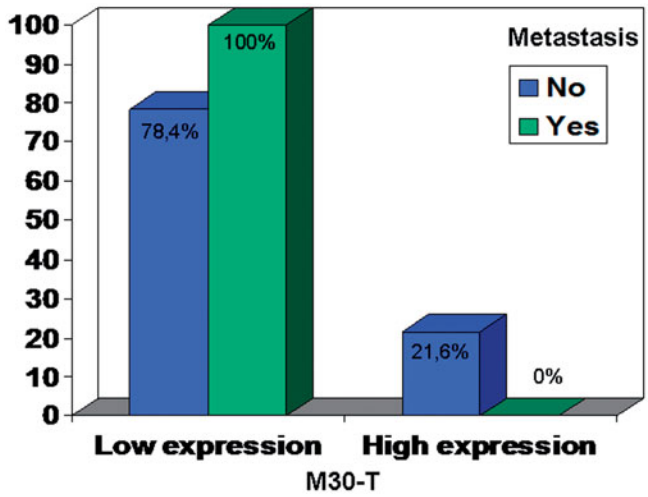

Figure 5. Expression of M30 at liver metastasis.

hand, no metastatic tissue showed positive expression of M30 ($p = 0.027$), as being depicted in Figure 5.

According to the reference study, stage D tumors as well as metastatic tissues did underexpress the Fas protein when compared to earlier colon cancer stages (1). Although the vast majority of cancer cells in apoptotic phase do overexpress the Fas protein (9 out of 11), the high expression of Fas protein is not actually followed by statistic significant increase in M30 expression. These data may implicate a potential tumor resistance against the Fas-mediated apoptosis in the carcinogenesis phase (Table 3).

Colon cancer and liver metastasis specimens were concurrently available from eight patients. The M30 expression was assessed and compared in corresponding primary and liver metastatic samples from all these cases and proved to be low at metastases as well as at primary cancers.

One interesting result is that the apoptosis of tumor-infiltrating lymphocytes (TILs), as estimated by M30 staining (M30-L), was statistically significantly higher ($p = 0.018$) in tumors that overexpress both Fas and FasL proteins (Table 4).

Unfortunately, the analysis of survival data gave up no clear conclusions, as most of the patients were treated in different centers, were enlisted in different follow-up protocols and the follow-up period was estimated for the first three postoperative years. Most of them were still alive, while some died from another cause. In that mean, there was not mentioned any significant difference in survival regarding the M30 expression status.

Discussion

Cytokeratins are proteins of keratin-containing intermediate filaments found in the intra-cytoplasmic cytoskeleton of epithelial tissue. Cytokeratin 18 (CK18, 45 kDa) is an acidic type I cytokeratin, consisting the main CK in hepatocytes, whereas its mutations are associated with cryptogenic liver cirrhosis (2). The detection of CK 18 can be made with specific antibodies, such as Ks18.04 (Ms IgG1), DC-10 (Ms IgG1), C04 (Ms IgG1) and DA-7 (Ms IgG1).

CK's modification by the action of caspases leads to expression of neoepitops, occurring at an early stage of apoptosis. The caspase-3-induced cleavage of epithelial cells' CK18 (CK18-Asp396) can be easily identified by a monoclonal antibody M30, highlighting the early apoptotic cells (3,4).

The methods commonly used to detect apoptotic cells are simple and electron microscopy, flow cytometry, 3 agarose gel DNA electrophoresis, in situ nick-end labeling (ISEL) and TdT-mediated dUTP nick-end labeling (TUNEL) (5).

The release of cleaved CK18 takes place early in the apoptotic process, before the annexin V or TUNEL become positive. The immunohistochemical detection of activated caspase-3 and cleaved CK18 show equal specificity, but greater sensitivity when compared to TUNEL (6,7).

The Fas and FasL protein system that activates the extrinsic pathway of apoptosis affects according to existing data on the biology and behavior of colorectal cancer cells (primary tumor and metastasis). The activity of immune cells of the body remains under Fas/FasL influence. Moreover, the cleaved CK18 serves to recognize the apoptotic cells. In that mean, detection of cleaved CK18 at pathologic specimens is helpful to assess the degree of apoptosis in various stages of cancer.

Backus et al. examined a sample of 63 patients with advanced colon cancer and concluded that apoptosis (assessed by M30 immunostaining) was more obvious at their primaries than at metastatic tissues (8). M30 immunostaining was also the apoptosis indicator in the study of Sugita et al. which showed a clear correlation between the number of FasL-producing macrophages that reside near the tumor and the percentage of tumor cells being in apoptotic phase (9).

The CK18-Asp396 and total CK18 plasma values were higher in patients with more advanced tumor stages, as proven in the study of Koelink et al. (10). These values are not tumor specific, as they represent the apoptosis of health cells as well. The plasma CK18-Asp396/CK18 proportion depends on the proportion between apoptosis and non-proteolytic necrosis. This ratio (CK18-Asp396/CK18) decreases with tumor progression, fact that is associated with worse prognosis especially for stages C/D. There is enough data to support that necrosis becomes more prevalent than apoptosis with tumor

progression (11), under the assumption of cancer cells escape from Fas-mediated apoptosis (12).

The plasma values of CK18-Asp396 are elevated after chemotherapy in hormone-resistant prostate or lung cancer as a result of the chemotherapy-induced apoptosis (13).

The percentage of tissue expression of M30 could be used as indicator of response to a specific therapy, such as the delivery of neoadjuvant chemoradiation for colorectal cancer, or even for determination of a therapy efficacy (14).

The study of Debucquoy et al (15) examined, beyond other factors, the tissue expression of M30 after neoadjuvant therapy administration in a sample of 99 patients. The patients with the best response showed higher tumor cells apoptotic index at the resection specimen.

No safe conclusion regarding survival could be extracted, as the follow-up period is relatively short for colon cancer.

The results of this study are in line with the majority of the relevant published literature (16,17). Tumor cells of colonic carcinomas of stage 4 or metastatic tissue showed limited expression rates of M30, which was statistically significant when compared to the other tumor stages. These findings suggest the development of resistance mechanisms against apoptosis during the tumor progression. Furthermore, the Fas overexpression was not necessarily accompanied by over-expression of M30 or apoptosis. Although these data, do imply tumor escape from Fas-mediated apoptosis and are also extracted by few other studies, the real pathophysiologic mechanism remains actually unknown (18,19).

On the other hand, the exclusive majority of tumor cells found in apoptosis (high M30 expression) did actually overexpress Fas protein. This pathway is therapeutically challenging, especially for early stages of colorectal tumors, where the Fas protein is typically overexpressed.

Future studies should focus on the specific role of apoptotic mechanisms such as Fas mechanism and each and exact apoptotic pathway to make these pathways prone to treatment strategies. Furthermore, other pathways similar to apoptosis like anoikis should be the target of future studies both in the clinical as well as in the experimental setting.

Table 3. Correlation of Fas to M30 expression.

	FAS				
	Low expression		High expression		
	N	%	*N*	%	p - χ^2 test
M30-T					
Low expression	23	41.1	33	58.9	0.189*
High expression	2	18.2	9	81.8	

*Fisher's exact test

Table 4. Apoptosis of TIL's in cases of Fas/FasL co-expression.

	High FAS & FASL co-expression				
	No		Yes		
	N	%	*N*	%	p*
M30-L					
Low expression	42	76.4	2	28.6	**0.018**
High expression	13	**23.6**	5	**71.4**	

*Fisher's exact test
Bold values indicate statistically significant values ($p < 0.05$)

Conclusions

The findings of this cohort study indicate that beside other factors, colorectal cancer progression is also facilitated by resistance to apoptosis. Low rate of tumor apoptosis is highly suggestive of the presence of metastasis and seems to be a negative prognostic factor. M30 staining could be performed in early stages of colorectal cancer, without detectable liver metastasis. In that mean, the detection of early apoptotic rate may become useful in minimizing the cases of tumor being understaged. Tumor underexpression of M30 should be considered as an indication for chemotherapy administration and closer patient's follow-up.

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Declaration of interest

The authors have no declarations of interest to report.

References

1. Kykalos S, Mathaiou S, Karayiannakis AJ, et al. Tissue expression of the proteins Fas and Fas ligand in colorectal cancer and liver metastases. J Gastrointest Cancer 2012;43:224–8.
2. Smith FJD. The molecular genetics of keratin disorders. Am J Clin Dermatol 2003;4:347–64.
3. Morsi HM, Leers MP, Jäger W, et al. The patterns of expression of an apoptosis-related CK18 neoepitope, the bcl-2 proto-oncogene, and the Ki67 proliferation marker in normal, hyperplastic, and malignant endometrium. Int J Gynecol Pathol 2000;19:118–26.
4. Kadyrov M, Kaufmann P, Huppertz B. Expression of a cytokeratin 18 neo-epitope is a specific marker for trophoblast apoptosis in human placenta. Placenta 2001;22:44–8.
5. ILoo DT. In situ detection of apoptosis by the TUNEL assay: an overview of techniques. Methods Mol Biol 2011;682:3–13.
6. Leers MP, Kölgen W, Björklund V, et al. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitopc exposed during early apoptosis. J Pathol 1999;187:567–72.
7. Duan WR, Garner DS, Williams SD, et al. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. J Pathol 2003;199:221–8.
8. Backus HJ, Van Groeningen CJ, Vos W, et al. Differential expression of cell cycle and apoptosis related proteins in colorectal mucosa, primary colon tumours, and liver metastases. J Clin Pathol 2002;55:206–11.
9. Sugita J, Ohtani H, Mizoi T, et al. Close association between Fas ligand (FasL; CD95L)-positive tumor-associated macrophages and apoptotic cancer cells along invasive margin of colorectal carcinoma: a proposal on tumor-host interactions. Jpn J Cancer Res 2002; 93:320–8.
10. Koelink PJ, Lamers CB, Hommes DW, Verspaget HW. Circulating cell death products predict clinical outcome of colorectal cancer patients. BMC Cancer 2009;9:88.
11. Kramer G, Erdal H, Mertens HJ, et al. Differentiation between cell death modes using measurements of different soluble forms of extracellular cytokeratin 18. Cancer Res 2004;64:1751–6.
12. Koornstra JJ, De Jong S, Hollema H, et al. Changes in apoptosis during the development of colorectal cancer: a systematic review of the literature. Crit Rev Oncol Hematol 2003;45:37–53.
13. Bivén K, Erdal H, Hägg M, et al. A novel assay for discovery and characterization of pro-apoptotic drugs and for monitoring apoptosis in patient sera. Apoptosis 2003;8:263–8.

14. Linder S, Havelka AM, Ueno T, Shoshan MC. Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. Cancer Lett 2004;214:1–9.
15. Debucquoy A, Goethals L, Libbrecht L, et al. Molecular and clinico-pathological markers in rectal cancer: a tissue micro-array study. Int J Colorectal Dis 2009;24:129–38.
16. Maher S, Toomey D, Condron C, Bouchier-Hayes D. Activation-induced cell death: the controversial role of Fas and Fas ligand in immune privilege and tumour counterattack. Immunol Cell Biol 2002;80:131–7.
17. Koornstra JJ, Rijcken FE, de Jong S, et al. Assessment of apoptosis by M30 immunoreactivity and the correlation with morphological criteria in normal colorectal mucosa, adenomas and carcinomas. Histopathology 2004;44:9–17.
18. Houston A, O'Connell J. The Fas signalling pathway and its role in the pathogenesis of cancer. Curr Opin Pharmacol 2004;4: 321–6.
19. O'Connell J, Bennett MW, Nally K, et al. Altered mechanisms of apoptosis in colon cancer: Fas resistance and counterattack in the tumor-immune conflict. Ann N Y Acad Sci 2000;910:178–95.