Loss of Smad4 expression predicts liver metastasis in human colorectal cancer

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Abstract. Distant metastases represent the major cause of death after curative surgery of colorectal cancer. The aim of this study was to evaluate the role of Smad4 and KRAS genetic alterations in colorectal metastases taking into account both the site (hepatic versus extrahepatic) and the time (synchronous versus metachronous) of recurrence. We examined the immunohistochemical expression of Smad4 and frequency of KRAS mutation in primary colorectal tumors and in their corresponding metastatic tissues. Loss of Smad4 expression was noted in 37% (26/71) of the primary tumors and the corresponding metastases. Absence of Smad4 protein was more frequently observed in hepatic metastases, whether they were metachronous or synchronous, than in extrahepatic metastases (p<0.005). The frequency of KRAS mutations was high in the synchronous and extrahepatic metachronous metastases (68-80%), but was significantly lower in the hepatic metachronous metastases (11%). Our results indicate that absence of Smad4 expression correlated significantly with liver metastases regardless of the time of their occurrence and represents a promising new biomarker to predict liver metastasis in colorectal cancer patients. Therefore, this group of patients could benefit from a specific and appropriate pre- and/or post-operative therapy.

Introduction

Distant metastases, by hematogenous or to a lesser extent by lymphatic vessels, represent the major cause of death after curative surgery of colorectal cancer (CRC). Liver is the most common site of CRC metastases compared to other organs such as lung, brain or bone (1). Hepatic metastases are present in 30-50% of patients, and liver is frequently the sole organ harboring metastases. To improve the prognosis of CRC, the most important considerations are the selection of high-risk patients to administer the appropriate therapy.

Several studies have attempted to find genetic markers that give accurate information on the prognostic impact for patient with CRC. Among these markers, KRAS and Smad4 (DPC4) have been extensively investigated. Oncogenic mutation in KRAS is one of the most common genetic alterations in colorectal cancer and considered an early event in colorectal tumorigenesis (2). Regarding the prognostic significance of KRAS gene mutations in colorectal cancer, there is a lack of agreement on how mutations relate to clinical factors. Some groups have suggested that the presence of any KRAS mutation conveys prognostic significance (3-6), whereas others have reached contrary conclusions (7-9).

Smad4 is expressed ubiquitously in different human organ systems. The gene product is an important cellular mediator of TGF-ß signals relevant for development and control of cell growth. Smad4 forms a complex with Smad2 and Smad3, and translocates to the nucleus where it activates transcription of multiple TGF-ß response genes (10). Smad4 mutations have been shown to be associated with the occurrence of juvenile polyposis (11) and frequent somatic mutations have been found as a late event in colorectal cancers, further suggesting an important role for this gene in colorectal carcinogenesis (12,13). Many studies showed that Smad4 alterations were more frequent in advanced colorectal disease and in metastatic cancers (13-18).

Some genetic abnormalities could be specifically required for the development of metastatic tumor cells in function of the recipient organ. In a recent study, we analyzed colorectal metachronous metastases and we found a good correlation between some of the genetic alterations identified in the tumor cells and the location of the metastases (19). However, the precise mechanism for the homing of colorectal metastases and the selected metastatic organ remains largely unknown.

In the present study, we examined the frequency of Smad4 and KRAS alterations in different groups of patients with CRC and developing metastases. We verified the role of these genetic changes in the different metastatic processes taking into account the mode of recurrence, regarding both the site (hepatic versus extrahepatic) and the time (synchronous versus metachronous), with the aim to select appropriate biomarkers useful for predicting metastases from colorectal cancers.

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Table I. Clinicopathological characteristics of patients who developed metastases after radical curative surgery for colorectal cancer or metastases present at the time of surgery.

| | Metachronous metastases | | Synchronous metastases | | |
	Extrahepatic (Group 1)	Hepatic (Group 2)	Extrahepatic (Group 3)	Hepatic (Group 4)	Total
Gender					
Male	10	11	5	13	39
Female	10	8	5	9	32
Age	46-81 (61.4)	50-84 (62)	46-77 (65.7)	51-80 (64.9)	46-84
Tumor location					
Proximal	4	4	1	8	17
Distal	15	11	6	12	44
Rectum	1	4	3	2	10
Differentiation					
Well	4	4	1	2	11
Moderate	15	11	7	17	50
Poor	1	4	2	3	10
Dukes' stage					
B	11	7	0	0	18
C	9	12	0	0	21
D	0	0	10	22	32
Adjuvant therapy					
Yes	7	7	8	21	43
No	13	12	2	1	28

Materials and methods

Patients and tissue specimens. The surgical specimens were obtained from a total of 91 patients who were treated surgically for primary sporadic CRC between 1993 to 2002. The cases were selected from the Pathology Department of the University of Modena and Reggio Emilia and from the Institute of Pathology of Lausanne. Fourteen patients received adjuvant post-operative 5-FU chemotherapy. Seventy-one patients developed metastases after (Groups 1 and 2) or at the time (Groups 3 and 4) of surgery.

Group 1: 20 patients with distant extrahepatic metastases resected 4-79 months after the primary tumor. The analyzed metastases were from the lung (14), the stomach (1), the thyroid (1) and the bone (4).

Group 2: 19 patients with distant liver metastases resected 4-51 months after the primary tumor.

Group 3: 10 patients with extrahepatic distant metastases to lung (6), bone (2) or brain (2), resected at the time of surgery.

Group 4: 22 patients with distant liver metastases resected at the time of surgery.

Group 5: 20 CRC patients with stage B and C. This group consists of patients disease-free at a 5-years follow-up obtained through direct contact, clinical charts and pathological records. There were 10 men and 10 women with a mean age of 64.4 years (range 45-79). The tumor was localized in the proximal

colon in 6 cases, distal colon in 9 cases and rectum in 5 cases.

Single-strand conformation polymorphism (SSCP) analysis of the KRAS gene. Genomic DNA was extracted from fixed materials using the DNeasy tissue kit (Qiagen, Hilden, Germany). The DNA was eluted from the column with 60 μl of elution buffer and kept at -20˚C until use. Exon 1 of the KRAS gene was amplified by PCR and mutation were detected by SSCP, as previously described (19).

Immunohistochemistry. Sections (4 μm) were cut from paraffin blocks and deparaffinized using standard methods. Slides were treated with sodium citrate and steamed for 30 min at 80˚C. After cooling for 5 min, the slides were labeled with a monoclonal antibody specific to Smad4 (clone B8, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100. The antibody was detected using a biotinylated secondary antibody and 3,3'-diaminobenzidine as the chromogen. Then, the slides were hematoxylin counterstained.

For Smad4, positive labeling was defined as strong to moderate staining of the cytoplasm of cells, with focal expression in the nuclei. Absence of expression was interpreted as negative. Alveolar and bronchiolar cells in the lung, hepatocytes in the liver, fibroblasts and lymphocytes, all showing moderate to strong expression of the Smad4 gene, served as
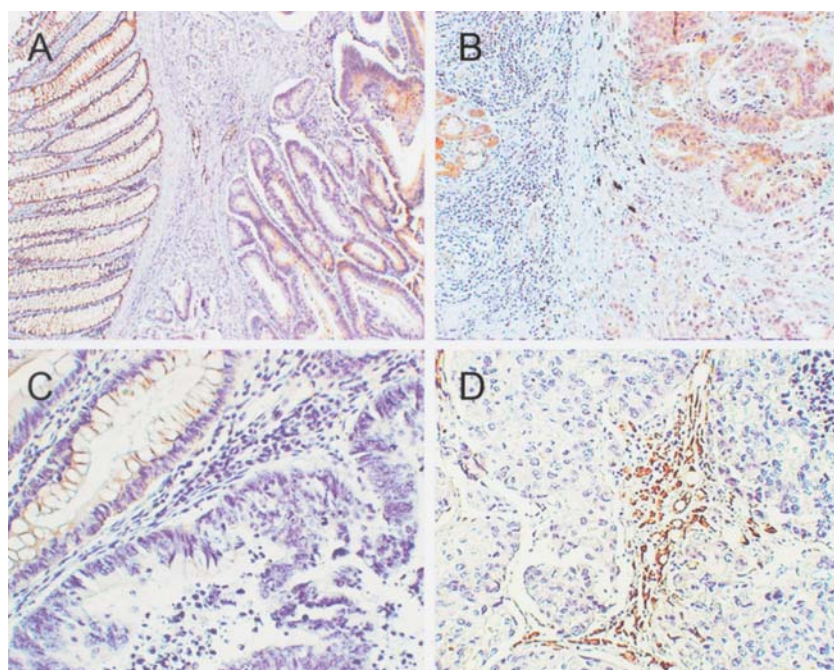
Figure 1. Smad4 immunohistochemistry performed on primary CRC (A and C) and their corresponding metastases (B and D). Positive cytoplasmic expression of Smad4 observed in a primary CRC and its corresponding lung metastasis (A and B, original magnification x10). In another case, loss of Smad4 expression can be noted in the primary CRC and in the liver metastasis (C and D, original magnification x20).

Table II. Genetic alterations in metastases developed after radical curative surgery for colorectal cancer (metachronous metastases) or at the time of surgery (synchronous metastases).

| | Metachronous metastases | | Synchronous metastases | | |
	Extrahepatic (Group 1)	Hepatic (Group 2)	Extrahepatic (Group 3)	Hepatic (Group 4)	Total
KRAS mutation	75% (15/20)	11% (2/19)	80% (8/10)	68% (15/22)	56% (40/71)
Loss of Smad4	15% (3/20)	53% (10/19)	10% (1/10)	55% (12/22)	37% (26/71)

internal positive controls. The primary antibody was omitted in the negative controls.

Statistical analysis. The Fisher's exact test was used to evaluate the association between two dichotomous variables. A p-value of <0.05 was considered to indicate statistical significance.

Results

The clinical and pathological data of the 71 colorectal cancer patients, having developed either metachronous or synchronous metastasis, are summarized in Table I.

The expression of Smad4 was similar in the primary tumors and the corresponding metastases. Loss of Smad4 expression was noted in 37% (26/71) of the cases. Fig. 1 shows immunohistochemistry of Smad4 with either positive or negative cytoplasmic staining of primary tumor cells and corresponding metastatic tumor cells. Absence of Smad4 was more frequently observed in hepatic metastases, whether they were metachronous or synchronous (53 and

55%, respectively), than in metachronous and synchronous extrahepatic metastases (15 and 10%, respectively) (Table II). The difference was statistically significant ($p<0.005$). In the comparative group of 20 non-metastatic patients, Smad4 expression was absent in only 3 tumors (15%), an occurrence similar to those observed in the extrahepatic metastatic group.

SSCP analysis revealed 40 of 71 (56%) cases with shifted bands for the KRAS exon 1. In all but one case, the same mutation was identified in the primary tumor and in the corresponding metastasis. Mutations occurred preferentially at the second base of codon 12 (27/40, 68%) and were GGT to GTT or GAT or GCT, in 17, 6 and 4 cases, respectively. Other mutations were observed at the first base of codon 12 in 6 cases and at the second base of codon 13 (GGC to GAC) in the last 7 cases. KRAS mutation occurred at a high level in the extrahepatic synchronous and metachronous metastases (80 and 75%, respectively), as well as in hepatic synchronous metastases (68%). On the contrary, the level of KRAS mutation was significantly lower in hepatic metachronous metastases (11%, 2/19 cases) ($p<0.0001$) (Table II).

Discussion

In the present study, we investigated the association of Smad4 and KRAS alterations with the location (hepatic versus extrahepatic) and the time of recurrence (synchronous versus metachronous) of distant metastasis with the aim to identify powerful biomarkers for predicting metastasis of colorectal cancers. This could permit the selection of patients who may be candidates for an appropriate chemotherapy pre- and/or postoperative.

Loss of Smad4 expression was more frequently observed in synchronous and metachronous liver metastases than in other distant metastases. The expression of Smad4 was similar in the primary CRC and the corresponding metastases. As observed in tumors with extrahepatic metastases, loss of Smad4 expression was also uncommon in the primary CRC without distant metastases. Therefore, absence of Smad4 expression in the primary colorectal cancer cells was significantly correlated with liver metastasis occurrence.

Smad4 is a central component of the TGF-ß/Smad pathway. Deletion or degradation of Smad4 in tumors could specifically inhibit the tumor suppressor effect of TGF-ß (20). Smad4 alteration has been associated with specific loss of TGF-ß-induced growth resulting in increased angiogenesis and loss of epithelial integrity (21,22). Studies analyzing cancers at different stages show Smad4 inactivation to be associated with the advanced disease state in colorectal tumorigenesis (13,14). Our results confirm that Smad4 is downregulated during tumor progression. Nevertheless, many studies that evaluated the prognostic value of Smad4 expression using immonohisto-chemical techniques have found Smad4 expression decreased in metastatic cancers, but did not take into account the mode of recurrence (14,16,17). In our study, we focused our attention on the different types of metastasis regarding both the site and the time of recurrence. According to our data, Smad4 was associated with the occurrence of liver metastasis in CRC patients regardless of the time of the recurrence, suggesting that loss of Smad4 may specifically contribute to the establishment of colorectal cancer liver metastasis.

Metastasis consists of a series of sequential steps that include the shedding of cells from a primary tumor into the circulation, survival of the cells in the circulation, arrest in a new organ, extravasation into the surrounding tissue, initiation and maintenance of growth, and vascularization of the metastatic tumor (23). Proteome analyses have detected Smad4 targets relevant for tumor-stroma interactions; the Smad4-negative cells produced a major group of proteins of the extracellular matrix, characterized as anti-adhesive and invasion-promoting proteins (24). The stroma micro-environment of target organ for metastasis has the ability to produce adhesion molecules, integrins, stromal metallo-proteinases and angiogenic molecules that facilitate the anchorage of tumor cells and promote tumor growth and angiogenesis (25,26). It has been observed that alteration of TGF-ß, an angiostatic molecule, may increase vascularization of metastases and thus, promoting their growth (27). Therefore, we hypothesized that Smad4, through the TGF-ß/Smad pathway, could play a role either when the cancer cells leave the circulation to extravasate into the liver tissue or for the growing of liver micrometastases. Further studies will be

necessary to understand why neoplastic colon cells with altered Smad4 expression represent a population that is able to adapt and develop with a relatively high specificity in the liver.

Our study reported a high frequency of KRAS mutation in metachronous and synchronous extrahepatic metastases (75 and 80%, respectively), mainly in the lung; whereas the liver metastases showed KRAS mutated prevalently in the synchronous ones (68%). KRAS, at least as a biomarker, seems to play a main part in the metastatic process through blood circulation and thus, would be a good indicator of the potential aggressiveness of colorectal tumor cells (19,28). However, we found a small group (11%) of metachronous liver metastases with KRAS mutation suggesting that KRAS alteration is not mandatory for the development of hepatic metastases or at least of late metastases, and that Smad4 or other genes most likely drive the mechanism of metachronous hepatic metastases.

In conclusion, our data raise the possibility that absence of Smad4 expression, alone or in combination with other conventional pathological factors, could be a surrogate marker for liver metastasis after curative resection of Dukes' B and C CRCs. Altered expression of Smad4, and perhaps other members of the TGF-ß/Smad pathway, in biopsy samples of colorectal cancer may provide clinicians useful information to identify patients with high-risk of liver metastasis for initiating a targeted adjuvant therapy, immediately after or even before surgical treatment of the primary tumor. A large-scaled immunohistochemical study, as well as the analysis of the other genes implicated in the TGF-ß/Smad pathway, will be needed before a definite conclusion can be drawn.

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