Expression of MAC30 protein is related to survival and biological variables in primary and metastatic colorectal cancers

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Abstract. MAC30 is highly expressed in several types of tumors including colorectal cancers, however, its clinicopathological and biological significance in colorectal cancers is currently not known. The aim of our study was to investigate MAC30 expression in distant normal mucosa, adjacent normal mucosa, primary tumors and metastases of colorectal cancer, and to determine the relationship between MAC30 expression and clinicopathological and biological variables. MAC30 expression was immunohistochemically examined in distant normal mucosa (n=54), adjacent normal mucosa (n=123), primary tumors (n=217) and lymph node metastases (n=56) from colorectal cancer patients. MAC30 cytoplasmic expression was increased from distant normal mucosa to primary tumor and to metastasis ($p<0.0001$-0.04). Furthermore, 40% primary and 37% metastatic tumors showed stronger cytoplasmic expression of MAC30 at the tumor invasive margins compared to inner tumor areas. Strong cytoplasmic expression of MAC30 in the metastasis was related to a poor prognosis ($p=0.04$). MAC30 cytoplasmic expression was positively related to expression of proliferating cell nuclear antigen ($p=0.04$), p53 ($p=0.04$), nucleoporin 88 ($p=0.001$), legumain ($p=0.004$) and particularly interesting new cysteine-histidine rich protein ($p=0.004$). However, MAC30 expression in the nucleus and stroma did not have any clinicopathological and biological significance ($p>0.05$). In conclusion, MAC30 protein may play a role in development of colorectal cancer, and can be considered as a prognostic factor.

Introduction

The meningioma associated protein (MAC30) gene is located on 17q11.2, having a small segment of similarity to an apical gut membrane poly-protein of Haemonchus contortus to olfactory receptor 30 of Mus musculus and to cytochrome b in several organisms. However, there is no real sequence homology to any human gene (1).

MAC30 mRNA is expressed as a non-erythropoietic gene in the fetal liver, but not in the adult liver, suggesting a possible role in growth and differentiation of liver. The identification of MAC30 in the fetal liver is closely linked to the early hematopoietic period (10-12 weeks). This may suggest that MAC30 is one of the developmentally regulated proteins that are expressed in the hematopoietic development (2). MAC30 was first described to be overexpressed in meningiomas, and altered expression was also found in different types of human tumors (1-3). MAC30 is expressed at moderate levels in normal pancreatic acinar cells, strongly present in tubular complexes of chronic pancreatitis, and observed at low levels in most pancreatic cancer cells. The loss or reduction of MAC30 expression in pancreatic cancer suggests that this gene acts as a tumor suppressor in pancreatic cancer (3). In contrast to pancreatic cancer, the expression of MAC30 is stronger in breast, stomach and colon cancers than corresponding normal tissues (3), indicating the importance of MAC30 expression in different types of malignancies.

MAC30 mRNA expression is down-regulated in human fibroblasts in response to serum, which is known to provide growth factors to the cells which are mandatory for cell proliferation (4). MAC30 expression is also down-regulated by c-jun N-terminal kinase antisense oligonucleotides in human PC3 prostate carcinoma cells in conjugation with growth suppression and induction of apoptosis in these cells (5). MAC30 expression is down-regulated by p53, thus being a potential transcriptional target of p53 (6). In contrast, MAC30 expression is induced by other genes, such as BRCA1, which could lead to an up-regulation of MAC30 in certain human malignancies such as breast cancer (7).

Kayed *et al* (3) observed that MAC30 mRNA was over-expressed in colon cancers compared to normal colon mucosa. However, the relationship between MAC30 expression in primary colorectal tumors and metastases, and its clinicopathological and biological significance was not investigated. The aim of the present study was to investigate MAC30 expression in distant normal mucosa, adjacent normal mucosa,

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primary tumors and metastases in the lymph nodes of colorectal cancer, and further to determine the relationship between MAC30 expression and various clinicopathological and biological variables.

Materials and methods

Patients and tissue samples. Samples of paraffin-embedded tissue used for immunohistochemical staining were obtained from 217 patients with primary colorectal adenocarcinoma who underwent surgical resection at Linköping University Hospital, Linköping, and Vrinnevi Hospital, Norrköping, Sweden. The study also included 54 distant normal mucosa specimens, which were histologically free from tumor (51 corresponding to the primary tumors, i.e. distant normal mucosa and primary specimens from the same patients), taken from the margin of distant resection, 56 metastases in the regional lymph nodes (53 corresponding to the primary tumors) and 123 adjacent normal mucosa samples (normal mucosa adjacent to the corresponding primary tumor). The patient's gender, age, tumor location, and Dukes' stage were obtained from surgical and pathologic records from the Linköping University Hospital and Vrinnevi Hospital. The mean age was 71 years (range, 34-94). The growth pattern was classified as expansive or infiltrative, based on the patterns of growth and invasiveness. Differentiation was graded as good, moderate, or poor. Inflammatory infiltration was graded as weak or strong. The extent of necrosis was classified as <10% or >10%. All patients were followed up until the end of 2004, the mean and median follow-up time was 79 and 50 months (range, 0.07-273 months), by which time 94 patients had died from colorectal cancer. Proliferating cell nuclear antigen (PCNA), p53, nucleoporin 88 (Nup88), legumain, and particularly interesting new cysteine-histidine rich protein (PINCH) expression, determined by immunohistochemistry and Western blotting methods, were taken from previous studies carried out at our laboratory (8-12).

Immunohistochemical staining. The sections (5 μm) were incubated at 60˚C overnight, deparaffinized in xylene, and rehydrated with graded ethanol and distilled water. Thereafter the sections were placed in 0.01 M citrate buffer (pH 6.0) and then boiled for 10 min in a microwave oven at 750 W, and kept at room temperature for 30 min. In order to block the endogenous peroxidase activity, the sections were incubated with 3% H_2O_2-methanol for 20 min and washed with phosphate-buffered saline (PBS, pH 7.4). Then the sections were further treated with protein block solution (Dako) for 10 min in order to avoid non-specific binding of the antibody. After removing the blocking solution, the sections were incubated with a monoclonal antibody, MAC30 1-19 (3), diluted 1:5 in antibody diluent (Dako) at 4˚C overnight. The sections were then rinsed with PBS and incubated with an anti-rabbit/mouse labeled polymer HRP, coupled with peroxidase for 30 min at room temperature followed by rinsing with PBS buffer. The peroxidase reaction was performed for 1 min using a Dako chemate envision detection kit (Dako), by mixing a 50:1 ratio of substrate buffer and 3, 3'-diaminobenzidine chromogen (containing 0.05% 3, 3'-diaminobenzidine tetrahydrochloride in organic solvent). The sections were finally counterstained with hemotoxylin. The sections known to stain positively were included as negative and positive controls. For negative controls, the sections incubated with PBS instead of the primary antibody were not stained, whereas positive controls were stained.

The slides were microscopically examined and scored independently by two investigators without any clinical or pathological information. To avoid artifacts, the areas with poor morphology, margins of the sections, and necrosis were not considered. MAC30 expression was observed in the cytoplasm of normal epithelial cells and tumor cells. The staining intensity in the entire tumor area was scored as weak, moderate or strong. According to the similarities of clinicopathological features, in statistical analysis, weakly and moderately stained cases were considered as a weakly stained group and compared with the strongly stained group. MAC30 expression was also observed in the nucleus of normal epithelial cells and tumor cells, scored as negative or positive, irrespective of the intensity of staining. Cytoplasmic expression of MAC30 in the invasive margins of tumor was scored as equal, lower or higher expression when compared to the inner tumor area. Stromal expression of MAC30 in primary tumors was considered as weak or strong. Among a total of 327 cases evaluated, there was discordance in 42 sections in the first round of evaluation. These sections were re-evaluated by the two authors individually three times and matched. The final 8 discrepant sections were re-examined by dual microscope and a concurrent score was achieved.

Statistical analysis. The significance of the difference in the intensity of MAC30 expression between normal mucosa and primary tumor and metastasis was tested by χ^2 analysis or McNemar's method. The relationship between MAC30 expression and clinicopathological/biological factors was examined by the χ^2 method. The relationship between MAC30 expression and survival was tested using Cox's proportional hazard model with the STATISTICA 6.0 program. All p-values mentioned are two sided, and p-values <0.05 were considered as statistically significant.

Results

MAC30 expression in distant normal mucosa, adjacent normal mucosa, primary tumors and metastasis. MAC30 expression was examined in 54 distant normal mucosa samples, 217 primary tumors, of which 123 had adjacent normal mucosa, and 56 metastases in the lymph nodes. Fig. 1 displays MAC30 cytoplasmic expression in normal mucosa, primary tumor, and metastasis in the lymph node. Strong cytoplasmic expression of MAC30 was observed in 28% of distant normal mucosa specimens, 45% of adjacent normal mucosa specimens, 55% of primary tumors, and 86% of metastases. MAC30 cytoplasmic expression was significantly increased from distant to adjacent normal mucosa (p=0.03), from distant normal mucosa to primary tumors (p<0.0001), and from primary tumors to metastases (p<0.0001). The expression tended to be increased from adjacent normal mucosa to primary tumors (p=0.07).

Furthermore, in the matched cases (both distant normal mucosa and primary tumor specimens from the same patients) MAC30 cytoplasmic expression was also increased from

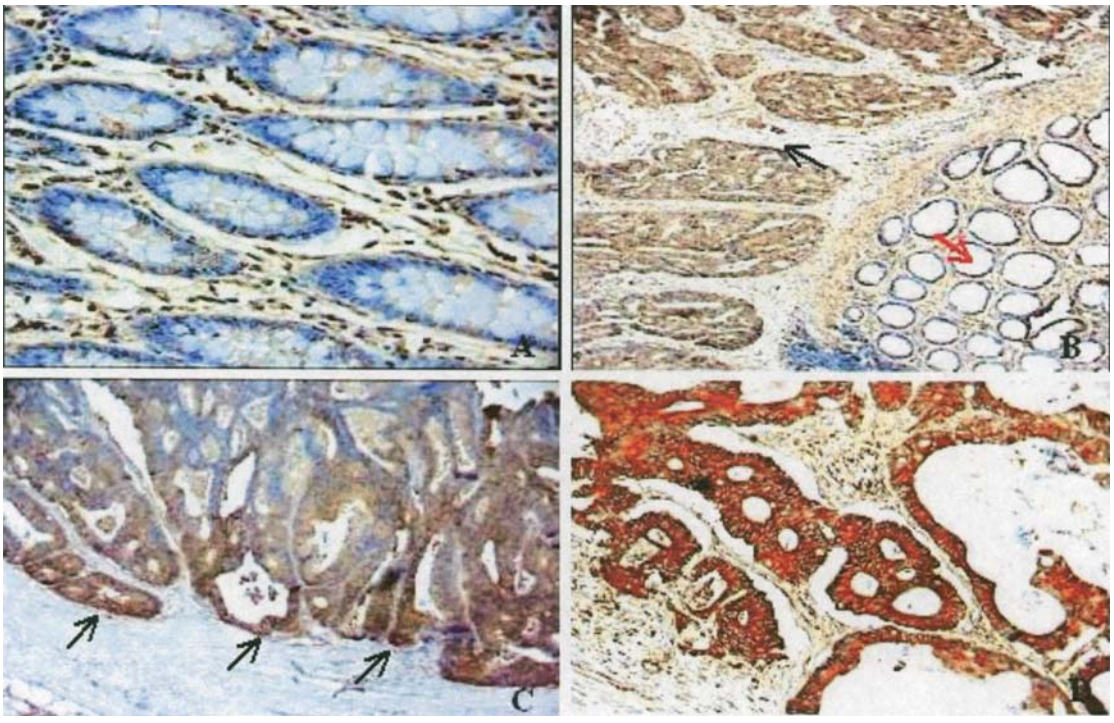

Figure 1. Cytoplasmic expression of MAC30 was weak in distant normal epithelial cells (A), moderate in primary tumor (black arrow) and weak in adjacent normal epithelial cells (red arrow) (B), stronger at the invasive margin (arrows) than in the inner part of the primary tumor (C), and highly strong in metastasis of the lymph node (D).

Table I. MAC30 cytoplasmic expression in distant normal mucosa in relation to the corresponding primary tumor.

	Primary tumor			
Distant normal mucosa	Weak (%)	Strong (%)	Total (%)	p-value
Weak	23 (45)	15 (29)	38 (74)	
Strong	5 (10)	8 (16)	13 (26)	0.04
Total (%)	28 (55)	23 (45)	51 (100)	

Table II. MAC30 cytoplasmic expression in primary tumor in relation to the corresponding metastasis in the lymph nodes.

	Metastasis			
Primary tumor	Weak (%)	Strong (%)	Total (%)	p-value
Weak	4 (7)	28 (53)	32 (60)	
Strong	3 (6)	18 (34)	21 (40)	<0.0001
Total (%)	7 (13)	46 (87)	53 (100)	

distant normal mucosa to primary tumors. As shown in Table I, among 51 cases, 15 (29%) showed weak expression in distant normal mucosa but strong expression in the corresponding primary tumors, whereas only 5 (10%) cases showed the opposite staining pattern (p=0.04). In the remaining cases (23+8, 61%) showed identical expression at both sites. MAC30 cytoplasmic expression was also increased from primary tumor to metastasis in the matched cases (primary and metastatic tumor from the same patients). As shown in Table II, 28 out of 53 cases (53%) showed weak expression in primary tumors but strong expression in the corresponding metastases, while only 3 (6%) cases showed the opposite staining pattern (p<0.0001). In the remaining cases (4+18, 41%) showed identical expression at both sites. However, there was no significant change in the expression between distant and adjacent normal mucosa (p=0.75, data not shown), as well as between adjacent normal mucosa and primary tumor (p=0.61, data not shown) in the matched cases. Considering the invasive margin, 41 (40%) primary tumors and 21 (37%) metastases showed stronger cytoplasmic expression at the invasive margins compared to inner tumor areas.

We also examined the nuclear and stromal expression of MAC30. Positive nuclear expression was observed in 20% of distant normal mucosa specimens, 6% of adjacent normal mucosa specimens and 11% of metastases, and there was no nuclear staining in primary tumors. Strong stromal expression of MAC30 was seen in 81% of primary tumors. We did not examine stromal expression in normal mucosa and metastasis due to small stromal areas. We further analyzed the relationship of these staining patterns (i.e. cytoplasmic, nuclear and stromal staining) in the different sites. In adjacent normal mucosa and metastasis, most of the cases that had strong cytoplasmic expression did not have nuclear expression (p<0.0001, data not shown). Among primary tumors, many cases had both

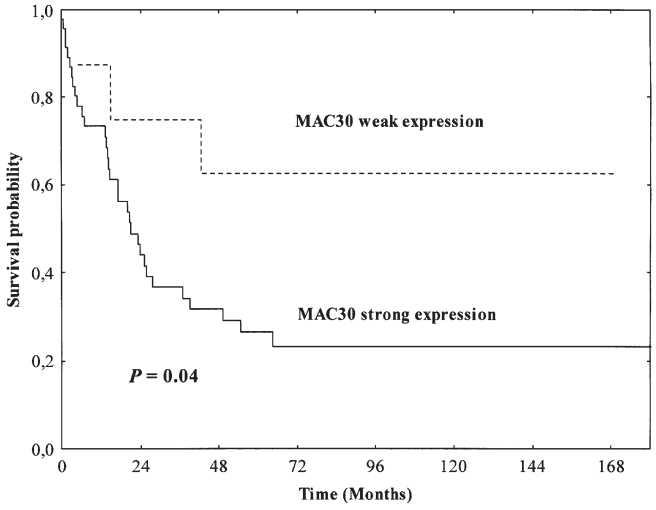

Figure 2. MAC30 cytoplamic expression in metastasis of the lymph node was related to survival in colorectal cancer patients.

Table III. MAC30 cytoplasmic expression in primary tumors in relation to biological variables in colorectal cancer.

	MAC30 expression		
Biological variables	Weak (%)	Strong (%)	p-value
PCNA			
<25%	38 (51)	37 (49)	0.04
≥25%	22 (33)	44 (67)	
p53			
Weak	55 (47)	64 (53)	0.04
Strong	5 (23)	44 (77)	
Nup88			
Weak	48 (49)	50 (51)	0.001
Strong	8 (19)	35 (81)	
Legumain			
Weak	26 (55)	21 (45)	0.004
Strong	21 (29)	52 (71)	
PINCH			
Weak	28 (53)	25 (47)	0.004
Strong	19 (27)	51 (73)	

weak cytoplasmic expression and strong stromal expression (p<0.0001, data not shown).

Relationships of MAC30 expression with clinicopathological variables and biological variables. MAC30 cytoplasmic expression in metastasis in the lymph node was related to survival, i.e. patients who had tumors with strong cytoplasmic expression showed a worse prognosis compared to patients who had weakly expressed tumor (p=0.04, Fig. 2). There was no significant relationship between cytoplasmic MAC30 expression in primary tumors and clinicopathological variables including gender, age, tumor location, Dukes' stage, growth pattern, differentiation, inflammatory infiltration, necrosis and survival (p>0.05, data not shown).

We analyzed MAC30 expression in the cytoplasm, nucleus and stroma in primary tumors in relation to biological variables including PCNA, p53, Nup88, legumain and PINCH. As shown in Table III, strong cytoplasmic expression of MAC30 positively correlated with the expression of PCNA (p=0.04), p53 (p=0.04), Nup88 (p=0.001), legumain (p=0.004) and PINCH (p=0.004).

MAC30 expression in the nucleus or stroma was not associated with any clinicopathological and biological variables (p>0.05, data not shown).

Discussion

A previous study showed that MAC30 is highly expressed in primary colon cancers compared to normal colon mucosa obtained from healthy individuals (3). In the present study, we demonstrated significantly increased MAC30 expression in the cytoplasm from distant normal mucosa to primary tumors, and further from primary tumors to metastases in both unmatched and matched cases. The expression tended to be increased from adjacent normal mucosa to primary tumors. We also observed strong MAC30 expression at the invasive margins of primary and metastatic tumors compared to inner tumor areas. More importantly, we found that strong cytoplasmic expression of MAC30 in metastasis of the lymph node was related to an unfavorable outcome of the patients. Taken together, these findings suggest that MAC30 may act as an oncogene in colorectal cancer and might play a role in tumor development and aggressiveness.

MAC30 expression positively correlated with the exprression of PCNA, p53, Nup88, legumain and PINCH on the same series of colorectal cancers studied previously by us (8-12). p53, as a tumor suppressor, and PCNA, as a member of cell cycle checkpoint proteins, play important roles in the inhibition of cell proliferation and induction of apoptosis (13). MAC30 is a primary transcriptional target of p53, hence MAC30 expression is down-regulated by p53 (6). One could speculate that overexpression of MAC30 in colorectal cancer is a result of p53 mutations in as much as there was a positive correlation between MAC30 expression and p53 overexpression (indicating p53 mutations). Nup88 (a member of nucleoporing complex proteins) abundant expression in tumor has high proliferative activity and low apoptotic activity, explained by the increased nucleocytoplasmic transport required for protein transportation in tumor cells (10,14-16). Legumain (a cysteine endopeptidase) expressed both intracellular and tumor-associated endothelial cells, where it is co-localized with integrins (17). Legumain overexpression possesses increased migratory and invasive activity *in vitro* and adopts an invasive and metastatic phenotype *in vivo*, inferring the significance of legumain in tumor invasion and metastasis (17). We previously demonstrated a positive relationship between overexpression of legumain and overexpression of p53 and PCNA (11). These findings together, the positive relationship of MAC30 cytoplasmic expression with PCNA, p53, Nup88 and legumain expression, indicate that MAC30 potentially interacts with the factors involved in

the cell proliferation, apoptosis and invasiveness of colorectal cancer.

PINCH is the protein involved in stromal related tumor development and aggressiveness. In colorectal cancer, the abundance of PINCH protein in the stroma increases from normal mucosa to primary tumor and to metastasis in the lymph nodes, and it is more intense at the invasive margin than in intratumoral stroma (12). PINCH plays a key role in the convergence point between integrin and growth factor signal transduction. There was a positive correlation between MAC30 and PINCH indicating that MAC30 might also play a role in the signal transduction pathway related to PINCH expression. The findings reported here suggest that MAC30 cytoplasmic expression was not only related to biological variables present in the cytoplasm (Nup88 and legumain) and nucleus (PCNA, p53 and Nup88) of tumor cells, but was also related to variables (legumain and PINCH) present in the stroma.

MAC30 expression was also present in the nucleus and stroma, but there was no correlation of MAC30 expression on these localizations with clinicopathological and biological variables. Several lines of evidence indicate that the observed nuclear and stromal MAC30 staining was specific. First, the utilized negative controls did not show nuclear or stromal staining. Secondly, there was strong cytoplasmic expression of MAC30 with absent nuclear expression in both the adjacent normal mucosa and metastasis. Thirdly, weak cytoplasmic expression was often associated with strong stromal expression in primary tumors. However, the significance of nuclear and stromal MAC30 localization is currently not known.

In conclusion, the cytoplasmic expression of MAC30 was much stronger in lymph node metastasis compared to primary tumor and normal mucosa, and was related to patient survival. Together with the higher expression of MAC30 at the invasive margin of primary and metastatic tumor, our findings suggest that MAC30 protein may play an important role in the development and aggressiveness of colorectal cancer.

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