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PINCH-2 expression in cancers involving serosal effusions using quantitative PCR

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Abstract

Objective—The *PINCH-2* gene was previously shown to be overexpressed in malignant mesothelioma compared with ovarian/peritoneal serous carcinoma in Affymetrix array analysis. The objective of the present study was to validate this finding at the mRNA and protein level.

Methods—Effusions (n = 91; 71 ovarian and 10 breast carcinomas, 10 malignant mesotheliomas) were assayed for *PINCH-2* mRNA expression using quantitative PCR. PINCH-2 protein expression was analysed in 37 effusions using flow cytometry.

Results—Quantitative PCR analysis showed significantly higher *PINCH-2* mRNA levels in mesotheliomas compared with carcinomas (P = 0.004). Values of <10 copies were found exclusively in carcinoma effusions (25.4% of ovarian and 50% of breast carcinomas). However, PINCH-2 protein expression by flow cytometry did not differ significantly between the three cancer types. No association was observed between *PINCH-2* levels and patient survival or expression of previously-studied molecules related to adhesion, metastasis and apoptosis inhibition in ovarian carcinoma.

Conclusions—Our data suggest that *PINCH-2* mRNA is overexpressed in malignant mesothelioma compared with carcinomas involving serosal cavities, and that low levels of this gene argue against the diagnosis of mesothelioma. The frequent PINCH-2 protein expression in all three studied cancers suggests a role for this molecule in cancer cell biology in effusions and merits further research.

Keywords

adenocarcinoma; cytology; flow cytometry; malignant mesothelioma; PINCH-2; quantitative PCR; serous effusions

Introduction

The serosal cavities are frequently affected by cancer, with the formation of both solid lesions and malignant effusions. The tumours that most commonly involve the peritoneal, pleural and pericardial spaces are metastases from carcinoma of the ovary (OC), breast or

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lung.[1] In addition, the serosal cavities are the site of origin of malignant mesothelioma (MM) and primary peritoneal carcinoma (PPC). Involvement of the serosal cavities by cancer cells is associated with poor patient outcome.[2]

The diagnostic immunohistochemistry panels that are currently used in effusion cytology and in surgical specimens obtained from the serosal cavities allow for correct diagnosis of the majority of tumours.[2-7] However, difficulties may still be encountered in determining the site of origin for metastatic adenocarcinoma, in differentiating reactive mesothelial cells from MM, and in the distinction between serous OC/PPC and MM.[2,4,5,7] Identifying molecular characteristics that are unique for one cancer type may aid in optimizing the diagnosis of serosal tumours, as well as in designing molecular therapies for specific malignancies.

We previously reported on the differential gene expression of 189 genes in OC/PPC compared with diffuse malignant peritoneal mesothelioma (DMPM) using cDNA microarray technology.[8] Among the differentially expressed genes, *PINCH-2* was identified as a potential marker of DMPM. We have no information at present regarding whether reactive mesothelial cells express PINCH-2.

The PINCH family consists of two members, PINCH-1 (also known as LIMS1) and PINCH-2. PINCH proteins contain five LIM cysteine-rich domains and a short c-terminal tail. [9] They interact with integrin-linked kinase (ILK) via the LIM1 sequence in a mutually exclusive manner and thereby regulate cell-extracellular matrix adhesion and motility.[10] PINCH-2 is expressed late during embryogenesis, but is widely expressed in adult tissues.[11] PINCH-2 is down-regulated via CpG island methylation in gastric cancer cell lines[12] but data regarding its expression and role in malignancy are to date limited.

The aim of our present study was to validate the gene expression array data for *PINCH-2*, obtained in analysis of 15 effusions, studying larger material using quantitative PCR (qPCR). In addition to DMPM and OC/PPC effusions, the qPCR material included pleural MM and breast carcinoma effusions. Additionally, we wished to validate our findings on protein level using flow cytometry.

Methods

Effusions

Specimens were submitted for routine diagnostic purposes to the Division of Pathology at the Norwegian Radium Hospital during the period 1998–2006. Effusions were centrifuged immediately after tapping and cell pellets were fresh-frozen in RPMI 1640 medium with 50% fetal calf serum and 20% dimethyl sulfoxide. Cell blocks were prepared using the thrombin clot method. Diagnoses were established based on smear and cell block morphology and immunohistochemistry.[13,14] The Regional Committee for Medical Research Ethics in Norway approved the study.

Quantitative PCR

A total of 91 effusions (71 OC/PPC, 10 breast carcinomas, 10 MM) were analysed for *PINCH-2* mRNA expression. OC/PPC effusions (n = 71; 54 peritoneal, 17 pleural) were obtained from 68 patients (three patients with two effusions each) diagnosed with advanced-stage (FIGO III-IV) OC (n = 58), PPC (n = 7) or the closely related serous carcinoma of the fallopian tube (n = 3). The majority of OC/PPC specimens (61/71; 85.9%) were of the serous type. Ten pleural effusions from patients diagnosed with histologically verified infiltrating duct carcinoma of the breast were analysed. The 10 MM effusions consisted of

seven pleural and three peritoneal specimens. All were from patients diagnosed with MM of the epithelioid or biphasic type in biopsy specimens.

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). mRNA isolation was performed using the Dynabeads Oligo (dT)25 kit (Dynal, Oslo, Norway). mRNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Primers for *PINCH-2* (NM_017980.3) were located at exon 7–8. Primer specificity was validated by running in parallel genomic DNA and cDNA as template, and viewing the results by gel electrophoresis. The assays were controlled for primer dimers using the NetPrimer software by PREMIER Biosoft, (Palo Alto, CA, USA) as well as for single nucleotide polymorphisms through the NCBI database. Primer efficiency was tested using Power SYBR® Green (Applied Biosystems) and a dilution series of synthetic oligonucleotides as template and subsequently as standard curve. The qPCR reaction was run using the Platinum® qPCR SuperMix-UDG with ROX solution (Invitrogen) and quantified on the Applied Biosystems 7900HT Sequence Detection System.

Primer and probe sequences were as follows: Sense: 5′-GCGAGACTCACTACAACCAGCTC-3′ Antisense: 5′-CCACATCGCCTTCAATCACA-3′ Probe: Fam 5′-CGGGGACGTCTGCTACAACTGCAGC-3′

Non fluorescent quencher—An array of 12 reference genes (TaqMan low density array human endogenous control panel; Applied Biosystems) was tested in order to identify the most uniformly expressed transcript in the effusion specimens. Based on this assay, *beta-glucuronidase* (*GUS*) was used as the housekeeping gene in these assays. The *GUS* primer and probe sequences have been published elsewhere.[15] Standard curves for the *GUS* assay were from Ipsogen (Marseille, France).

Flow cytometry

Thirty-seven fresh-frozen malignant effusions, consisting of 24 OC/PPC (three pleural, 21 peritoneal), six MM (three pleural, three peritoneal) and seven breast carcinomas (all pleural), were analysed for PINCH-2 protein expression. Four-colour flow cytometry was performed using the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with a 15 mW Argon-ion laser (488 nm) and a 12 mW red diode laser (635 nm), as recently described.[16] Briefly, carcinoma effusions were stained using the following antibodies: the epithelial antibodies Ber-EP4 (FITC-conjugated, clone Ber-EP4, Dako) Glostrup, Denmark and EpCAM (APC-conjugated, clone EBA-1, BD Biosciences); the leukocyte anti-CD45 antibody (PerCP-conjugated, clone 2D1, BD Biosciences); the anti-PINCH-2 rabbit polyclonal antibody;[10] IgG1-APC (clone X40, BD Biosciences); IgG1-FITC (clone DAK-GO1, Dako); and Polyclonal F(abÕ)2 goat anti-mouse IgG-PE (Dako). For MM effusion analysis, Ber-EP4 and EpCAM were replaced by an anti-epithelial membrane antigen (EMA) antibody (clone E-29, Dako). Control of instrument performance and time delay calibration were performed using FACSComp software version 4.1, Calibrite™ 3 beads and Calibrite™ APC beads (all from BD Biosciences), as previously described.[16] Fluorescein isothiocyanate (FITC, FL1, BP 530/30 nm), phycoerythrin (PE, FL2, BP 585/42 nm), peridium chlorophyll protein (PerCP, FL3, LP 670 nm) and allophycocyanin (APC, FL4, BP 661/16 nm) measurements were collected in the logarithmic mode. For each specimen, data from at least 10 000 cells were acquired.

Evaluation and scoring of flow cytometry immunophenotyping was undertaken using the FlowJo analysis software, version 8.7.3 (Tree Star Inc., Ashland, OR, USA). The percentage of tumour cells expressing PINCH-2 was scored. Expression in <1% of cells was scored as

negative. The OC cell line OVCAR-3, shown to express PINCH-2 in analysis of different OC and breast carcinoma cell lines, was used as positive control in all experiments.

Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 16.0, SPSS, Chicago, IL, USA).

Probability of <0.05 was considered significant. The association between tumour type (OC/PPC, breast carcinoma or MM) and PINCH-2 mRNA levels by qPCR was studied using the Kruskal-Wallis H test. The same test was used for analysis of the relationship between PINCH-2 protein expression and tumour type using flow cytometry. The relationship between PINCH-2 mRNA expression level in OC/PPC and the expression of previously studied molecules related to adhesion and apoptosis was studied using the Mann-Whitney *U*-test (relationship to categorical variables) or a two-sided *t*-test (relationship to continuous variables).

Clinicopathological data for the OC/PPC patient cohort were previously detailed.[17] The relationship between *PINCH-2* mRNA levels and clinicopathologic parameters (patient age, histological grade, FIGO stage, residual disease volume, previous administration of chemotherapy, and response to chemotherapy at diagnosis and at first disease recurrence) in OC/PPC was studied using the Mann-Whitney *U*-test. Univariate analysis of overall survival and progression-free survival was performed using the Kaplan–Meier method and log-rank test. For the latter analysis, *PINCH-2* mRNA levels were grouped as high versus low based on cut-off at the median value.

Results

PINCH-2 mRNA expression is higher in MM compared with OC/PPC and breast carcinoma

PINCH-2 mRNA was expressed in all 91 effusions, irrespective of tumour type, using qPCR. However, considerable differences were observed with respect to copy number (Figure 1). *PINCH-2* copy number values were as follows: OC/PPC, range = 1.17–453.06, median = 33.17; breast carcinoma, range = 1.57–75.52, median = 9.53; MM, range = 12.45–1456.91, median = 97.58. Values of the reference gene *GUS* showed little variation among samples.

Statistical analysis showed significantly higher *PINCH-2* expression in MM compared with the two other cancers ($P = 0.004$). Values of <10 copies were found exclusively in carcinoma effusions (25.4% of OC/PPC and 50% of breast carcinomas).

PINCH-2 protein is expressed in MM, OC/PPC and breast carcinoma

Flow cytometry analysis confirmed the morphological diagnosis of OC/PPC or breast carcinoma by demonstrating Ber-EP4- and EpCAM-expressing cells in all carcinoma effusions, whereas MM cells expressed EMA (Figure 2). PINCH-2 protein was localized to tumour cells in all 37 effusions (Figure 2). Expression extent was as follows: OC/PPC, range = 1–78% of cells, median = 15; breast carcinoma, range = 1–77% of cells, median = 8; MM, range = 9–92% of cells, median = 41. Despite the higher expression in MM, this difference failed to reach significance in statistical analysis (mean rank = 25.17, 18.25 and 16.29 for MM, OC/PPC and breast carcinoma, respectively; P = 0.286).

PINCH-2 mRNA levels in OC/PPC are not associated with those of adhesion-related molecules or with clinical parameters

Pinch proteins have previously been shown to regulate adhesion, epithelial-to-mesenchymal transition (EMT) and apoptosis in various cellular systems.[9-11,18-25] We therefore analysed the potential relationship between *PINCH-2* mRNA levels and the expression level of previously-studied integrin subunits,[26,27] E-cadherin and negative E-cadherin regulators involved in EMT (Snail, Slug and SIP-1),[28,29] gap junction proteins of the claudin family,[30] the inhibitor of apoptosis (IAP) members XIAP and Survivin[31] and NFjB p65.[32]. The number of specimens in these analyses ranged between 27 and 60. No association was found between *PINCH-2* mRNA levels and any of these molecules ($P > 0.05$; Table 1).

Finally, in view of the marked differences among OC/PPC cases with respect to *PINCH-2* mRNA levels, we investigated whether expression of this mRNA is related to clinicopathological parameters, including overall survival and progression-free survival, in OC/PPC. No significant association with any of these parameters was found, although a trend was seen for lower *PINCH-2* mRNA levels in disease recurrence (post-chemotherapy) effusions compared with specimens obtained at diagnosis (pre-chemotherapy; $P = 0.13$), which was related to platinum ($P = 0.1$), but not to paclitaxel ($P = 0.81$), treatment. Higher *PINCH-2* mRNA levels were additionally associated with overall survival of 30 months compared with 36 months for patients with effusions with lower than median expression level. However, this difference too was not statistically significant ($P = 0.12$; data not shown).

Discussion

Achieving high accuracy in diagnosing tumours involving the serosal cavities is made difficult by several factors, including the large number of origins for metastatic carcinomas involving this anatomic site, the antigens shared by MM and carcinomas, and the morphological changes undergone by reactive mesothelial cells in various benign conditions. The paucity of consistently reliable markers for the differentiation between MM and adenocarcinoma is exemplified in the case of serous OC/PPC, due to overlapping clinical presentation (especially within the peritoneal cavity), morphological resemblance, and co-expression or lack of expression of multiple markers. Markers that are expressed by both tumours in surgical specimens include podoplanin, calretinin, CK5/6, WT1 and mesothelin, while both tumours are CEA negative [reviewed in 4-5], and similar difficulties are encountered in effusion cytology.[14,33] In order to expand the diagnostic panel available to date, we applied gene expression array technology to the differential diagnosis between OC/PPC and DMPM.[8] In follow-up studies based on the array findings, we have identified several new potential markers that are significantly more highly expressed in OC/PPC compared with MM and/or reactive mesothelial cells, including the gap junction protein claudin-3,[34] MUC-4,[35] cyclin E[36] and folate receptors,[17] as well as a new MM marker, tenascin-X.[37] In the present study, we investigated the diagnostic role of PINCH-2 as a potential MM marker.

Significantly higher levels of *PINCH-2* mRNA were found in MM effusions compared with OC/PPC, in agreement with the Affymetrix array data,[8] with comparable differences between MM and breast carcinoma effusions. While no high cut-off levels that were diagnostic for MM were found, low cut-off expression levels below which the diagnosis of MM was unlikely were observed. While applying this cut-off will not result in correct classification of the majority of OC/PPC using the *PINCH-2* assay alone, our recent observations with respect to *TNXB*[37] and the folate receptors *FOLR1* and *FOLR3*[17] suggest that the combination of several genes in the qPCR assay may be highly informative. We are

currently studying several additional genes that may differentiate OC/PPC from MM, with the aim of designing such a qPCR panel in the near future.

The diagnostic role of PINCH-2 was subsequently studied at the protein level. Flow cytometry analysis of PINCH-2 protein expression showed higher expression in MM compared with OC/PPC and breast carcinoma, in agreement with the mRNA expression data. However, this difference was of a moderate degree, as reflected by the overlapping expression extent among the three cancer types, suggesting that this assay has little diagnostic value in the differential diagnosis between these three malignancies. Nevertheless, the high expression of PINCH-2 in effusions suggests a biological role for this protein in metastatic cancer cells, a possibility that we attempted to explore by analysing the relationship between this molecule and previously studied molecules with a role in adhesion, metastasis and regulation of apoptosis. This analysis was applied to the OC/PPC specimens analysed using qPCR, as this tumour category was the largest and best characterized one in our material.

The expression of ILK, one of the central molecular partners of PINCH proteins[9,10,18-20,23,24] has not been investigated in our material to date. However, we have previously studied the expression of the α6, αv and β1 integrin subunits, which are molecular partners of ILK, in OC/PPC.[26,27] Analysis of the association between *PINCH-2* mRNA levels and expression of these integrin subunits did not reveal any significant association. Similarly, no relationship was found with the presence and levels of E-cadherin, Snail, Slug, SIP-1, claudins, IAP members or NFjB p65 in our material. Further research is necessary in order to characterize the molecular partners of PINCH-2 in serosal cancers, primarily through a study of ILK and PINCH-2 expression in a large series of MM and OC/PPC, as well as *in vitro*.

MM is an aggressive tumour with extremely poor outcome in the majority of cases. As we observed large variation in *PINCH-2* mRNA levels in OC/PPC effusions, we hypothesized that patients with effusions characterized by a high level of this gene may have poor outcome. However, no significant association between *PINCH-2* mRNA levels and clinicopathological parameters, including survival, were found, suggesting that *PINCH-2* is not a strong prognosticator in this cancer. While the number of MM specimens analysed in this study does not allow evaluation of the clinical role of this molecule in MM, future investigation of this issue may be of interest.

In conclusion, qPCR analysis confirmed the higher levels of *PINCH-2* mRNA in MM compared with OC/PPC and breast carcinoma effusions, suggesting a diagnostic value for this molecule as part of a larger gene panel. PINCH-2 protein is frequently expressed in cancer cells in effusions, but further research is required in order to identify its potential molecular partners and clinical role in this setting.

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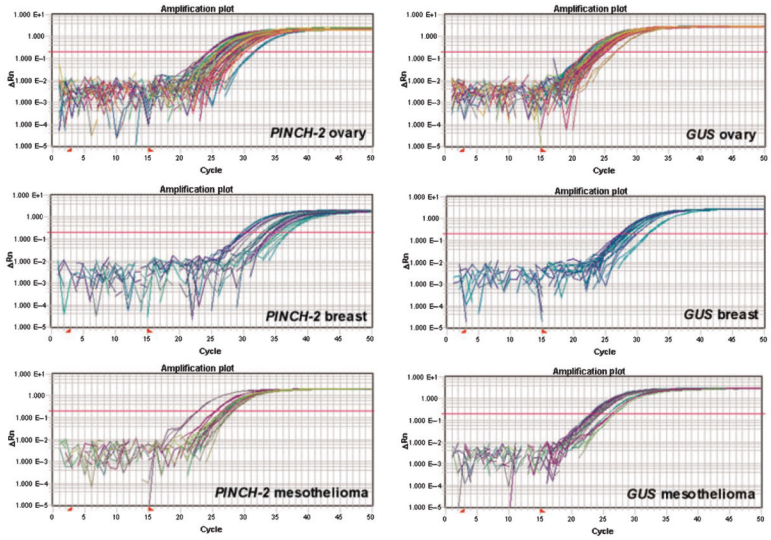

Figure 1.

PINCH-2 mRNA expression in serous effusions. Quantitative PCR amplification plots for *PINCH-2* mRNA in a representative series of 47 effusions (29 ovarian carcinomas, 9 malignant mesotheliomas, 9 breast carcinomas). Cases were run in triplicate. The cycle threshold value (curve crossing the red horizontal line) is clearly lower for MM compared with ovarian and breast carcinoma effusions, denoting higher mRNA expression in the former tumor. Final values were established as ratio to level of the reference gene *GUS* (see also text).

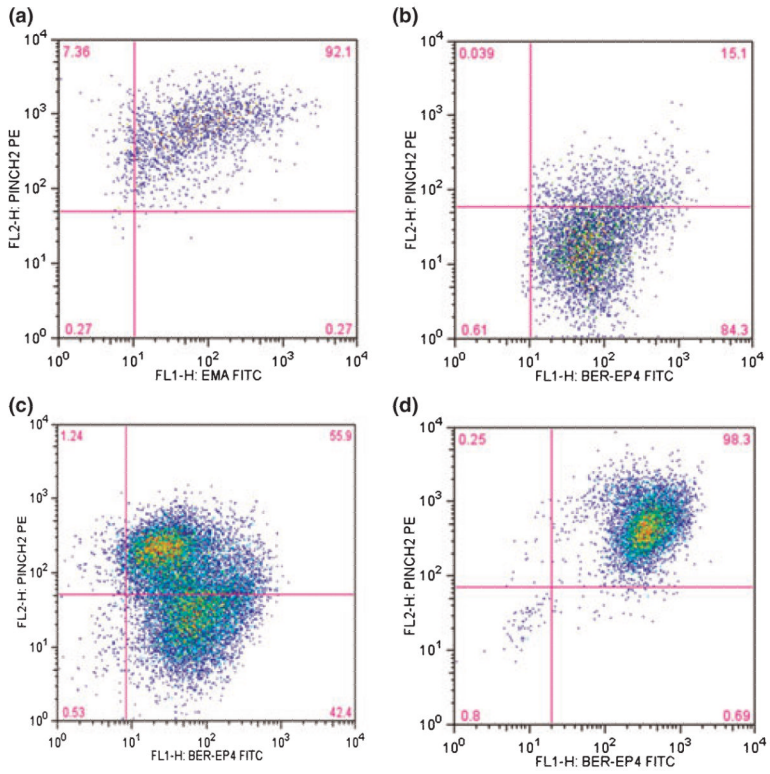

Figure 2.

PINCH-2 protein expression in serous effusions. Quantitative flow cytometric analysis of PINCH-2 protein levels, representative example for each of the three specimen types and control cells. (a) Malignant mesothelioma effusion. The majority of tumour cells (92.1%) are co-labelled by the epithelial membrane antigen (EMA) and PINCH-2 antibodies. (b) Ovarian carcinoma effusion. Co-labelling of 15.1% of tumour cells by the Ber-EP4 epithelial marker and PINCH-2. (c) Breast carcinoma effusion. Co-labelling of 55.9% of tumour cells by the Ber-EP4 and PINCH-2 antibodies. (d) The ovarian carcinoma cell line (OVCAR-3) expresses PINCH-2 in 98.3% of cells.

Table 1

Association between *PINCH-2* mRNA levels and expression of previously studied cancer-associated molecules in ovarian carcinoma

Molecule	Ref.	Expression level	No. of cases	*P*-value
α6 integrin	26	mRNA	30	0.35
αv integrin	27	Protein	28	0.35
β1 integrin	27	Protein	27	0.26
E-cadherin	28,29	mRNA	28	0.29
Snail	28,29	Protein	27	0.97
Snail	28,29	mRNA	28	0.56
SIP1	28,29	mRNA	28	0.73
Slug	28,29	mRNA	27	0.51
Claudin-1	30	Protein	50	0.98
Claudin-3	30	Protein	50	0.18
Claudin-7	30	Protein	50	0.99
XIAP	31	Protein	53	0.14
Survivin (cytoplasmic)	31	Protein	53	0.35
Survivin (nuclear)	31	Protein	53	0.35
NFκB p65 (nuclear)	32	Protein	60	0.99