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DIFFERENTIAL EXPRESSION OF PKD1 AND PKD2 IN GASTRIC CANCER AND ANALYSIS OF PKD1 AND PKD2 FUNCTION IN THE MODEL SYSTEM

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Aim: **To study the differential expression of PKD1 and PKD2 in primary gastric cancer samples and to examine the role of PKD1 and PKD2 protein kinases in regulation of gastric tumor cell biology in the model system.** ***Methods:*** **Tumor samples of different histological variants of primary gastric cancer were analyzed. PKD1 and PKD2 expression levels in tumor samples were accessed by Western blot analysis and quantitative polymerase chain reaction (Q-PCR). As a model system we have used gastric adenocarcinoma cell line AGS sublines constitutively transfected by pcDNA3.1 coding PKD1 or PKD2, or empty pcDNA3.1 vector. These cell lines were analyzed by Western blot, Q-PCR, MTT and proliferation assays, *in vitro* scratch and Transwell assays, clonogenic assay.** ***Results:*** **It was found that primary gastric tumors possess different levels of PKD1 and PKD2 expression on mRNA and protein levels. Low level of PKD1 expression on protein and mRNA level was detected in low differentiated adenocarcinoma and ring cell gastric cancer — disorders with poor clinical prognosis. The high level of PKD2 expression was also found in gastric tumors with poor prognosis: low differentiated adenocarcinoma and adenogen cancer. To find out whether differential expression of PKD1 and PKD2 could affect biology of gastric tumor cells *in vitro*, we used a model system based on AGS cell line that constitutively expressed PKD1 or overexpressed PKD2. PKD1 transfection led to the inhibition of cell proliferation, migration and colony formation, in the meanwhile, the PKD2 overexpression enhanced proliferation, migration and colony formation capacities of AGS cells.** ***Conclusions*****: Our data suggest that both downregulation of PKD1 or upregulation of PKD2 expression may determine the behavior of gastric tumor cells, which promotes invasive phenotype and could result in general poor prognosis.**

Key Words: **PKD1, PKD2, levels of expression, gastric cancer, cell proliferation, cell migration.**

Kinases of protein kinase D (PKD) family are expressed ubiquitously in different human tissues. PKDs could be activated by growth factors, antigen stimulation and oxidative stress, the processes that usually are observed during tumor progression [1]. PKDs regulate cell-cell contacts by affecting cell adhesion [2, 3]. These kinases are involved in the regulation of cell proliferation and apoptosis and also participate in epigenetic regulation of gene expression. The presence of the nuclear translocation signal peptide may allow PKD translocation to the nucleus and phosphorylation of its nuclear targets, such as transcription factor NF-kB, histone deacetylases and histone chaperone SET [4–6]. Depending on PKD localization site, this kinase can be implicated in the regulation of a variety of cellular and subcellular processes, such as Golgi function and organization, receptor signalling, apoptotic or antiapoptotic signalling, tumor cell invasion. PKD family of protein kinases can be involved in the regulation of tumor cells survival [7–9]. Nevertheless, the role of different PKD isoforms in these processes in normal and malignant cells is not fully clarified. Recently the differential expression of PKD genes was found in primary tumors of different histogenesis: gastric cancer, breast cancer, prostate cancer, lymphoproliferative disorders [2, 10–13]. Thus, the studies of differential expression and activity of PKD1 and PKD2 in the context of tumor invasiveness and prognosis could be of interest for translational research in oncology.

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Abbreviations used: MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-kB – nuclear factor kB; PKC – protein kinase C; PKD – protein kinase D; Q-PCR – quantitative polymerase chain reaction.

MATERIALS AND METHODS

The protein levels of PKD1 and PKD2 were evaluated in 38 primary tumor samples and surrounding tissues from various histological variants of gastric cancer that are characterized by different prognosis. Samples have been received from the patients cured in Kyiv City Oncological Hospital (Kyiv, Ukraine), and written consent was obtained from each patient before enrolling into the study. The study was approved by Ethical Committee of R.E.Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine. To access the protein level of PKD1/2 we used Western blot analysis [13]. For Western blot analysis we used anti-PKCμ rabbit antibody that detects both PKD1 and PKD2 (Clone D-20, Santa Cruz, USA) and anti-actin rabbit antibody (Santa Cruz, USA), secondary goat anti-rabbit antibody conjugated with peroxidase (Santa Cruz, USA).

mRNA level of PKD1 and PKD2 kinases was evaluated for 28 tumor samples of gastric cancer by quantitative polymerase chain reaction (Q-PCR). For Q-PCR analysis we used Applied Biosystems 7500 System SDS (USA). RNA from gastric tissue samples was isolated using Tri Reagent (Sigma, USA). RNA were reverse transcribed using an M-MLV Reverse Transcriptase and Ribonuclease Inhibitor RNaseOUT™ (Invit-

rogen, USA) and oligo(dT)$_{18}$ primer (Fermentas, USA). Genetic changes and expression level were analyzed by Q-PCR with SYBR Green (Fermentas, USA). Gene expression ratios were normalized using a housekeeping gene β2-microglobulin. Following primers were used for PKD1: forward — AATGAATGAGGAGGGTAGGG, reverse — GCTAGGTGCATTGTCTTGAG; for PKD2: forward — TGTGTCCCATTGGTGTTGTC, reverse — TTTTATTCCCTACCCTCCTC; for β2-microglobulin: forward — CCGTGTGAACCATGTGACTTTGTC, reverse — TGCGGCATCTTCAAACCTCCATGATG. Relative quantification of the obtained results was performed using $2^{-\Delta\Delta Ct}$ method [14]. Gene expression ratios for tumor samples were compared with PKD1 and PKD2 mRNA expression level in the signet-ring cell gastric carcinoma sample, which was chosen because of the lowest PKD1 and PKD2 expression levels among all analyzed gastric tumor samples. Gene expression ratios for transfected cells were compared with parental cell line AGS.

For *in vitro* studies the gastric adenocarcinoma cell line AGS was used. Cells were cultivated in IMDM media (Sigma, USA) supplemented with 10% of fetal bovine serum (Sigma, USA) and antibiotics. AGS cells were transfected with following plasmids: pcDNA3.1, pcDNA3.1-PKD1 or pcDNA3.1-FLAG-PKD2. We used standard protocol of Ca-phosphate transfection followed by clone selection on G418 (0.6 mg/ml). After selection on G418 for 14 days, transfected cells were cloned and sublines that constitutively express PKD1 and PKD2 were obtained: AGS-PKD1, AGS-PKD2 and AGS-pcDNA3.1.

To explore the characteristics of AGS transfectants we have used an array of standard *in vitro* assays, including dye exclusion method with 0.4% trypan blue, MTT assay (colorimetric assay with 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), cell migration scratch assay, Transwell and clonogenic assays [15–18].

The results of biological assays represent the mean ± s.d. derived from at least three different experiments. Statistical significance of differences was evaluated by Student's t-test.

RESULTS AND DISCUSSION

In current study we have explored PKD1 and PKD2 expression on protein and mRNA levels in primary gastric cancer samples. To confirm our preliminary data on differential level of PKD1/2 expression in gastric tumors tissues shown by immunohistochemistry [13], we have performed Western blot analysis and Q-PCR. Western blot analysis has shown both PKD1 (120 kDa) and PKD2 (105 kDa) expression in the majority of tumor samples. It should be noted that the low level of PKD1 expression was detected in low differentiated adenocarcinoma and signet-ring cell gastric cancer (Fig. 1, tracks 1–3, and data not shown). Altogether, in 6 out from 38 samples (15.8%) the level of PKD1 expression was much lower than PKD2. Moreover, in one case of adenogen cancer PKD1 expression was not detected on protein level.

To study the expression of PKD1 and PKD2 on mRNA level we applied Q-PCR analysis. In samples 2, 5, 7–9 of tumors with poor prognosis, the level of PKD1 expression was considerably low in comparison with other tumor samples (Fig. 2). The highest level of PKD2 expression was shown for adenogen tumor (Fig. 2, sample 6) and low differentiated adenocarcinoma (Fig. 2, sample 2) that also had poor prognosis. At the same time in gastric tissues surrounding both low and moderately differentiated adenocarcinomas (Fig. 2, samples 1 and 3) PKD1 and PKD2 were expressed on higher levels than in corresponding tumors (Fig. 2, samples 2 and 4). It should be noted that in tumors with more favorable prognosis (moderately differentiated adenocarcinoma, cardioesophageal cancer and gastric adenoma, Fig. 2, samples 4, 10 and 11, respectively) the expression level of PKD1 mRNA was higher than in other cases.

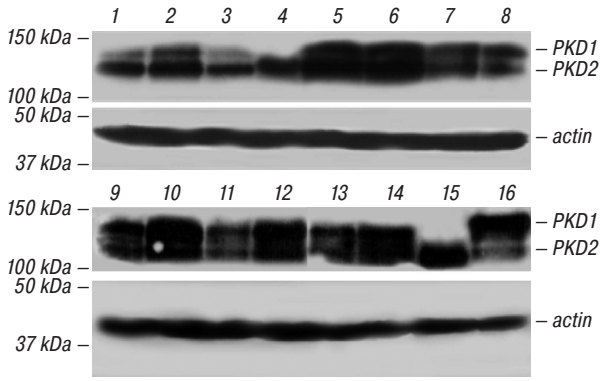

Fig. 1. Western blot analysis of cancer cells from different histological variants of primary gastric cancer. 1 — low differentiated adenocarcinoma, 2 — normal gastric tissues surrounding low differentiated adenocarcinoma, 3 — signet-ring cell gastric carcinoma, 4 — adenogen tumor, 5, 6, — gastric adenoma, 7, 8 — non differentiated cancer, 9, 13 — normal gastric tissues surrounding moderately differentiated adenocarcinoma, 10, 12, 14 — moderately differentiated adenocarcinoma, 11 — carcinoid, 15 — cells lysate of HEK293T-PKD2, 16 — cells lysate of HEK293T-PKD1

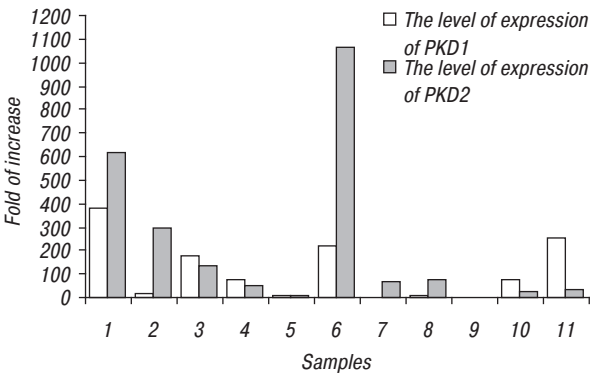

Fig. 2. Results of the Q-PCR on mRNA from tumor samples of different histological variants of primary gastric cancer. White columns — PKD1 expression ratio, black — the PKD2 expression ratio, both in comparison with PKD1 and PKD2 expression level in the sample of signet-ring cell gastric cancer, which demonstrated the lowest PKD1 and PKD2 expression levels. 1 — normal gastric tissues at low differentiated adenocarcinoma, 2 — low differentiated adenocarcinoma, 3 — normal gastric tissues at moderately differentiated adenocarcinoma, 4 — moderately differentiated adenocarcinoma, 5 — non differentiated gastric cancer, 6 — adenogen tumor, 7 — adenogen tumor (with metastasis), 8 — carcinoid cancer, 9 — signet-ring cell gastric cancer, 10 — cardioesophageal gastric cancer, 11 — gastric adenoma, Data on PKD2 expression level was normalized to expression of β2-microglobulin. *Exactly, the value on the level of PKD2 expression in sample 6 is significantly larger than the one shown in Fig.2 (sample 6: it is equal to 8012).

Thus, we demonstrated the differential expression of PKD1 and PKD2 in various histological variants of primary gastric cancer and surrounding tissues on mRNA and protein levels.

PKD2 is the major serine/threonine protein kinase of PKD family, which is expressed in human gastric adenocarcinoma cell line AGS. In AGS cells PKD2 is activated by gastrin and is a downstream target of PKCs [19]. PKD1 was not detectable in these cells on protein level (Fig. 3, *a*, track 4). To examine the role of PKD1 and PKD2 protein kinases in regulation of gastric tumor cell biology we developed a AGS sublines expressing PKD1 or overexpressing PKD2. The expression levels of protein kinases PKD1 and PKD2 in constitutive transfectants was checked by Western blot (Fig. 3, *a*) using antibody against human PKCμ (Clone D-20, Santa Cruz, USA), which recognizes both PKD1 and PKD2. In our study, AGS cells were transfected with following plasmids: pcDNA3.1, pcDNA3.1-PKD1 or pcDNA3.1-FLAG-PKD2. Since FLAG peptide is about 7 kDa and PKD2 — 105 kDa the molecular weight of transfected PKD2 was expected to be 112 kDa (Fig. 3, *a*, tracks 1 and 2). PKD1 cDNA in pcDNA3.1 vector resulted in 120 kDa protein expression (Fig. 3, *a*, track 5).

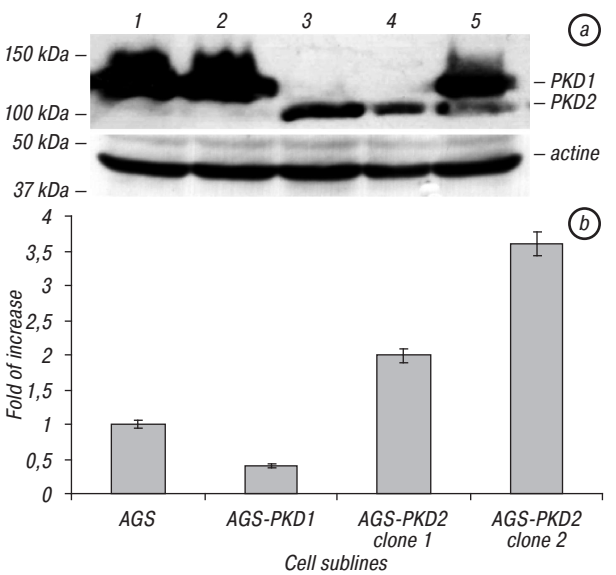

Fig. 3. The level of PKD1 and PKD2 expression in AGS sublines. *a*. Western blot analysis of PKD1 and PKD2 expression in cell lysates. 1, 2 — AGS-PKD2 (two different colonies with over expression of protein kinase PKD2), 3 — AGS with empty pcDNA3.1 plasmid (transfection control), 4 — AGS, 5 — AGS-PKD1. Western blot of actin expression served as a loading control (lower panel). *b*. Results of Q-PCR of PKD2 in AGS cell line and transfectants AGS-PKD1 and AGS-PKD2. The results of three independent experiments. Data on PKD2 expression level was normalized to expression of β2-microglobulin

Also we have checked mRNA levels of PKD1 and PKD2 in AGS-PKD1 and AGS-PKD2 sublines using Q-PCR analysis (Fig. 3, *b*). There was a prominent difference between the level of PKD2 mRNA in AGS-PKD2 cells, which overexpress PKD2, when compared with AGS wild type cells (see Fig. 3, *b*). One of the clones tested expressed twice more of PKD2 coding mRNA, and other clone had 3.6 times more PKD2 coding mRNA than parental AGS cell line, reflecting the efficiency of transfection.

Also we have found that expression of PKD1 upon transfection in AGS cell line lead to the significant reduction of endogenous PKD2 mRNA and protein expression (see Fig. 3, *b*). Mechanisms and nature of this phenomenon require further study, but now we have shown in this study that expression of PKD1 kinase could influence the level of PKD2 expression.

Using Western blot analysis we have selected two sublines of each transfectants with different level of protein kinases PKD1 and PKD2 expression when compared with parenteral AGS and AGS with empty plasmid (AGS-pcDNA3.1). These sublines (AGS-PKD2 and AGS-PKD1) were used to study the role of PKD1 and PKD2 isoforms in regulation of gastric tumor cell biology. These cell sublines were used for morphological study, comparative analysis in MTT and proliferation assays, and also tests for cell migration and survival.

Morphological examination of the transfectants AGS-PKD1 and AGS-PKD2 demonstrated that these cells acquired morphological features different from the parental AGS cells. AGS-PKD1 cells were of polygonal shape, and increased in size in comparison with sublines with constitutive expression of PKD2, AGS-pcDNA3.1 and parental AGS cells (Fig. 4, *a*). It was observed that colonies of AGS-PKD1 cells had sufficiently more compact edge (Fig. 4, *b*), while colonies of transfectant cells with constitutive expression of AGS-PKD2 had irregular edge. Moreover, AGS-PKD2 cells were round-shaped (Fig. 4, *c*).

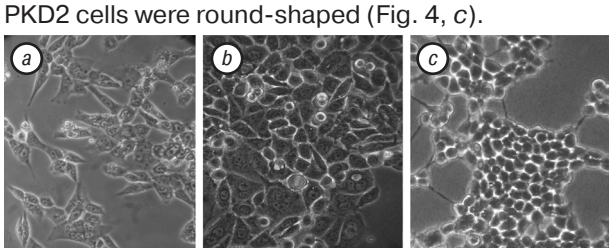

Fig. 4. The morphological differences of AGS sublines. *a* — control cell line AGS, *b* — transfectant AGS-PKD1, *c* — transfectant AGS-PKD2. x80

To explore the cell survival and proliferation activity the transfected cells were stained with 0.4% trypan blue dye. Tested cells were cultured in 24-well plates in IMDM medium in concentration of $7x10^4$ cell/ml/well in triplicates. After 24, 48 and 72 h of cultivation the number of alive and dead cells was calculated. The transfection of AGS cell line with PKD1 or PKD2 did not significantly affect the survival of cells: the number of dead cells in each of the studied sublines was the same within 72 h of cultivation (from 3.7% to 7.5%). At the same time we have revealed the significant differences in the number of alive cells for AGS-PKD1 and AGS-PKD2 sublines. After 24 h of cultivation the number of AGS-PKD2 cells increased 4.6 times, while the number of control AGS cells — only 2.5 times. At the same time the rate of AGS-PKD1 subline proliferation was lower than of control AGS and AGS-pcDNA3.1 cells (Fig. 5, *a*, lower curve). The kinetics studies up to 72 hs showed

even more prominent difference. Taken together, AGS-PKD1 cell were characterized by slight inhibition of cell proliferation and AGS-PKD2 — with strongly enhanced proliferation rate, in comparison with AGS and AGS-pcDNA3.1 sublines.

We also applied MTT assay to evaluate cell proliferation activity of transfected cells. With this approach we also observed the same difference between AGS-PKD1 and AGS-PKD2 sublines and AGS parental cells in 24 hs of cultivation (Fig. 5, *b*). As shown on Fig. 5, *b*, AGS-PKD2 line had much higher proliferation activity than AGS-PKD1. Thus, PKD1 expression led to the inhibition of cell proliferation, and, in contrary, PKD2 overexpression resulted in the enhancement of cell proliferation.

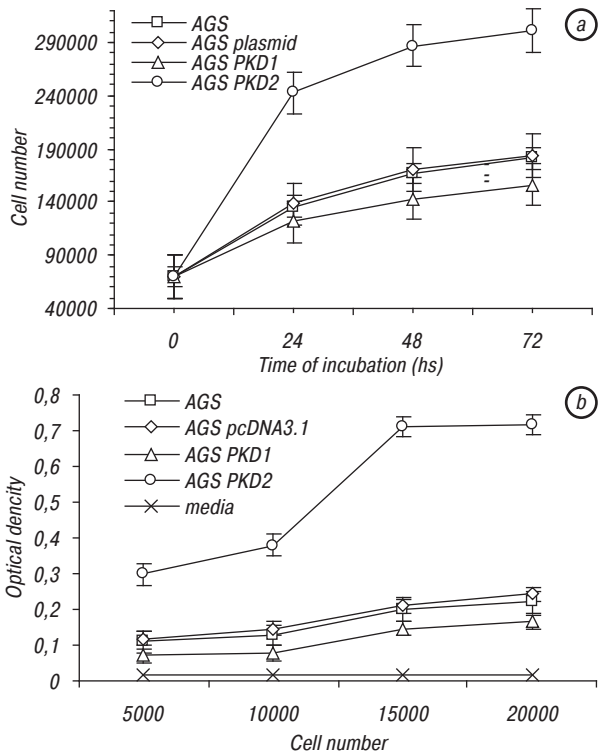

Fig. 5. Evaluation of proliferative activity of AGS, AGS-pcNDA3.1, AGS-PKD1 and AGS-PKD2 sublines. *a* — kinetics of cell number changes in cultures of AGS sublines. Results of three independent experiments. *b* — MTT assay after 24 h of cultivation of AGS sublines. Results of three independent experiments

Since it is known that PKD1 kinase is involved in regulation of cell adhesion by phosphorylation of β-catenin, integrins and cortaktin [20, 21], we carried out *in vitro* scratch and Transwell assays in order to access the migration properties of gastric cancer cell sublines that express PKD1 or PKD2. In *in vitro* scratch assay with cell cultures, expressing only PKD2 (AGS, AGS-pcDNA3.1 and AGS-PKD2), the migration of cells on the free surface of the plastic was observed already after 4 h. At the same time, for AGS-PKD1 cells, migration was not registered for the same time period (Fig. 6, *a*). This trend was maintained for 12, 24 and 48 h of observation. Even after 48 h in culture the free surface area of the scratch was only slightly decreased for AGS-PKD1 cells, while AGS-PKD2 cells culture formed 100% confluent monolayer.

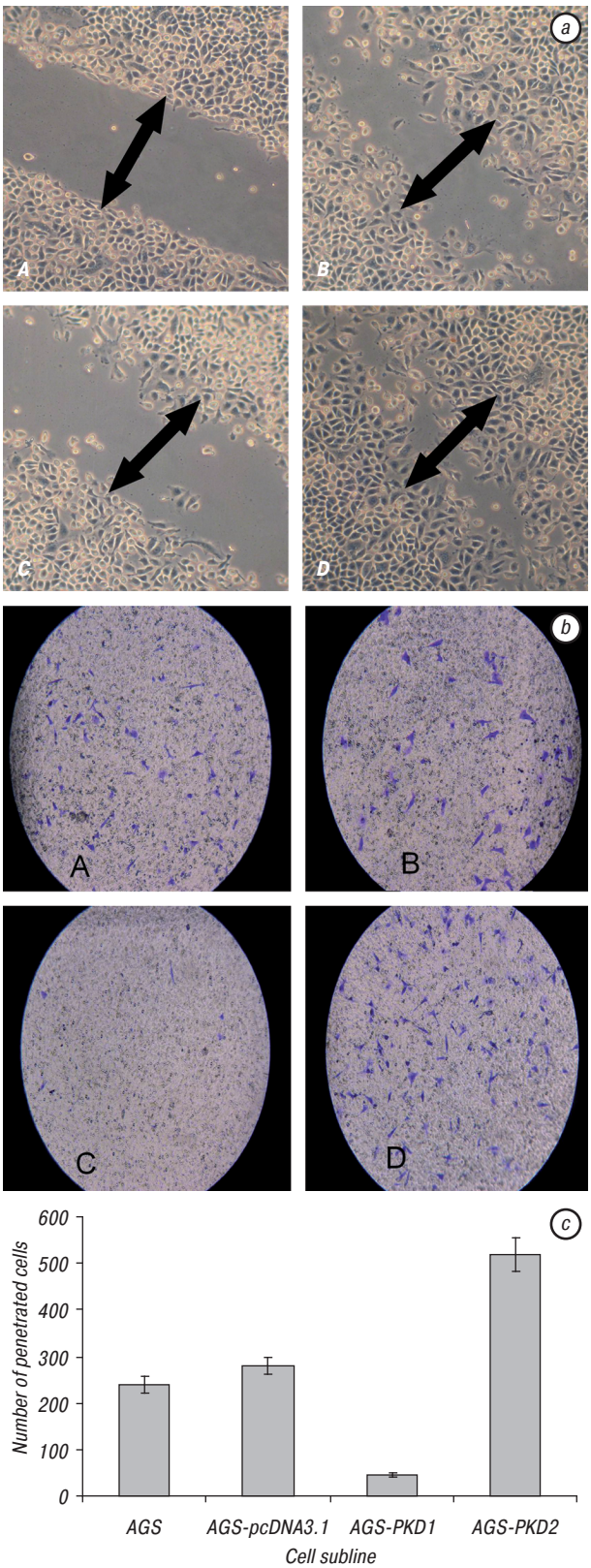

Fig. 6. Evaluation of migration capacity of AGS cell sublines. *a*. *In vitro* scratch assay. A — AGS cell line at once after applying stripes (as control). B, C, D — all sublines after 24 h of incubation. B — AGS cell line, C — AGS-PKD1, D — AGS-PKD2. x 40. The arrow shows the initial size of scratch. (AGS-pcDNA3.1 — data not shown). *b*. Transwell assay. The representative fields on membrane after 4 h of incubation. One of 5 evaluated fields for each cell subline. x40. Staining with crystal violet, cells are shown in blue, A — AGS, B — AGS-pcDNA3.1, C — AGS-PKD1, D — AGS-PKD2. *c*. Transwell assay. The level of cell migration activity estimated as a number of penetrated cells for 4 h in 5 microscopic fields at x40

Further performed Transwell assay revealed that such behavior of transfectant cells could be explained by the different migration capacity of AGS transfectants. The AGS-PKD2 transfectants had the highest level of migration activity. However, migration capacity of AGS-PKD1 cells was twice lower then of AGS and AGS-pcDNA3.1 control cells (Fig. 6, *b*, *c*).

To evaluate colony formation ability of AGS sublines we have used clonogenic assay, which reflects the potential tumorogenic capacity of cells. After two weeks of cultivation AGS cells gave rise to 128 colonies: 112 colonies with up to 20 cells/per colony and 16 colonies — more than 20 cells/per colony. In cultures of AGS-PKD1 transfectant all 72 colonies were less than 20 cells/colony. In the contrast, for AGS-PKD2 transfectant we have detected 408 colonies including 176 colonies with more than 20 cells/colony. Numbers of colonies formed by control cultures transfected with empty plasmid were close to parental AGS line. Thus, PKD1 transfection decreases colony formation capacity of AGS, while PKD2 overexpression dramatically enhances colony formation (Fig. 7).

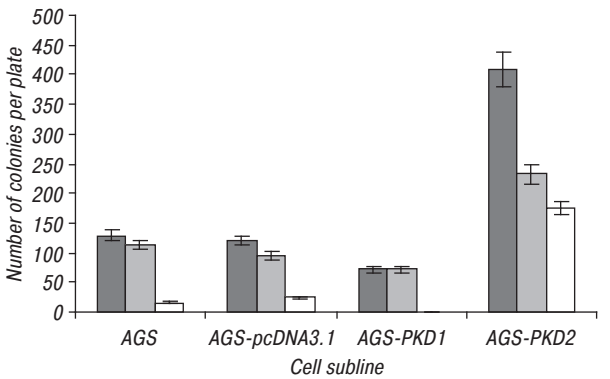

Fig. 7. Clonogenic capacity of AGS sublines. Colony formation assay in soft agar. White columns — number of colonies with up to 20 cells, grey — number of colonies with more than 20 cells, black — all colonies. Results of three independent experiments

Our study provided new evidences of PKD1 and PKD2 differential expression in gastric cancer cells and their distinct roles in regulation of tumor cell biology. We have demonstrated that primary gastric tumors possess different levels of PKD1 and PKD2 expression on mRNA and protein levels that greatly complement the data on PKD1 mRNA expression previously obtained by Kim *et al.* [11]. We also found that low PKD1 expression levels (both on mRNA and protein level) are the feature of particular histological variants of gastric cancer: signet-ring cell gastric carcinoma, undifferentiated gastric cancer, carcinoid and adenogen tumor with metastasis.

To find the potential biological consequences of differential PKD1 and PKD2 expression in gastric cancer cells we have created experimental system based on AGS cells line. We have shown that the expression of PKD1 upon transfection in AGS cell line lead to the significant reduction of endogenous PKD2 mRNA expression. Various tests were performed, including MTT and proliferation assays, *in vitro* scratch, Transwell and clonogenic assays, which revealed clear difference in morphological features, proliferation, migration and colony formation of sublines overexpressing PKD2 or expressing PKD1. Overall, PKD1 transfection led to the inhibition of cell proliferation, migration and colony formation. Meanwhile, PKD2 overexpression enhanced cell proliferation, cellular migration and colony formation capacities. Thus, our data clearly show that both down-regulation of PKD1 or upregulation of PKD2 expression may determine the behavior of tumor cells that resulted in invasive phenotype and in general poor prognosis.

Since differential expression of PKD1 and PKD2 protein kinases affects biological features of malignant cells in experimental system *in vitro*, heterogeneity in PKD1 and PKD2 expression of primary gastric tumor samples may influence the proliferative activity and migration capacity of tumor cells *in vivo*. Therefore, the studies of differential expression and activity of PKD1 and PKD2 in the context of tumor manifestation and prognosis of the disease could be the perspective subject of translational research in oncology. This will contribute to the development of new approaches to differential diagnostics of tumor and target therapy, and also reveal prognostic factors for the prediction of clinical outcome.

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