The microtubule-associated protein MAPRE2 is involved in perineural invasion of pancreatic cancer cells

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Abstract. Perineural invasion of tumor cells is a characteristic feature of human pancreatic cancer. Unrevealing the molecular mechanisms that enable cancer cells to invade and grow along nerves is important for the development of novel therapeutic strategies in this disease. We have previously identified transcriptional changes in highly nerve invasive pancreatic cancer cells. Here we further analyzed one of the identified deregulated genes, MAPRE2, a microtubule-associated protein. MAPRE2 expression was significantly increased in high versus less nerve invasive pancreatic cancer cells, and changes of MAPRE2 expression resulted in altered actin distribution in these cells. MAPRE2 was predominately expressed in normal pancreatic acinar cells but absent in ductal cells. In pancreatic cancer, there was strong cytoplasmic and occasionally nuclear expression of MAPRE2 in the cancer cells themselves. Increased MAPRE2 mRNA levels in bulk pancreatic cancer tissues tended to be associated with reduced postoperative survival of pancreatic cancer patients. In conclusion, MAPRE2 is highly expressed in pancreatic cancer cells, and seems to be involved in perineural invasion. Therefore, targeting this microtubule-associated protein might be a promising approach in the therapy of pancreatic cancer.

Introduction

Perineural invasion of cancer cells is one of the key characteristics of pancreatic cancer (1,2). Growth along and into pancreatic and peri-pancreatic nerves is involved in the pain syndrome associated with pancreatic cancer and a likely cause of the microscopic incomplete resection and the subsequent frequent local recurrence of the disease (3,4). Defining molecular mechanisms that allow pancreatic cancer cells to grow along and into nerves is therefore important for the development of novel therapeutic strategies in pancreatic cancer, a disease whose incidence virtually mirrors its mortality rate (5). We have previously identified and described in detail the transcriptome signature of perineural invasion in pancreatic cancer by generating highly nerve invasive pancreatic cancer cells (6). One of the genes that displayed a significant upregulation in pancreatic cancer cells with a high potential for nerve invasion was MAPRE2.

The MAPRE gene family consists of three members (MAPRE1, 2, and 3) that code for three microtubule associated proteins (also termed EB1, EB2/RP1, and EB3/EBF3) (7). These microtubule-associated proteins regulate microtubule functions and dynamics. Microtubules, which are essential for eukaryotic cells, are polymers of α and ß tubulin (8-10). They are important for many cellular processes such as e.g., cell division, cytoplasmatic organization, maintenance of cell polarity, chromosome segregation, and cell migration (8-10). Cell locomotion, including invasion of foreign tissue territories, is closely associated with the dynamic morphology of the cell and especially with the dynamics of its cytoskeletal and adhesive structures (8-10). In view of the importance of microtubule organization on cell migration and invasion (11,12), here we further analyze MAPRE2, a previously identified gene that is potentially involved in perineural invasion.

Materials and methods

Cell culture. Pancreatic cancer cells were routinely grown in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium). Cells were maintained at 37˚C in a humid chamber with 5% CO_2 and 95% air atmosphere.

Tissue sampling. Pancreatic tissue specimens were obtained from patients who underwent pancreatic resection or through an organ donor program from previously healthy individuals. The Human Subjects Committee of the University of Heidelberg, and the Technische Universität München, Germany, approved all studies. Written informed consent was obtained from all patients.

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Real-time quantitative polymerase chain reaction (QRT-PCR). All reagents and equipment for mRNA/cDNA preparation were supplied by Roche Applied Science (Mannheim, Germany). mRNA was prepared by automated isolation using the MagNA pure LC instrument and isolation kit I (for cells) and kit II (for tissues). cDNA was prepared using the first-strand cDNA synthesis kit for RT-PCR (AMV) according to the manufacturer's instructions. QRT-PCR was carried out using the LightCycler FastStart DNA SYBR Green kit. The number of specific transcripts was normalized to the housekeeping gene cyclophilin B (cpb) and presented as copies/10,000 copies cpb. All primers were obtained from Search-LC (Heidelberg, Germany).

Immunoblotting. Protein levels were assessed by immunoblotting as described previously (13,14), using a specific mouse monoclonal MAPRE2 (EB2) antibody (Abcam, Cambridge, UK). Densitometry analysis of the blots was performed using the ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence. Cells were grown in complete medium overnight in 8-well chambers, washed with PBS, fixed with 2% formaldehyde/PBS for 15 min at RT, permeabilized with 0.2% Triton X-100/PBS for 15 min, and subsequently blocked with 3% BSA/PBS for 45 min. Slides were then incubated overnight with the primary mouse monoclonal MAPRE2 (EB2) antibody (Abcam, Cambridge, UK) at 4˚C. After that, slides were washed with PBS and incubated with fluorescent-labeled secondary antibodies for 1 h, washed, and incubated with Alexa Fluor® 488 phalloidin (Molecular Probes, Inc. Eugene, OR). Slides were then mounted with DAPI and anti-fading medium (Gel/mount™ , Abcam). Microscopic analysis was performed using Leica fluorescent Microscope (Leica Microsystems GmbH, Heidelberg, Germany).

Immunohistochemistry. Paraffin-embedded nerves and human pancreatic tissue sections (3-μm thick) were subjected to immunostaining as described previously (13,14). Sections were deparaffinized in Roticlear (Carl Roth GmbH, Karlsruhe, Germany) and rehydrated in progressively decreasing concentrations of ethanol. Antigen retrieval was performed by boiling with citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was quenched by incubating the slides in methanol containing 3% hydrogen peroxide. The sections were incubated at 4˚C overnight with the primary mouse monoclonal MAPRE2 antibody (Abcam). The slides were rinsed with washing buffer and incubated with EnVision+ System Labeled polymer HRP anti-rabbit antibody (Dako Corp., Carpinteria, CA) for 30 min at room temperature. Tissue sections were then washed in washing buffer and subjected to 100 μl DAB-chromogen substrate mixture (Dako), followed by counterstaining with hematoxylin. Sections were washed, dehydrated in progressively increasing concentrations of ethanol, and mounted with xylene-based mounting medium. Slides were visualized using the Axioplan 2 imaging microscope (Carl Zeiss Lichtmicroskopie, Göttingen, Germany). Additionally, to confirm the specificity of the primary antibodies, tissue sections were incubated in the absence of the primary antibody and with negative control rabbit IgG. Under these conditions, no specific immunostaining was detected.

Statistical analysis. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Survival analysis was carried out using the Kaplan-Meier method for estimation of event rates and the log-rank test for survival comparisons between patient groups.

Results

As described previously (6), an *ex vivo* perineural invasion assay was designed using rat vagal nerves and pancreatic cancer cell lines. Three nerve-invasive clones (NP1, 2 and 3) of Panc-1, Colo357 and T3M4 pancreatic cancer cell lines were obtained from these experiments. MAPRE2 up-regulation was evident in the nerve invasive clones of all three pancreatic cancer cell lines (Fig. 1A), confirming our micro-array data (6). Although expression of these genes in all NP clones was not significantly different, the tendency of MAPRE2 up-regulation from NP0 to NP3 was obvious. MAPRE2 up-regulation in nerve invasive clones was also evident on the protein level comparing NP0 versus NP3 clones. A MAPRE2 specific band of approximately 37 kDa was clearly visible in the examined cells (Fig. 1A, lower panel).

Expression analysis in nine cultured pancreatic cancer cell lines demonstrated variable expression of MAPRE2 mRNA in all cell lines ranging from 6-239 copies/10,000 cpb copies (Fig. 1B). Immunofluorescence analysis of cultured pancreatic cancer cell lines demonstrated predominant cytoplasmic localization of MAPRE2 in these cells (Fig. 1B, lower panel).

To analyze whether increased MAPRE2 expression in highly nerve invasive cells would alter the cellular localization of MAPRE2 and actin distribution, immunofluorescence analysis was carried out in NP0 (Fig. 2A and B) versus NP3 (Fig. 2C and D) Panc-1 pancreatic cancer cells. This analysis revealed increased granular cytoplasmic MAPRE2 expression with dominant peri-nuclear localization in NP3 cells compared to NP0 cells. In addition, MAPRE2 displayed co-localization with filamentous actin in NP3 cells, where actin organization was markedly increased with intense formation of cortical and transverse stress fibers (Fig. 2C and D). In contrast NP0 cells exhibited apical actin expression with strong accumulation in filopodia-like structures (Fig. 2A and B).

Next, we determined MAPRE2 mRNA expression levels in the normal pancreas, chronic pancreatitis and pancreatic cancer tissues. To this end, bulk tissue samples were analyzed from 59 pancreatic cancer patients (31 female, 28 male, median age 66 years). According to the 6th edition of the UICC classification, there were 1 stage IB, 11 stage IIA, and 47 stage IIB tumors. In addition, tissue samples from 20 chronic pancreatitis patients (4 female, 16 male, median age 47 years) and 20 healthy organ donors (7 female, 13 male, median age 49 years) were analyzed (Fig. 3A). This analysis revealed median (95% CI) MAPRE2 mRNA levels in the normal pancreas of 124 (101-156) copies/10,000 cpb copies, in chronic pancreatitis of 153 (113-229) copies/10,000 cpb copies. In contrast, MAPRE2 mRNA levels in pancreatic cancer were 77 (74-168) copies/10,000 cpb copies. The

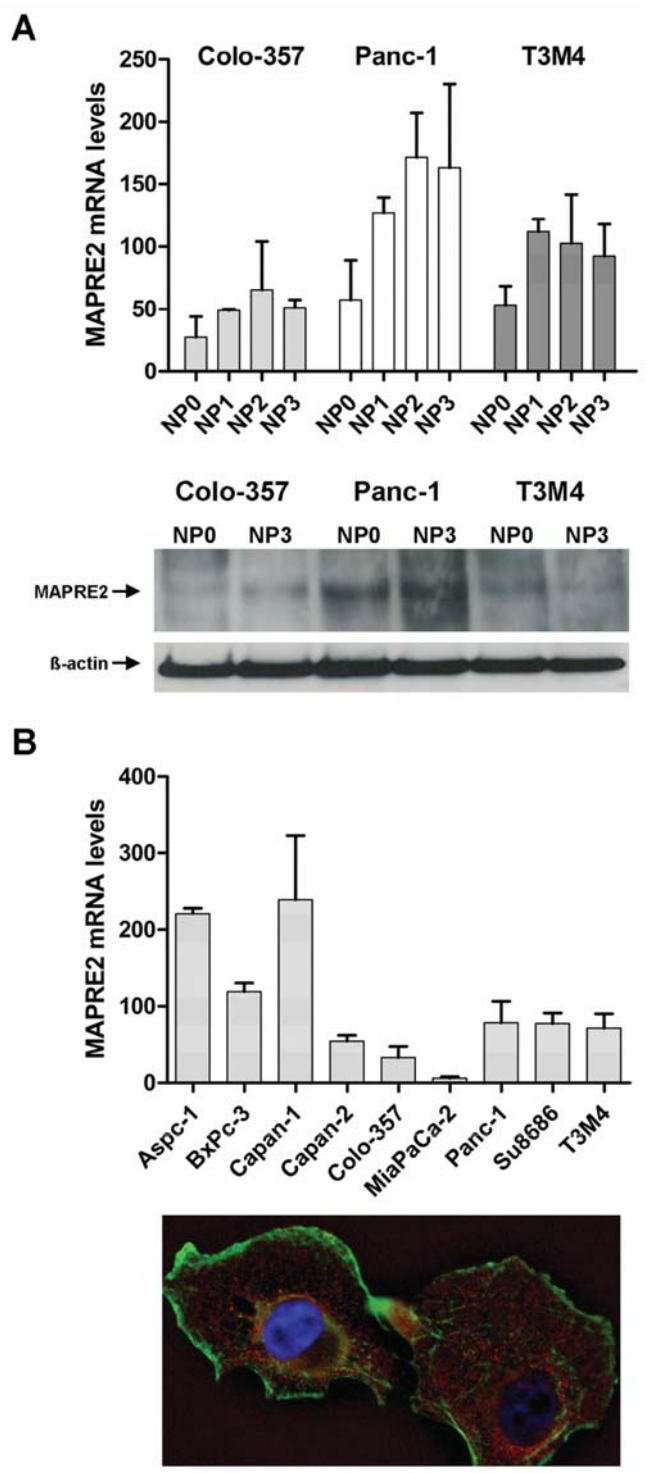

Figure 1. (A) Expression of MAPRE2 mRNA and protein in the indicated nerve passage cells using QRT-PCR (upper panel) and immunoblot analysis (lower panel) as described in Materials and methods. Data are presented as mean (± SD) from three independent experiments. (B) Expression of MAPRE2 mRNA in cultured pancreatic cancer cell lines (upper panel). Data are presented as mean (± SD) from three independent experiments. Immunofluorescence analysis of MAPRE2 localization in Panc-1 pancreatic cancer cells (lower panel). Red, MAPRE2; green, actin; blue, nuclear staining.

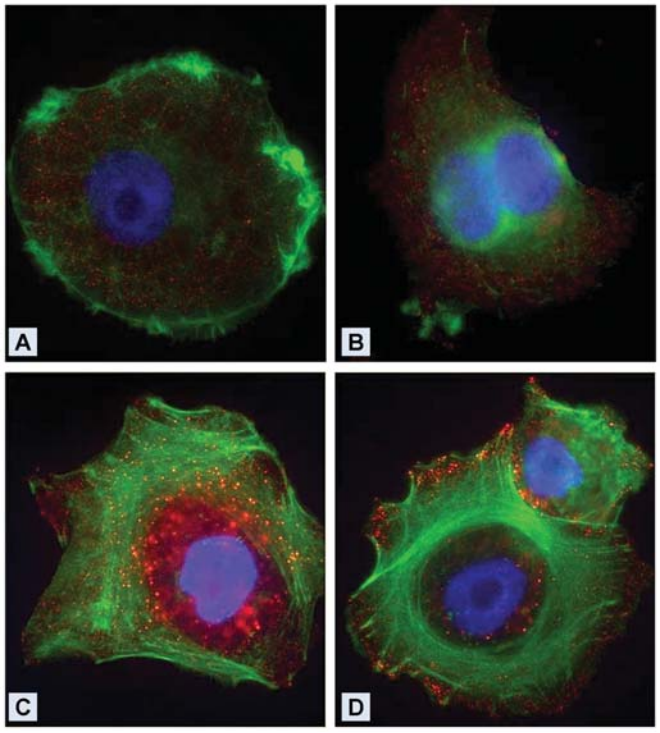

Figure 2. Immunofluorescence analysis of MAPRE2 localization in NP0 (A and B) and NP3 (C and D) Panc-1 pancreatic cancer cells as described in Materials and methods. Red, MAPRE2; green, actin; blue, nuclear staining.

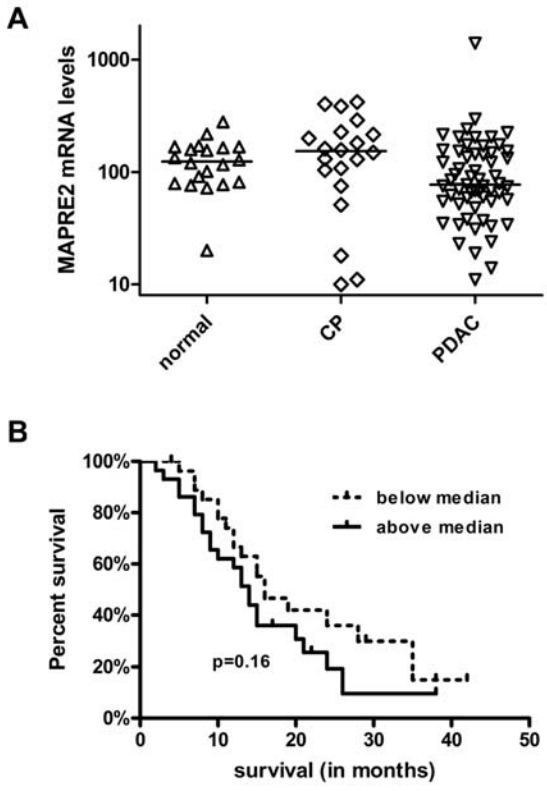

Figure 3. (A) Expression of MAPRE2 mRNA in normal pancreatic tissues (n=20), as well as chronic pancreatitis (CP) (n=20) and pancreatic cancer (n=59) bulk tissues using QRT-PCR, as described in Materials and methods. Horizontal lines represent the mean expression level. (B) The median value of MAPRE2 expression was taken as cut-off to define groups with high (interrupted line) and low (continuous line) MAPRE2 mRNA levels. The median survival of patients in these groups was compared using the Kaplan-Meier analysis and the log-rank test.

median MAPRE2 value in pancreatic cancer patients was taken as a cut-off to compare patients with high/low MAPRE2 mRNA levels using the Kaplan-Meier method. High MAPRE2 mRNA levels indicated a tendency for a worsened prognosis (p=0.16; Fig. 3B).

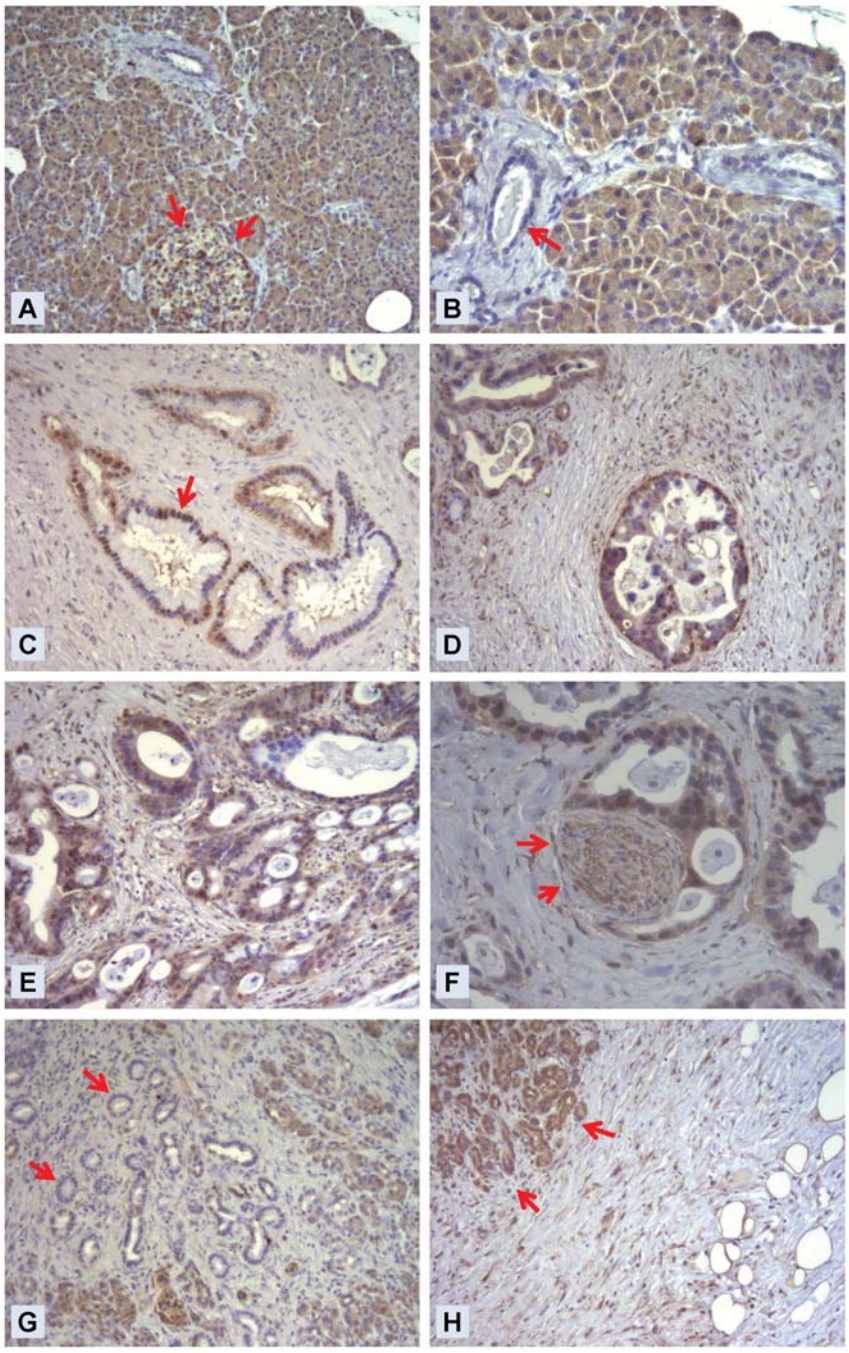

Figure 4. Immunohistochemistry in pancreatic tissues using a specific MAPRE2 antibody as described in Materials and methods. (A and B) Normal pancreatic tissues; arrows in (A) depict an islet; the arrow in (B) depicts a normal pancreatic duct with absent MAPRE2 staining. (C-F) Pancreatic cancer tissues; note the strong cytoplasmic and occasionally nuclear (C, arrow) staining. (F) Perineural invasion of pancreatic cancer cells (arrows point towards the nerve). (G and H) Chronic pancreatitis-like lesions adjacent to the tumor mass. Arrows depict tubular complexes (G) and degenerating acinar cells (H).

Next, the exact localization of MAPRE2 was determined in human pancreatic tissue specimen. In the normal pancreas, MAPRE2 was present in the cytoplasm of pancreatic acinar cells and in some islet cells (Fig. 4A and B). In contrast, MAPRE2 was below the level of detection in normal pancreatic ductal cells (Fig. 4B). These results are in line with the observed relatively high MAPRE2 mRNA levels in bulk normal pancreatic tissues. In pancreatic cancer tissues, MAPRE2 was present in the cytoplasm and occasionally in the nucleus of pancreatic cancer cells (Fig. 4C-E). MAPRE2 expression was also present in those cancer cells that grew into and along nerves (Fig. 4F), however, there was no obvious difference in the expression levels of those cells that invaded nerves and those that did not (Fig. 4C-F). In chronic pancreatitis as well as in chronic pancreatitis-like lesions next to pancreatic cancer, MAPRE2 was strongly present in degenerating acinar cells, and to a much lesser extent in tubular complexes (Fig. 4G and H).

Discussion

Many cellular proteins such as the MAPRE family of microtubule-associated proteins regulate microtubule functions (15,16). Human MAPRE1 (EB1), MAPRE2 (EB2/RP1),

and MAPRE3 (EB3/EBF3) have been shown to associate with microtubules both *in vivo* and *in vitro* (17-19). These microtubule associated proteins display specific localization to growing plus ends of the microtubules, thereby regulating their dynamics (19). Microtubule plus ends exhibit a specific behavior called dynamic instability, which is fundamentally important to the way cells respond to their environment (8,20). Rapid microtubule dynamics play a decisive role during mitosis especially the metaphase, as well as during cell migration and orientation (8,20). For example, it has been shown that the plus ends of growing microtubules target nearby focal adhesions and then may promote their dissolution in conjunction with retraction of the cell edge (21,22).

Besides their physiological role, proteins of the MAPRE family have been shown to play a role during carcinogenesis. For example, both MAPRE1 and MAPRE3 might act as potential oncogenes by activating the ß-catenin pathway thereby promoting growth and inhibiting apoptosis (23,24). In addition, MAPRE1 has been shown to be differentially expressed in different human tumors such as for example hepatocellular cancer, esophageal cancer, and head-and-neck squamous cell carcinoma (25-27).

Previously (6) and in the present study we have identified MAPRE2 as a potential factor in perineural invasion of pancreatic cancer cells. MAPRE2 levels were increased in the three established highly nerve invasive pancreatic cancer cell clones in comparison to the respective parental cells. Analysis of pancreatic cancer tissues demonstrated nuclear and cytoplasmic expression of MAPRE2 in pancreatic cancer cells *in vivo*, suggesting that this gene is involved in both cytokinesis and migration of pancreatic cancer cells. MAPRE2 expression was also evident in acinar cells of the normal pancreas, indicating a physiological role in acinar cell function. Interestingly, we have previously shown that KIF14, a member of the kinesin superfamily of microtubule-associated motors that play important roles in intracellular transport and cell division, is as a suppressor of perineural invasion, since loss of KIF14 expression is observed in the majority of neural/perineural invading cancer cells and since its down-regulation in pancreatic cancer cells results in enhanced perineural invasion (6). The identification of different microtubule associated proteins underscores the importance of this system in perineural invasion, and most likely invasion in general, of pancreatic cancer cells.

Even though MAPRE proteins have similar amino acid sequences, and all associate with microtubules, there are important differences (17,28). For example, MAPRE1 and MAPRE3 bind to APC but MAPRE2 does not (7). In addition, MAPRE1 is expressed at constant levels in a variety of examined cell lines, whereas MAPRE2 and MAPRE3 vary among different cell lines (7). Our finding of up-regulation of MAPRE2 but not MAPRE1 or MAPRE3 in nerve invasive pancreatic cancer cells, further supports specific functional differences of these closely related proteins.

Drugs that interfere with microtubule assembling and remodeling, such as vinca alkaloids that act as microtubule destabilizers, and taxanes that act as microtubule stabilizers, have been used as anti-mitotic agents in treating cancer (29). A large number of other agents that act on the microtubules at different sites with a variety of structures are presently being developed and tested in clinical and pre-clinical trials (29-31). The rationale behind this is that disruption of the spindle function with agents that suppress microtubule dynamics and either increase or destroy microtubules blocks cell cycle progression in many cells at the transition from prometa/meta to anaphase, the mitotic check-point. Subsequently, this mitotic block induces apoptosis in different cell types. Since the microtubular system is also important for the maintenance of cell shape and directional motility, drugs targeting microtubules not only have the capacity to act as anti-mitotic, but also anti-migratory by inducing loss of polarity, and by modulating focal contacts with the matrix.

In conclusion, MAPRE2 seems to be involved in perineural invasion of pancreatic cancer cells, most likely by interfering with the microtubule dynamics and organization. Targeting this protein might not only act anti-mitotically but might also block perineural invasion, and could therefore be a promising approach in the future treatment of pancreatic cancer.

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