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MAP17 overexpression is a common characteristic of carcinomas

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We undertook a large-scale genetic screen to identify genes able to alter the cellular response to physiological signals and provide selective advantage once tumorigenesis has begun. We identified MAP17, a small 17 kDa non-glycosylated membrane protein previously identified, being overexpressed in carcinomas. We found that MAP17 is overexpressed in a great variety of human carcinomas. Immunohistochemical analysis of MAP17 during cancer progression shows, at least in prostate and ovarian carcinomas, that overexpression of the protein strongly correlates with tumoral progression ($P < 0.0001$). Many tumor cells also express MAP17 and its expression does not correlate with expression of SCL, a neighbor gene reported to be co-expressed in some hematopoietic cell lines. SCL neither is expressed in most MAP17-positive tumors, indicating the independent transcription of MAP17, at least in carcinomas. We cloned 5′ genomic region to MAP17 and described the minimal promoter necessary to produce independent activation of MAP17. Moreover, we have found that MAP17 promoter is activated by oncogenes. Taken together, our data show an independent activation of MAP17 promoter that can be driven by oncogenes and that might explain the common overexpression of MAP17 in human carcinomas.

Introduction

Although multiple oncogenes that activate signaling pathways directly involved in cell survival or proliferation have been discovered in the last decades, many other genes that provide advantage to the tumoral cells making them insensitive to physiological signals or altering their normal physiology are still to be found. Functional genetic screens using retroviral delivery of high complexity cDNA libraries constitute a valuable tool to discover new genes involved in the appropriate phenotypic characteristic of the tumorigenic process (1–3). A genome-wide retroviral cDNA screen to search for genes that confer selective advantage during tumorigenesis to cancer cells allowed us to identify MAP17 (4), a small non-glycosylated membrane-associated protein of 17 kDa that locates to the plasma membrane and the Golgi apparatus (4,5). The protein sequence showed a hydrophobic N-terminus of 13 aminoacids encoding a PDZ-binding domain and two *trans*-membrane regions (6–8). MAP17 was first described by differential display overexpressed in carcinomas (6,7). Transfection of full-length wild-type MAP17 into HT29 colon carcinoma cells decreased cell proliferation *in vitro* and tumor growth *in vivo* (7). MAP17 binds several PDZ domain-containing proteins, including PDZK1, NHRF proteins and NaPi-IIa. Overexpression of MAP17 into opossum kidney cells participates, together with NHRF3 and NHRF4, in NaPi-IIa internalization to the *trans*-golgi network (8). In a transgenic mouse model, MAP17 hepatic overexpression resulted in PDZK1 liver deficiency, suggesting that MAP17 is an endogenous regulator of PDZK1 turnover (9). MAP17 acts as an atypical anchoring site for PDZK1 and interacts with the NaPi-IIa–PDZK1 protein complex in renal proximal tubular cells (10). The physiological role of MAP17 in proximal tubules is not known but it stimulates specific Na-dependent transport of mannose and glucose in *Xenopus* oocytes (5) and some human cells (4). MAP17 share regulatory elements with the stem cell leukemia gene (SCL, TAL-1), a basic HLH protein essential in the formation of the hematopoietic lineages (11,12), although, major expression of MAP17 has been found only in kidney among normal tissues.

However, the known data do not provide an explanation why MAP17 could be overexpressed in some carcinoma tumors. In the present work, we studied MAP17 expression through tumors of different origin and found it overexpressed in correlation with the advanced stage of ovarian and prostate carcinoma. Moreover, we found evidence of independent transcription from neighbor SCL gene. Finally, we report independent activation of MAP17 promoter by oncogenes providing a hypothesis to explain its broader distribution among carcinomas.

Abbreviations: RT–PCR, reverse transcription–polymerase chain reaction; TMA, tissue microarray.

Materials and methods

Cell culture

Tumor cells, from the American Type Culture Collection, were maintained each in the recommended medium with glutamax (Gibco) and containing 10% fetal bovine serum (Sigma), penicillin, streptomycin and fungizone.

Reverse transcription–polymerase chain reaction

Total RNA was purified using the TRI-REAGENT (Molecular Research Center, Cincinnati, OH). Reverse transcription (RT) was performed with 5 μg of mRNA using MMLV reverse transcriptase (Promega) and oligodT primer according to the manufacturer's recommendations. The following primers were used to amplify specific cDNA regions: MAP17 forward 5′-CAGCCATGTCGGCCCTCA-3′ and reverse 5′-TTATTTCACAGAAATTAGGGCC-3′; SCL forward 5′-CCGCATGGTGCAGCTGAGT-3′ and reverse 5′-CCCATCACCGAGGGCC-3′; b-actin forward 5′-AGGCCAACCGCGAGAAGATGAC-3′ and reverse 5′-GAAGTCCAGGGCGACGTAGCA-3′. cDNA was subjected to polymerase chain reaction (PCR) under the following conditions (five cycles: 95°C, 1 s; 58°C, 45 min; 72°C, 2 s; 30 cycles: 95°C, 1 s; 62°C, 45 min; 72°C, 2 s), and products were analyzed by 1% agarose gel electrophoresis.

Monoclonal MAP17 antibody was generated from bacterial purified GST-MAP17 protein. Several clonal antibodies were tested for specificity and validated by antigen competition (see supplementary material 1 available at *Carcinogenesis* online). Similar results were obtained with at least other three anti-MAP17 monoclonal antibodies.

RNA array hybridization

Cancer Profiling Array membranes (BD Biosciences) were pre-hybridized with ExpressHyb Hybridization solution during 4 h at 65°C. The appropriate probe (full-length MAP17 cDNA, full-length ubiquitin cDNA, and 654 bp SCL cDNA) was then labeled by PCR with redivue dCTP32 (Amersham). The labeled probe was then purified from free hot nucleotides with a Sepharose G-50 column NickTM (Amersham). The purified probe was then denatured for 3 min at 100°C and added to the hybridization solution. The hybridization was performed overnight at 65°C. Then the membrane was washed at 65°C twice with 2× SSPE, 0.1% sodium dodecyl sulfate; once with 1× SSPE, 0.1% sodium dodecyl sulfate and once more with 0.1× SSPE, 0.1% sodium dodecyl sulfate. The membrane was then exposed to a Biomax MS film (Kodak). As control, the same procedure was repeated with the recommended standard probe for ubiquitin.

Q-RT–PCR

Patients. Normal tissue and tumor tissue from 20 patients with colon carcinoma, 20 patients with prostate carcinoma and 20 patients with lung carcinoma were randomly chosen from the tumor bank at the Pathology Department of Vall d'Hebrón Hospital (Barcelona, Spain). Biopsy samples are routinely collected, quickly frozen and stored at −80°C immediately after surgery. All tumors were histologically examined to confirm the diagnosis of carcinoma. All procedures of the present study have been approved by the Ethics Committee of the Vall d'Hebron Hospital.

Real-time quantitative RT–PCR. Total RNA was extracted from normal and tumor tissue with the RNAeasy mini kit (Qiagen, Hilden, Germany). The RNA nano Lab Chip kit (Agilent, Palo Alto, CA) was used to quantify and determine the integrity of the isolated total RNA. cDNA synthesis was done using random primers with SuperScriptTM II reverse transcriptase (Invitrogen, Carlsbad, CA), and aliquots were stored at −20°C.

Quantitative real-time TaqMan RT–PCR technology (Applied Biosystems, Foster City, CA) was used to determine the differential expression of the selected genes. Relative quantification analysis was performed with the ABI PRISM 7700 instrument (Applied Biosystems). Data were analyzed with sequence detection software (Applied Biosystems). The PCR cycling program consisted of denaturing at 95°C for 10 min and 50 cycles at 95°C for 15 s, and annealing and elongation at 60°C for 1 min.

The primers and TaqMan probe (Applied Biosystems) used to analyze MAP17 were ref. Hs00173779.

Cyclophilin (ref. 4326316E), an endogenous control, was used to normalize variations in cDNA quantities from different samples. Each reaction was performed in triplicate with cDNA from normal and tumor tissue from each patient studied. A blank sample (no DNA) was included in each experiment. A new RNA extraction was randomly performed from the original tissue of some samples and reproducible quantitative real-time PCR results were obtained (data not shown).

Tissue microarray immunohistochemistry

To corroborate the *in vitro* results, immunohistochemical studies were performed in prostate specimens included in a tissue microarray (TMA). This TMA was constructed by standard method using paraffin-embedded tumoral samples kindly provided by the Spanish National Cancer Tumor Bank Network and contains representative samples of prostatic carcinoma, prostatic intraepithelial neoplasia, and benign prostate.

Three-micrometer slices were sectioned from the TMA block and applied to special immunochemistry coated slides (DAKO, Glostrup, Denmark). The slides were baked overnight in a 56°C oven, deparaffinized in xylene for 20 min, rehydrated through a graded ethanol series, and washed with phosphate-buffered saline. A heat-induced epitope retrieval step was performed in a solution of sodium citrate buffer, pH 6.5. The slide was then heated for 2 min in a conventional pressure cooker and after heating was incubated with proteinase K for 10 min and rinsed in cool running water for 5 min. Endogenous peroxide activity was quenched with 1.5% hydrogen peroxide (DAKO) in methanol for 10 min and incubation with the primary antibodies αMAP17 (1:250) was performed (40 min). After incubation, immunodetection was carried out with EnVision (DAKO) visualization system using diaminobenzidine chromogen as substrate, according to manufacturer's instructions.

Immunostaining was performed in a TechMate 500 automatic immunostaining device (DAKO).

Characterization of the antibody is provided in supplementary material 1 available at *Carcinogenesis* online.

Dual-luciferase assays

MDA-MB-468 or Hek293T cells were seeded in six-well plates and cultured during 24 h at 37°C. Then, the medium was changed, and 2 h later, the cells were transiently transfected overnight with the appropriate DNAs (and additionally with phRG-TK vector, a renilla luciferase reporter) using the calcium phosphate protocol. Cells were then washed from the calcium phosphate crystals with phosphate-buffered saline and, if necessary, a glycerol shock (Dulbecco's modified Eagle's medium supplemented with 10% glycerol) was performed during 1 min. Fresh medium was added to the transfected cells and cultured at 37°C. Both cell types were harvested 48 h after transfection and lysed with 500 µl of 1× Passive Lysis Buffer (Promega). The lysates were then centrifuged for 30 s at 13 200 r.p.m., 4°C. Twenty microliters of each sample was then transferred to a 96-well plate and firefly luciferase activity measured with the automated addition of 50 µl of luciferase substrate (Promega) by a Victor II (PerkinElmer Wallac Oy) reader. Second, after the automated addition of 50 µl of Stop & Glo reagent, renilla luciferase activity was measured by the Victor II reader. Firefly luciferase activity was then normalized with Renilla luciferase activity.

Results

MAP17 is overexpressed in tumors from different origins

MAP17 has been reported to be overexpressed in certain carcinomas (6,7). Therefore, we set up to confirm whether MAP17 expression is a marker for tumorigenesis. To assess the overexpression of MAP17 protein in tumors, we analyzed by immunohistochemistry samples from lung, colon, and ovarian tumors. Twenty lung tumors were analyzed, comprising squamous carcinoma, adenocarcinoma, and non-specified Non-small cell lung carcinoma. From these, three squamous and two adenocarcinomas were positive for MAP17

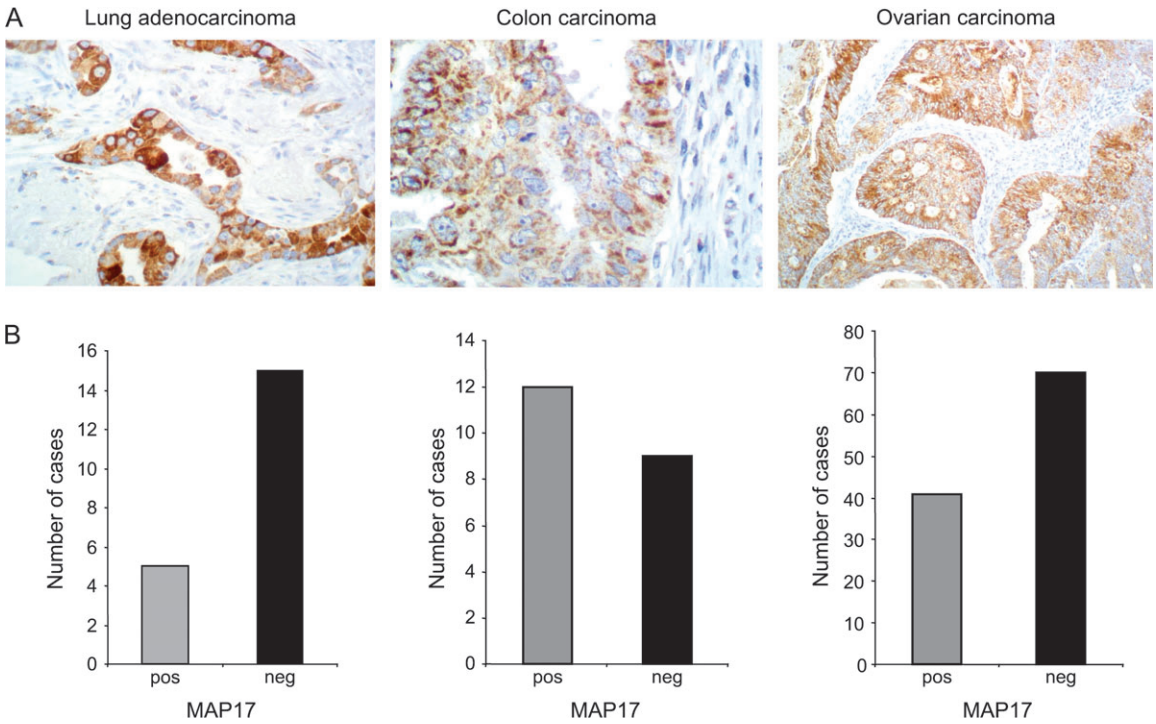

Fig. 1. Immunostaining of tumor samples shows a significant percentage of tumors with high expression of MAP17. Immunohistologic staining of MAP17 in representative samples of lung adenocarcinoma, colon carcinoma and ovarian carcinoma. A section (3 µm) of paraffin-embedded tumor TMA was stained using a monoclonal antibody raised against MAP17. (**A**) Picture shows a representative MAP17-positive tumor. (**B**) Graph shows the cases of each tumor analyzed discriminating among positives and negatives for MAP17 staining. See text for details.

staining (Figure 1A), most of them in T2 or T3 stage. We did also analyze 21 colon carcinomas, from which 12 were positive (57%) (Figure 1B). Finally, we analyzed 111 ovarian carcinomas with different degree of malignity, 41 of them staining positive for MAP17 (36.9%) (Figure 2B). Most MAP17-positive ovarian tumors were malignant tumors (Chi-square test, $P < 0.0001$). Non-tumoral tissue did not show MAP17 staining in lung, colon or ovarian samples.

To confirm whether MAP17 overexpression correlated with an advanced tumor grade, we analyzed the levels of MAP17 expression in samples representing different stages of the same tumor. To that end, we analyzed prostate tissue from prostate cancer patients. We selected tissue from eight patients with different tumor grades, ranging from normal ducts and hyperplasia to advanced carcinoma, and generated a tissue array with all the samples from these patients to homogenize the results. The array was stained with anti-MAP17 antibodies. The analysis of the samples showed that MAP17 expression positively correlated with the stage of the tumor (Figure 2).

Normal prostate ductal cells showed no staining for MAP17, indicating low levels of this protein (Figure 2). However, prostatic intraepithelial neoplasias found in the prostate of the same patient showed increased levels of MAP17 (Figure 2). Moreover, adenocarcinoma lesions from the same patient showed the higher levels of MAP17 staining (Figure 2). This positive correlation between MAP17 levels and the degree of the prostate lesion was observed in seven out of eight patients. The last patient did not show positive staining for MAP17 at any stage.

Our data confirm MAP17 overexpression in different types of cancer and, in some cases such in ovarian and prostate tumors, MAP17 level correlates with the advanced stage of the tumor.

To test whether the MAP17 overexpression in tumoral tissue was produced at transcription level, we performed quantitative analysis of the MAP17 mRNA levels in three selected tumor types: colon, lung and prostate (three types where MAP17 was found to be frequently overexpressed, see Figures 1 and 2). From each tumor type, 20 patients were selected, mRNA extracted from tumoral and normal tissue and MAP17 mRNA quantified in each sample by Q-RT–PCR. In colon cancer, 7 out of 20 patients (35%) showed a clear increase of MAP17 (Figure 3A). This percentage was higher in lung cancer, where 13 out of 20 tumors (65%) showed increased MAP17 expression respect to the normal tissue. In prostate, 9 out of 20 tumors (45%)

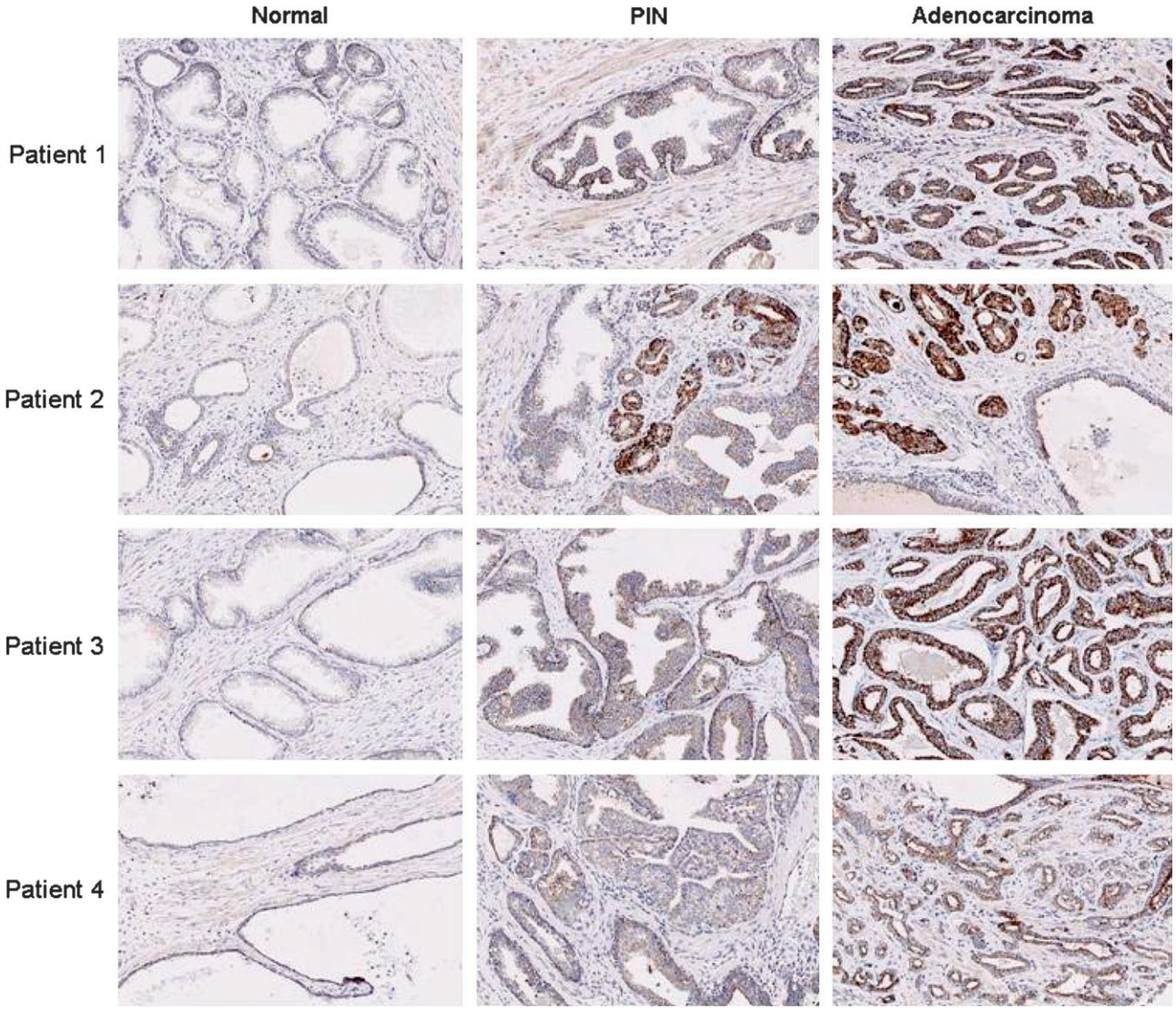

Fig. 2. MAP17 levels correlate with the degree of the tumor in prostatic lesions. Immunohistologic staining of MAP17 in representative samples of benign prostate, prostatic intraepithelial neoplasia (PIN) and prostatic carcinoma. A section (3 μm) of paraffin-embedded prostatic TMA was stained using a monoclonal antibody raised against MAP17. Note the high expression of MAP17 protein in the cytoplasm of carcinomas when compared with benign prostatic cases. Magnification 20×.

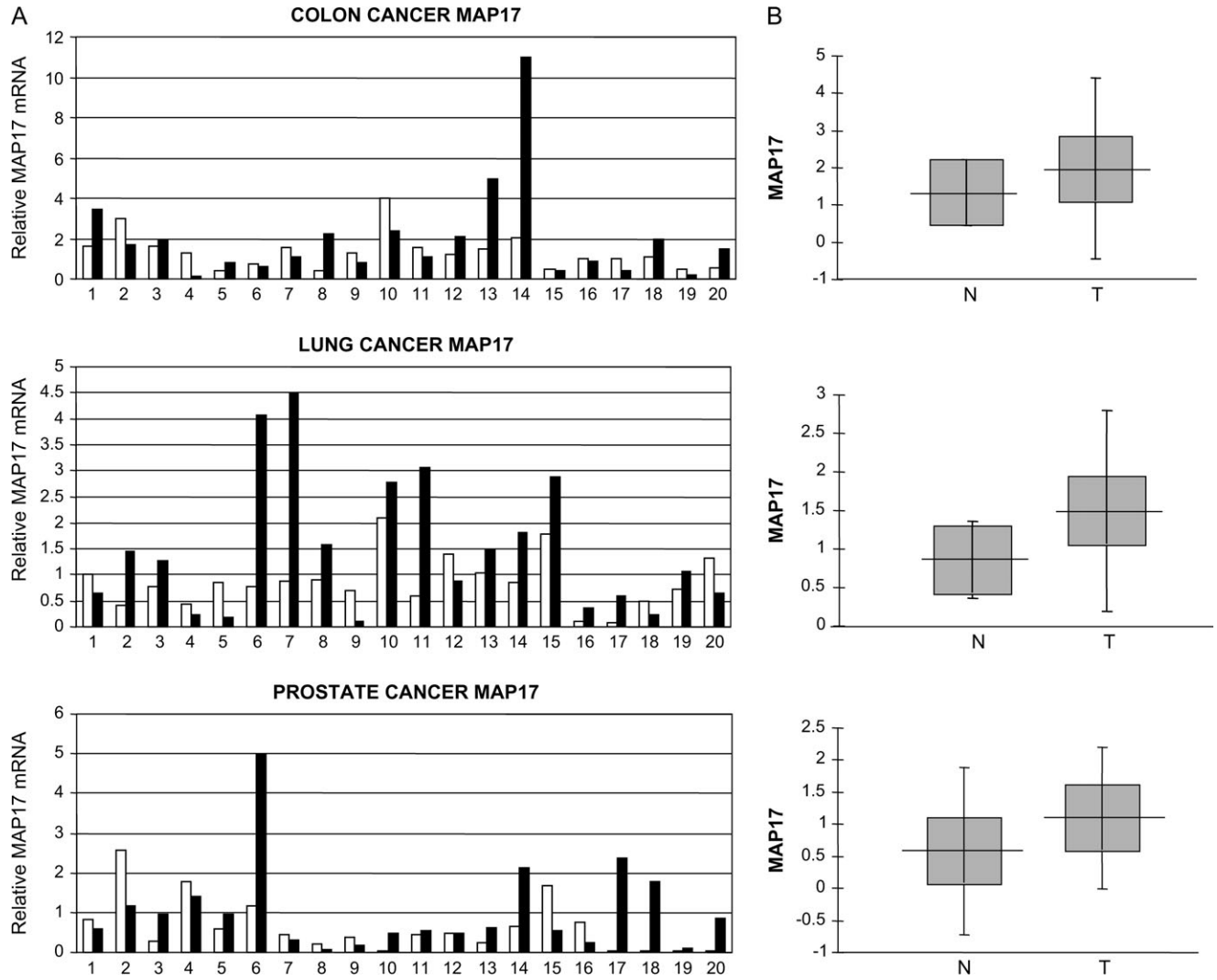

Fig. 3. Quantitative determination of MAP17 mRNA in tumor samples. We quantified MAP17 mRNA levels in three selected tumor types, including colon, lung and prostate, by quantitative PCR. mRNA from tumor and normal tissue was extracted from 20 patients of each tumor type. MAP17 mRNA levels were quantified in each sample by quantitative PCR as detailed in Materials and Methods. (**A**) Analysis of quantitative PCR of each individual sample compared with its non-tumoral match. (**B**) Averaged amount of MAP17 mRNA present in normal non-tumoral samples of the 20 patients and compared with the averaged amount present in tumor samples.

showed increased levels of MAP17 mRNA (Figure 3A). We compared the average amount of MAP17 mRNA in non-tumoral samples with the average amount in tumor samples of the 20 patients: in the tumors MAP17 mRNA levels were higher than in normal tissue (Figure 3B).

MAP17 overexpression in carcinoma is independent of SCL/Tal-1 gene

It has been reported that MAP17 share some regulatory elements, at least in cells of hematopoietic lineage with SCL (11,12); therefore, we tested whether in carcinoma we observed a coordinated mRNA over-expression of both genes. To that end, we first checked MAP17 mRNA expression in several arrays of paired normal/tumoral tissue samples from the same patients. A cDNA probe from MAP17 was radioactively labeled and hybridized with the arrayed tumoral/normal RNA samples. As loading control, arrays were hybridized with an ubiquitin-specific probe. Figure 4A shows a resume of the array hybridization experiments. Most tumor types had marked increase of MAP17 expression, as compared with the normal tissue from the same patient. An especially high percentage of the tumoral samples from ovary, colon, stomach, cervix and thyroid gland showed increase of the expression of MAP17 (Figure 4A and B). Then, we used the same arrays to test SCL expression. We amplified a 654 bp fragment located in the exons 1–3 from human SCL mRNA by RT–PCR. After sequencing, this fragment was used to generate the probe to hybridize the tumoral array. As Figure 4A shows, tumor tissue express the same amount of SCL mRNA than the non-tumoral tissue in almost all samples. Overexpression of MAP17 is not coordinated with higher levels of expression of SCL in any sample (Figure 4). These results indicated that MAP17 overexpression is fully independent of SCL.

To further confirm the independent expression of MAP17 and SCL, we have analyzed the presence of MAP17 in a panel of tumor cell lines and correlated to SCL expression. We extracted mRNA from exponentially growing cells and analyzed MAP17 mRNA presence by specific RT–PCR (Figure 5). Non-tumoral cells used as control (IMR90, WI38, HaCat and HMVEC) do not express MAP17. Among tumoral cells, 7 out of 28 (25%) express MAP17 mRNA. We analyzed SCL mRNA expression in several cell lines correlating MAP17 and SCL expression (Figure 5). We found that MAP17 and SCL are also fully independent events in tumor cells in culture.

These data confirm an independent overexpression of MAP17 in tumoral tissue and suggest that MAP17 can constitute an excellent marker for tumorigenesis.

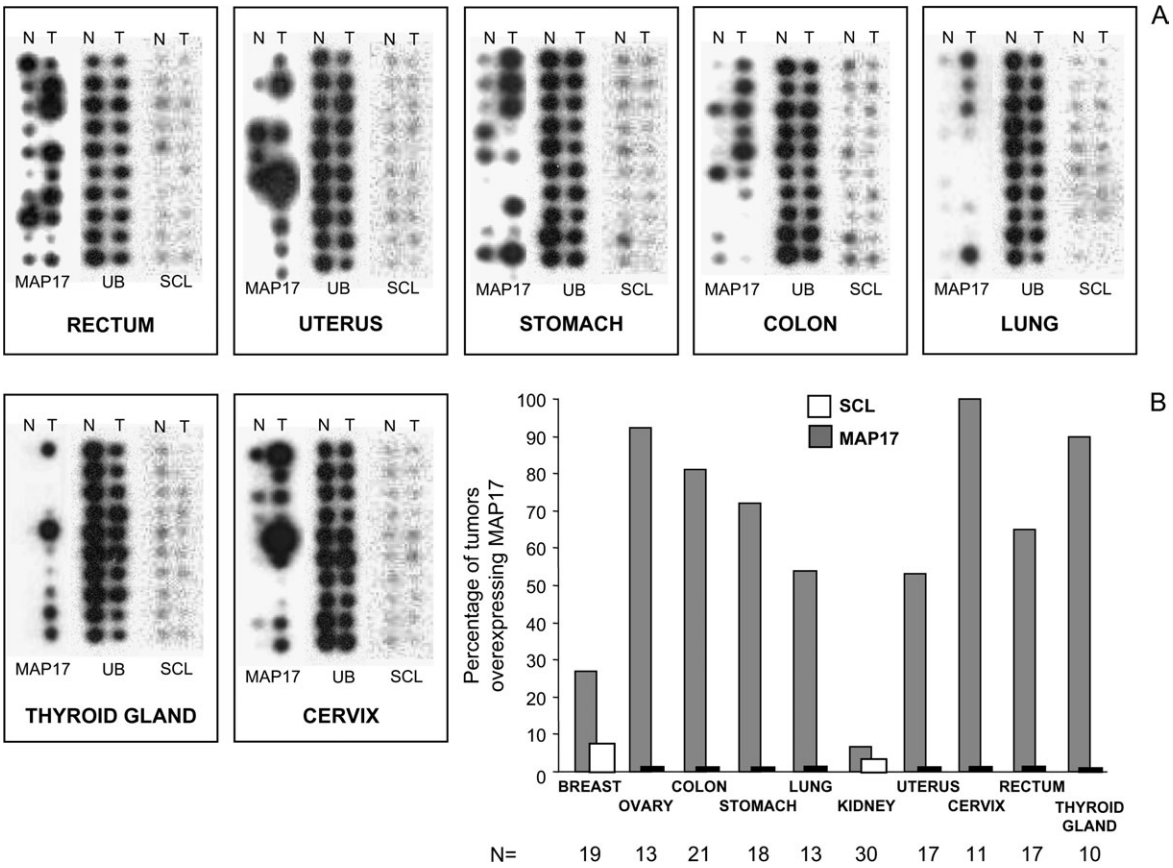

Fig. 4. MAP17 mRNA is increased in multiple tumor samples. (**A and B**) Cancer Profiling Array membranes (BD Biosciences) with paired non-tumoral/tumoral samples from the same patient were hybridized with P^{32}-labeled probes of the MAP17 and SCL genes to determine the respective amount of MAP17 mRNA. (**A**) shows representative hybridizations. The figure (**B**) shows the relative amount of samples overexpressing MAP17 for each type of tumor in relation to their paired normal tissue sample.

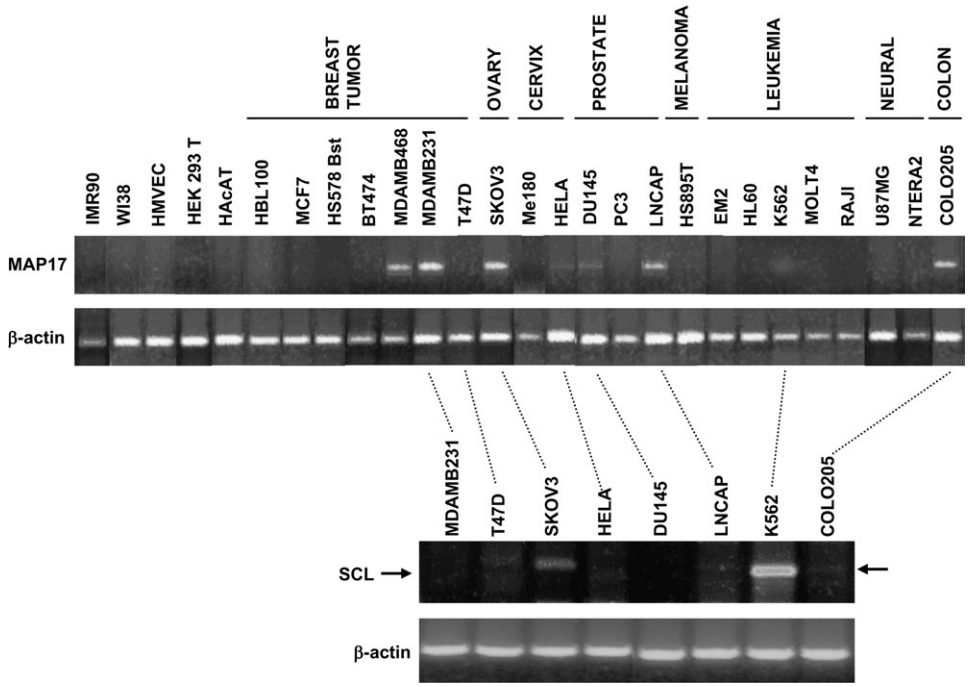

Fig. 5. MAP17 and SCL mRNA expression in multiple tumor cell lines. Expression of MAP17 and SCL in a panel of tumor cell lines analyzed by RT–PCR. Upper panel shows presence of MAP17 mRNA in all cell lines analyzed by RT–PCR. The bottom panel shows results of RT–PCR with SCL-specific primers (see Materials and Methods). Similar results were obtained in at least other three independent experiments.

MAP17 promoter is activated by oncogenes

MAP17 is commonly overexpressed in human carcinomas; therefore, we sough to test mechanisms responsible for its broad overexpression in tumors. To that end, we cloned 2.3 kb of the genomic DNA located 5′ of the MAP17 gene, and subcloned driving the luciferase reporter gene. We made 15 independent constructs containing different fragments of the 2.3 kb genomic DNA (Figure 6A). We introduced each one of these reporter plasmid in a MAP17-positive cell line MDA-MB-468, and measured the activity of the reporter (Figure 6A). We confirmed the activity of promoter contained in the fragment of 2.3 kb genomic DNA and reduced the minimal promoter to 600 bp 5′ of MAP17 gene (fragment G). Then we used this two constructs, full-length fragment (fragment F3) and the minimal promoter (fragment G) to evaluate the activation of MAP17 transcription by oncogenes. To assess whether different oncogenes induce MAP17 transcriptional activity, we measured the expression of the reporter gene driven by MAP17 promoter fragments ectopically co-expressed with the different oncogenes in MAP17-null HEK293T cells. Most oncogenes tested induced MAP17 promoter activation to certain degree (Figure 6B). However, this was not an universal feature of al genes tested since genes such as cyclin E did not induced MAP17 promoter activation (Figure 6B).

Taken together, our data show an independent activation of MAP17 promoter that can be driven by oncogenes and that might explain the common overexpression of MAP17 in human carcinomas

Discussion

MAP17 is a small non-glycosylated membrane-associated protein of 17 Kda that locates at the plasma membrane and the Golgi apparatus (4,5). MAP17 acts as an atypical anchoring site for PDZK1 and interacts with the NaPi-IIa–PDZK1 protein complex in renal proximal tubular cells (10). The physiological role of MAP17 in proximal tubules is not known but MAP17 stimulates specific Na-dependent transport of mannose and glucose in *Xenopus* oocytes (5) and some human cell lines (4).

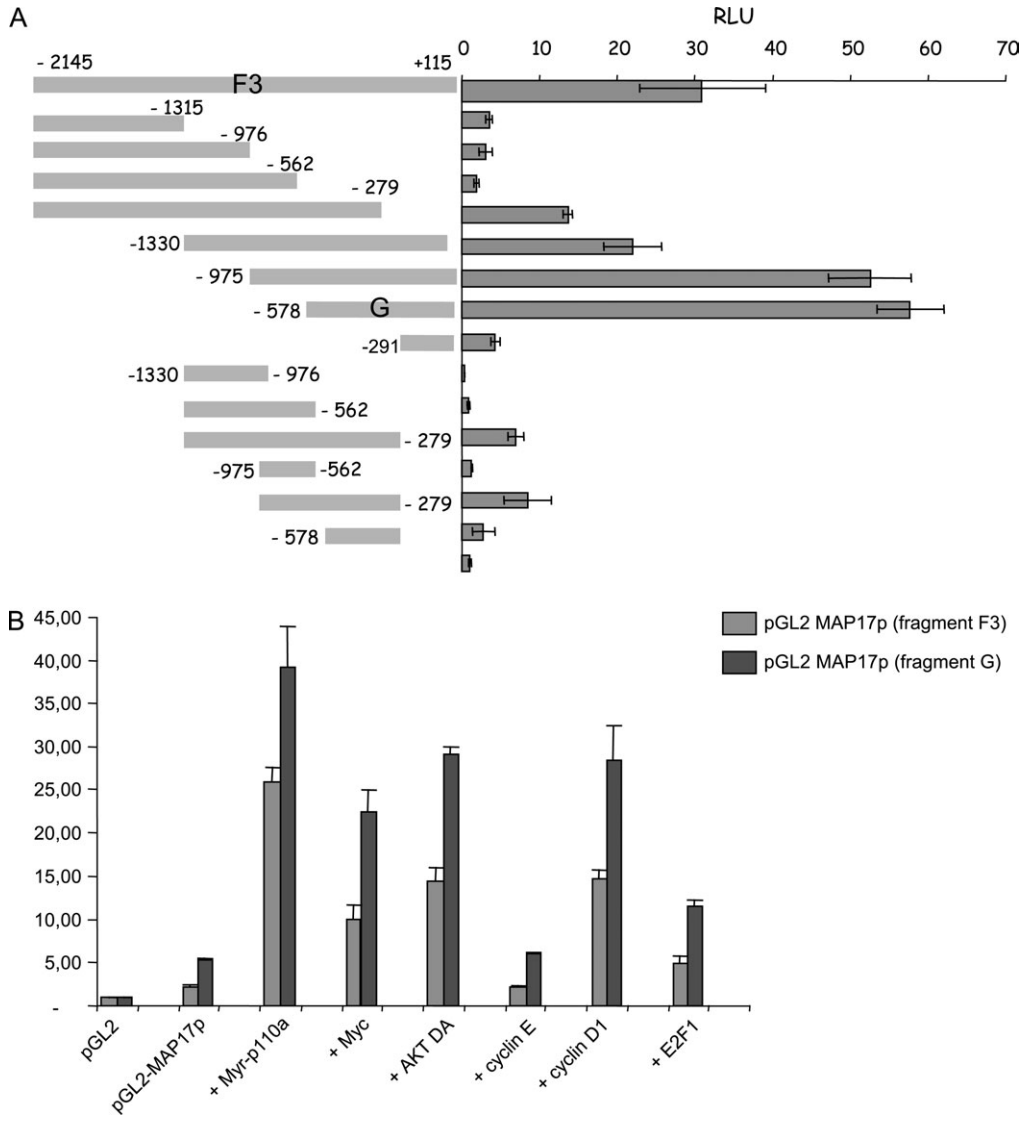

Fig. 6. MAP17 promoter is activated by oncogenes. (**A**) Identification of the MAP17 minimum promoter. 5′ MAP17 human genomic DNA (2.3 kb) was cloned and sequenced. Then different fragments (as indicated in the left part of the figure) were selected and cloned driving luciferase reporter gene in PGL3 vector. Each of these reporter plasmid was introduced into MDA-MB-468 (MAP17-positive cells) and luciferase measured (right part of the panel). (**B**) MAP17 promoter is activated by oncogenes. Hek293T cells were co-transfected with a reporter plasmid with the luciferase gene under MAP17 promoters (either F3 or G fragments, see above) and a plasmid carrying the indicated oncogene under LTR promoter. Control line one (pGL3-MAP17p) carries the reporter construct plus an empty plasmid. Dual-luciferase activity was measured as indicated in Material and Methods.

MAP17 is highly expressed in renal proximal tubular cells and has been described previously to be associated with carcinomas (6,7). We have performed an in deep analysis of MAP17 overexpression in carcinomas by immunohistochemistry and mRNA expression. We have found that MAP17 protein is overexpressed in a large percentage of the tumors analyzed and significantly correlated with the tumor grade in ovarian and prostate carcinomas. The analysis of mRNA levels by quantitative PCR or by hybridization comparing tumoral versus non-tumoral tissues of the same patient show an even higher percentage of tumor samples with MAP17 overexpression. In tumors such as ovary, colon, stomach, cervix and thyroid gland, the percentage of overexpression in tumor samples is higher than 70%, whereas in lung, uterus and rectum is around 50%. Although more samples need to be analyzed to confirm these high rates, the data suggest that MAP17 overexpression is the most common marker of tumorigenesis in carcinomas.

This could be due to the ability of MAP17 promoter to be activated by oncogenes. Tumorigenic progression involves progressive genetic alterations triggering oncogenic cascades (13,14). Advances stages of tumors might accumulate more oncogenic alterations that result in more probability of MAP17 promoter activation and increased transcription. This hypothesis will explain the correlation of MAP17 overexpression with advanced tumor stages.

The physiological relevance of MAP17 activation is unclear. In our hands, tumor cells that overexpress wild-type MAP17 show an increased tumoral phenotype, with enhanced proliferative capabilities both in presence or absence of contact inhibition, decreased apoptotic sensitivity and increased migration (MuGuijarro, J.F.M. Leal, C. Blanco-Aparicio, S. Alonso, J. Fominaya, M. Lleonart, J. Castellvi, S. Ramon y Cajal & A. Carnero). The increased tumorigenic properties induced by MAP17 are associated with an increase in Reactive Oxygen Species production, and the treatment of MAP17-expressing cells with antioxidants results in a reduction in the tumorigenic properties of these cells. We have overexpressed MAP17 in human tumor cells and found that these cells are more sensitive to certain treatments such as cisplatin (M.V.Guijarro and A.Carnero, unpublished data). Future studies might clarify whether MAP17 could be a good marker to predict treatment response.

In summary, generalized MAP17 overexpression in human carcinomas indicates that MAP17 can be a good marker for tumorigenesis and, at least in ovarian and prostate carcinoma, for malignant progression. Our results indicate that this protein is probably to play an important role in carcinogenesis.

Supplementary material

Supplementary material can be found at http://carcin.oxfordjournals.org/

Acknowledgements

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Conflict of Interest Statement: None declared.

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