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Proteomic Profiling of Signaling Proteins in Ten Different Tumor Cell Lines

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Abstract. *Normal cell development requires a coordinated and organised reaction and adaptation to the constantly changing environment. Cells achieve this by a network of signaling pathways comprising proteins that serve as molecular switches. Subversion of these intracellular signaling pathways is implicated in several diseases, including cancer. To better understand the mechanisms of this process and to identify potential biomarkers and/or therapeutic targets at the protein level, we performed two-dimensional electrophoresis (2-DE) and mass spectrometry in ten different tumor cell lines. Following separation by high resolution 2-DE, a series of seventy signaling proteins were unambiguously identified that were differentially expressed in different cell lines. Signaling proteins of immense significance in cancer biology including two proteins of the 14-3-3 protein family, growth factor receptor bound protein 2, Cdc25B phosphatase, disheveled associated activator of morphogenesis-1, putative ORF1, zyxin, phosphatidylethanolamine-binding protein, Rho/Rab GDP-dissociation inhibitors, Stam binding protein, SH3 domain GRB2-like protein B2, Cullin homolog 3, Coronin-1B, calcium binding proteins and enzymes with signaling function displayed tumor cell line-specific expression. Other signaling proteins of importance, such as maspin, nucleoside diphosphate kinase-A, Ser/Thr kinases, Ser/Thr phosphatases, septins, annexins and receptor for hyaluronic acid-mediated motility, however, showed tumor cell line-associated expression. These data highlight that there might be specific and shared signaling pathways that are activated in the chain of events leading to tumor formation. Moreover, the data open up the possibility of developing new prognostic markers, as well as widening the avenue of cancer chemotherapy.*

Abbreviations: 2-DE, two-dimensional gel electrophoresis; AKAP, A-kinase anchoring protein; AMSH, associated molecule with the SH3 domain of Stam; ATCC, American Type Culture Collection; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate); CREC, Cab45, reticulocalbin, endoplasmic reticulum calcium binding protein, calumenin; DAAM, disheveled associated activator of morphogenesis; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal regulated kinase; GAPs, GTPase activating factor; GDI, GDP dissociation inhibitor; GRB, growth factor receptor bound protein; GEF, guanine nucleotide exchange factor; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; MAPK, mitogen activated protein kinase; JNK, Jun-amino terminal kinase; MAPKK, mitogen activated protein kinase kinase; NDK, nucleoside diphosphate kinase; NFAT, nuclear factor of activated T cells; PAK, p-21 activated kinase; PBS, phosphate-buffered saline; RGS, regulatory of G-protein signaling; RHAMM, receptor for hyaluronic acid mediated motility; SH, Src homology; PP, protein phosphatase; TGF-beta/BMP, transforming growth factor-beta/bone morphogentic protein; VDAC, voltage dependent anion channel.

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Cells interpret and respond to changes in their microenvironment by making use of a complex intracellular communication network process collectively referred to as signal transduction. Signaling molecules such as growth factors, hormones, cytokines and other molecules interact with specific cellular receptors that translate and funnel messages into intracellular signaling cascades. These cascades require scaffold proteins and the generation of second messengers that translate the general signals to specific biological responses by coupling the receptor to downstream effectors, which, in turn, convey the signal from the receptor to the target point within the cell (1). The scaffold proteins could be non-catalytic and docking proteins containing SH2/SH3 (SH, src homology) domains, such as *Grb2*; intracellular second messengers include cyclic AMP and calcium; and effector molecules could be small monomeric GTP-binding proteins (Ras GTPases), cytoplasmic Ser/Thr kinases and non-receptor-associated tyrosine kinases.

Most signal transduction events evoke particular changes in gene expression, which allow cells to respond to biological commands and changes in their surrounding. The

large category of signal transducing proteins associated with membrane-bound receptors is formed by G-proteins, which include heterotrimeric G-proteins (2) and small G-proteins (3). The G-proteins are located on the inner surface of the cell membrane and have a common feature of switching and cycling between an inactive GDP-bound form and an active GTP-bound form. Whilst heterotrimeric, G-proteins couple signals received from activated plasma membrane receptors mainly to cytoplasmic second messengers; small G-proteins transduce signals into the cytoplasm *via* effector pathways that regulate cell growth, differentiation and apoptosis (4).

Cancer development depends on aberrant activation of signal transduction pathways that: (a) control cell growth and survival, (b) play important roles in embryonic development, (c) defy regulatory processes and (d) lead to uncontrolled signal casacade activity (5). Phosphorylation of proteins is the major intracellular regulatory mechanism in eukaryotic cells and the level of phosphorylation is tightly regulated by the concerted action of protein kinases and protein phosphatases (6). Thus, dysregulation in the activity of any one of the players may lead to cellular transformation (7). The cell cycle is one of the rigorously controlled cellular proceses and its correct execution is essential for the maintenance of genomic integrity and, therefore, for tumor suppression. Checkpoint mechanisms guarantee that the next cell cycle phase is only entered after error-free completion of the previous stage (8). An understanding of the mechanisms responsible for the control of normal proliferation and differentiation of the various cell types that make up the human body thus will undoubtedly allow greater insights into the abnormal growth of malignant cells. Particular attention is now focused on the signals that act as positive mediators and negative regulators of cell growth and function in normal and abnormal cells (9). In this regard, many studies have been published that demonstrate the relevance of a certain signaling molecule in tumor biology. However, most studies use either a single cell line or related cell lines. In the present study, an attempt was made to provide a comprehensive expressional profile of signaling molecules in ten different cell lines by implementing the proteomic approach using two-dimensional electrophoresis (2-DE) coupled with matrix-assisted laser desorption/ionization-mass spectrometry (MALDI/ MS). Our goal was to identify potential tumor-specific and/or tumor-associated biomarker candidates and possible novel molecular targets for cancer therapy.

Materials and Methods

Cell culture. Ten different tumor cell lines were purchased from the American Type Culture Collection (ATCC). The cell lines and their ATCC no. are given in Table I.

Table I. *Identification of investigated cell lines (ATCC)[1].*

Name	ATCC no.	Cancer type
Saos-2	HTB-85	Osteosarcoma
SK-N-SH	HTB-11	Neuroblastoma
HCT-116	CCL-247	Colorectal carcinoma
CaOva3	HTB-75	Adenocarcinoma of ovary
A-549	CCL-185	Alveolar cell carcinoma of lung
HL-60	CCL-240	Promyelocytic leukemia
A-673	CRL-1598	Rhabdomyosarcoma
A-375	CRL-1619	Malignant melanoma
MCF-7	HTB-22	Adenocarcinoma of mammary gland
Hela	CCL-2	Adenocarcinoma of cervix

[1]Data on cell lines published previously (10).

The SK-N-SH and Hela cervix cell lines were cultured in Minimum Essential Medium (Eagle) with 2 mM L-glutamine and Earle's Basic Salt Solution (BSS) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate, with 10% fetal bovine serum (FBS). The same conditions were used to culture the MCF-7 cell line, except for supplementing 10% FBS with 90% 0.01 mg/ml bovine insulin. The HCT 116 and Saos-2 cell lines were cultured in McCoy's 5a medium with 90% 1.5 mM L-gluatmine and 10% FBS. The A-549 cell line was cultured with Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 10% FBS. The HL-60 cell line was cultured with Iscove's modified Dulbecco's medium with 4 mM L-glutamine adjusted to contain 80% 1.5 g/L sodium bicarbonate and 20% FBS. The A-673, CaOva-3 and A-375 cell lines were cultured in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5g/L glucose with 10% FBS.

The cell cultures were maintained in a humidified atmosphere of 5% v/v CO_2 in air at 37˚C.

Sample preparation. Harvested cells were washed three times with 10 mL PBS (phosphate buffered saline) (Gibco BRL, Gaithersburg, MD, USA) and centrifuged for 10 min at 800 g at room temperature. The supernatant was discarded and the pellet was suspended in 1.0 ml of sample buffer consisting of 40 mM Tris, 7 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (Sigma), 65 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (ethylenediaminetraacetic acid) (Merck), protease inhibitors complete (Roche, Basel, Switzerland) and 1 mM phenylmethyl-sulfonyl chloride. The suspension was sonicated for approximately 3 sec. After homogenisation, the samples were left at room temperature for 1h and centrifuged at 14,000 rpm for 1h. The supernatant was transferred into an Ultrafree-4 centrfugal filter unit (Millipore, Bedford, MA, USA), for desalting and concentrating the proteins. The protein content of the supernatant was quantified by the Bradford protein assay system (10). The standard curve was generated using bovine serum albumin and absorbance was measured at 595 nm.

Two-dimensional gel electrophoresis (2-DE). Samples prepared from each cell line were subjected to 2-DE as described elsewhere (11). One mg protein was applied on immobilized pH 3-10 nonlinear

gradient strips in sample cups at their basic and acidic ends. Focusing was started at 200 V and the voltage was gradually increased to 5000 V at a rate of 3 V/min and then kept constant for a further 24 h (approximately 180,000 Vh totally). After the first dimension, the strips (13 cm) were equilibrated for 15 min in the buffer containing 6 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. After equilibration, the strips were loaded on 9-16% gradient sodium dodecylsulfate polyacrylamide gels for second-dimensional separation. The gels (180 x 200 x 1.5 mm) were run at 40 mA. Immediately after the second dimension run, the gels were fixed for 18 h in 50% methanol, containing 10% acetic acid, then stained with Colloidal Coomassie Blue (Novex, San Diego, CA, USA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Biorad Laboratories, Hercules, CA, USA) covering the range 10-250 kDa. pI values were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water and the gels were scanned with an ImageScanner (Amersham Bioscience).

Electronic images of the gels were recorded using Adobe Photoshop and Microsoft Power Point Softwares.

Matrix-assisted laser desorption/ionization mass spectrometry. Spots were excised with a spot picker (PROTEINEER sp™, Bruker Daltonics, Germany), placed into 96-well microtiter plates and in-gel digestion and sample preparation for MALDI analysis were performed by an automated procedure (PROTEINEER dp™, Bruker Daltonics) (12, 13). Briefly, the spots were excised and washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, the gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. The dried gel pieces were reswollen with 40 ng/μl trypsin (Roche Diagnostics, Penzberg, Germany) in enzyme buffer (consisting of 5 mM octyl β-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated for 4 h at 30˚C. Peptide extraction was performed with 10 μl of 1% TFA in 5 mM OGP. The extracted peptides were directly applied onto a target (AnchorChip™, Bruker Daltonics) that was loaded with α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) matrix thinlayer. The mass spectrometer used in this work was an Ultraflex™ TOF/TOF (Bruker Daltonics) operated in the reflector for MALDI-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF/TOF with a fully automated mode using the FlexControl™ software. An accelerating voltage of 25 kV was used for PMF. Calibration of the instrument was performed externally with $[M+H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin and adrenocorticotropic hormones (clip 1-17 and clip 18-39). Each spectrum was produced by accumulating data from 200 consecutive laser shots. Those samples which were analysed by PMF from MALDI-TOF were additionally analysed using LIFT-TOF/TOF MS/MS from the same target. A maximum of three precursor ions per sample were chosen for MS/MS analysis. In the TOF1 stage, all ions were accelerated to 8 kV under conditions promoting metastable fragmentation. After selection of jointly migrating parent and fragment ions in a timed ion gate, the ions were lifted by 19 kV to high potential energy in the LIFT cell. After further acceleration of the fragment ions in the second ion source, their masses could be simultaneously analysed in the reflector with high sensitivity. PMF and LIFT spectra were interpreted with the Mascot software (Matrix Science Ltd, London, UK). Database searches, through Mascot, using combined PMF and MS/MS datasets were performed *via* BioTools 2.2 software (Bruker Daltonics). A mass tolerance of 100 ppm and two missing cleavage sites for PMF and MS/MS tolerance of 0.5 Da and one missing cleavage site for MS/MS search were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as the criterion for correct identification. The algorithm used for determining the probability of a false-positive match with a given mass spectrum is described elsewhere (14).

Results

Proteins derived from different tumor cell lines were separated by 2-DE and the protein spots were visualised with Coomassie Blue staining. The spots were picked, processed following in-gel digestion and identified by mass spectrometry. Bioinformatics tools were also used to mine databases to match the peptide mass with the theoretical peptide mass. Internal standards were used to correct the measured peptide mass, thus reducing the windows of mass tolerance and increasing the confidence of identification. A number of differentially expressed signaling proteins were identified and presented in Table II with their SWISS-PROT accession number, relative molecular weight, pI values and mass spectroscopical data (observed molecular weight, total score and number of peptide matches). Figures 1-10 represent maps of the identified signaling proteins in the individual cell lines where 1 mg of total protein was applied. The maps revealed tumor-specific as well as tumor-associated expression patterns for signaling proteins.

G-proteins. Various G-proteins that belong to the heterotrimeric G-protein and small G-protein subfamilies, and their interacting proteins, had been identified by 2-DE/MALDI-MS. The heterotrimeric G-proteins and their interacting proteins, except for *guanine nucleotide-binding protein beta subunit 2-like 1* (HL60-specific), did not show cell line-specific expression. *Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1 and RGS (regulatory of G-protein signaling)-19-interacting protein 1* were expressed in two different cell lines. Compared to the heterotrimeric G-proteins, although the small G- proteins appeared to display promiscous expression, they showed cell line-specific expression. Whilst *Rho GDI-2* (HL60) (Figure 6) and *Rab GDI-alpha* (SK-N-SH) (Figure 2) were expressed cell line-specifically, respectively, *Rab GDI-beta, Ran specific GTPase activating protein, Rho GDI-1, GTP binding nuclear protein Ran, Ras GTPase activating protein-1 and Coronin-1B* were expressed in two to five cell lines.

Signaling enzymes and their interacting proteins. This group comprises enzymes involved in signaling pathways, including kinases, phosphatases, proteases, lipases and ATPases and their interacting proteins. Not unlike the G-proteins group,

Table II. *Mass spectroscopical identification of signaling proteins in ten different investigated tumor cell lines. (+, detected; -, undetected, each + represents one spot)*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
P16885	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma 2	147,937	6.3	6.8	-	-	-	-	-	-	+ OMW*1: 47,225 TS*2: 74 PM*3: 23	-	-	-
P61981	14-3-3 protein gamma	28,171	4.8	4.9 4.6	-	-	-	-	-	-	-	+ OMW: 28,325 TS: 112 PM: 19	-	+ OMW: 28,352 TS: 67 PM: 11
P31947	14-3-3 protein sigma	27,774	4.7	4.3	-	-	-	-	-	-	-	-	-	+ OMW: 27,871 TS: 172 PM: 32
P63104	14-3-3 protein zeta/delta	27,745	4.7	4.5	-	+ OMW: 25,069 TS: 117 PM: 16	-	-	-	-	-	-	-	-
Q9Y3F5	A6 related protein	39,548	6.4 6.9	7.0	+ OMW: 39,751 TS: 112 PM: 15	++ OMW: 39,751 TS: 74 PM: 12	-	-	-	-	-	-	-	-
Q01518	Adenyl cyclase-associated protein	51,542	8.1	7.1 7.2 7.4	-	-	-	+ OMW: 51,926 TS:72 PM: 16	-	-	++ OMW: 51,926 TS: 247 PM: 41	-	+ OMW: 51,926 TS: 125 PM: 21	-
Q96DH3	Alpha isoform of regulatory subunit A, protein phosphatase 2 Similar to isoform of (P30153) (99% identity)	65,309	5.0	5.0	-	-	-	-	-	-	-	-	+ OMW: 66,065 TS: 305 PM: 43	-

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Table II *continued*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
O00170	Aryl hydrocarbon-receptor interacting protein	37,664	6.1	6.9	-	-	-	-	-	-	+ OMW: 38,096 TS: 97 PM: 15	-	-	-
Q99996	A-kinase anchor protein 9	453,667	5.0	6.9 7.0	-	-	-	+ OMW: 455,725 TS: 138 PM: 57	-	-	-	-	-	+ OMW: 455,725 TS: 101 PM: 44
O95630	AMSH (synonomous: STAM binding protein)	48,077	5.9	6.3	-	-	-	-	-	-	-	-	+ OMW: 48,617 TS: 116 PM: 18	-
P04083	Annexin A1	38,583	6.6	7.0 7.1 7.2 6.5 6.6	+ OMW: 38,918 TS: 119 PM: 19	+ OMW: 38,787 TS: 101 PM: 17	+++ OMW: 38,787 TS: 286 PM: 35	++ OMW: 35,246 TS: 246 PM: 19	-	-	+++ OMW: 35,246 TS: 156 PM: 20	+ OMW: 38,787 TS: 191 PM: 20	-	++ OMW: 38,787 TS: 277 PM: 32
				6.7 6.8 6.9 5.1							OMW: 38,787 TS: 237 PM: 26			
P07356	Annexin A2	38,545	7.5	6.6 7.0 7.2 7.3	-	+ OMW: 38,677 TS: 86 PM: 14	-	-	++ OMW: 41,670 TS: 142 PM:22 OMW: 38,677 TS: 131 PM: 18	-	-	-	-	+ OMW: 38,677 TS: 181 PM: 23
P12429	Annexin A3	36,375	5.6	5.9 5.6 6.3	-	-	-	+ OMW: 36,393 TS: 108 PM: 16	+ OMW: 36,393 TS: 300 PM: 32	-	-	-	+ OMW: 36,393 TS: 135 PM: 18	+ OMW: 36,393 TS: 332 PM: 36

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Table II *continued*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
P09525	Annexin A4	35,752	5.9	5.9 5.7	-	+ OMW: 35,957 TS: 94 PM: 18	-	-	-	+ OMW: 35,957 TS: 92 PM: 22	-	-	+ OMW: 35,957 TS: 257 PM: 26	-
P08758	Annexin A5	35,806	4.9	4.8 4.5 4.6 4.7 5.2	-	+ OMW: 35,125 TS: 166 PM: 29		+ OMW: 35,125 TS: 279 PM: 33	+ OMW: 35,125 TS: 145 PM: 20	-	+ OMW: 35,125 TS: 261 PM: 37	+ OMW: 35,840 TS: 283 PM: 35	-	+ OMW: 35,125 TS: 146 PM: 37
P20073	Annexin A7	50,316	6.3	6.9 6.3 6.8 7.0	-	-	-	-	+ OMW: 50,569 TS: 135 PM: 23	-	+ OMW: 50,569 TS: 96 PM: 21	-	+ OMW: 50,569 TS: 139 PM: 24	+ OMW: 50,569 TS: 215 PM: 36
Q9P129	Calcium-binding transporter [Fragment]	45,819	5.3	5.9 6.2	-	-	-	-	-	-	-	-	++ OMW: 46,075 TS: 158 PM: 25	-
P20807	Calpain 3	94,254	5.8	7.3	-	-	-	-	-	-	+ OMW: 94,364 TS: 65 PM: 17	-	-	-
O43852	Calumenin, precursor	37,107	4.5	4.4 4.3	+ OMW: 37,164 TS: 76 PM: 14	-	-	-	-	+ OMW: 37,198 TS: 123 PM: 19	+ OMW: 37,198 TS: 124 PM: 18	-	-	-
P07339	Cathepsin D	44,552	6.1	5.1 5.3 5.8	++ OMW: 26457 TS: 148 PM: 19	-	-	-	-	++ OMW: 26457 TS: 170 PM: 24	-	-	+ OMW: 45,037 TS: 226 PM: 34	-
O43550	Cell division cycle protein 25B phosphatase [Fragment]	33,858	6.3	5.1 8.8	-	-	-	-	-	-	+ OMW : 34,179 TS : 66 PM: 11	-	-	-

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Table II *continued*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
O00299	Chloride intracellular channel protein 1	26,792	5.1	4.9 5.1 5.2 5.3 5.4 4.7	++ OMW: 23,813 TS: 95 PM: 8 OMW: 27,249 TS: 166 PM: 18	-	-	-	+ OMW: 27,249 TS: 172 PM: 18	+ OMW: 27,248 TS: 152 PM: 17	+ OMW: 27,248 TS: 201 PM: 21	+ OMW : 27,249 TS: 172 PM: 19	-	+ OMW: 27,248 TS: 220 PM: 21
O95833	Chloride intracellular channel protein 3	26,648	6.0	6.4	-	-	-	+ OMW: 23,611 TS: 82 PM: 10	-	-	-	-	-	-
Q9Y677	COP9 complex subunit 4	46,198	5.6	5.6	-	+ OMW: 46,454 TS: 101 PM: 20	-	-	-	-	-	-	+ OMW: 46,454 TS: 289 PM: 37	-
Q9BR76	Coronin-1B	54,235	5.6	5.8	-	+ OMW: 54,885 TS: 87 PM: 184	-	-	-	-	-	-	+ OMW : 54,885 TS: 145 PM: 20	-
Q13618	Cullin homolog 3	88,930	8.7	6.5	-	-	-	-	-	-	-	-	+ OMW: 89,249 TS: 73 PM: 18	-
Q9UP65	Cytosolic phospholipase A2 gamma	60,949	6.5	7.3	-	-	-	+ OMW: 61,309 TS: 63 PM: 15	-	-	-	-	-.	-
Q16555	Dihydropyrimidinase related protein-2	62,294	6.0	5.9	-	-	-	-	+ OMW: 62,711 TS: 165 PM: 18	-	-	-	-	-

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Table II *continued*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
Q14195	Dihydropyrimidinase related protein 3	61,963	6.0	6.2	-	+ OMW: 62,323 TS: 108 PM: 24	-	-	-	-	-	-	-	-
Q9Y4D1	Disheveled associated activator of morphogenesis 1	123,473	6.8	7.3	-	-	-	-	-	-	-	-	-	+ OMW: 124,737 TS: 117 PM: 29
P78527	DNA-dependent protein kinase catalytic subunit	469,089	6.8	7.0	-	-	-	-	-	-	-	+ OMW: 470,227 TS: 69 PM: 37	-	-
P36507	Dual specificity mitogen-activated protein kinase kinase 2	44,424	6.1	6.5 6.3 6.8	-	-	-	-	-	+ OMW: 44,681 TS: 73 PM: 14	-	-	-	+ OMW: 44,681 TS: 146 PM: 24
P62993	Growth factor receptor-bound protein 2	25,206	5.9	6.2	-	-	-	-	-	-	-	-	-	+ OMW: 21,489 TS: 123 PM: 15
P62826	GTP-binding nuclear protein Ran	24,423	7.0	7.0 7.1 7.2 7.3	-	+ OMW: 19,524 TS: 72 PM: 10	-	+ OMW: 20,728 TS: 78 PM: 12	+ OMW: 20,728 TS: 149 PM: 16	-	-	-	-	++ OMW: 20,728 TS: 182 PM: 20 OMW: 19,524 TS: 144 PM: 16
P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	37,377	5.6	5.5 5.6	+ OMW: 38,151 TS: 67 PM: 14	-	-	-	-	-	-	-	+ OMW: 38,151 TS: 84 PM: 11	-

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Table II *continued*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
P63244	Guanine nucleotide-binding protein beta subunit 2-like 1	35,077	7.6	7.3	-	-	-	-	-	+ OMW: 35,511 TS: 119 PM: 19	-	-	-	-
O75330	Hyaluronan mediated motility receptor	84,031	5.6	6.3 4.8 6.2	-	-	-	-	+ OMW: 84,545 TS: 73 PM: 13	-	-	++ OMW: 84,557 TS: 102 PM: 22	+ OMW: 84,545 TS: 82 PM: 17	-
P36952	Maspin [Precursor]	42,138	5.7	5.7 6.3	-	-	+ OMW: 42,568 TS: 114 PM: 16	-	-	-	-	-	-	+ OMW: 42,568 TS: 183 PM: 21
Q09666	Neuroblast differentiation associate protein AHNAK [fragment]	312,493	6.3	7.3 6.9 6.5 5.8	-	+ OMW: 312,580 TS: 61 PM: 35	-	++ OMW: 180,065 TS: 104 PM: 35	-	+ OMW: 180,065 TS: 65 PM: 26	-	-	-	-
Q8N4C6	Ninein	243,290	5.0	6.5 6.1	-	+ OMW: 23,937 TS: 64 PM: 9	-	-	-	+ OMW: 240,477 TS: 90 PM: 33	-	-	-	-
P15531	Nucleoside diphosphate kinase A	17,149	5.8	6.0 6.5 5.3 5.8 5.9	+ OMW: 17,309 TS: 108 PM: 12	+ OMW: 17,309 TS: 73 PM: 11	-	+ OMW: 17,309 TS: 94 PM: 13	+ OMW: 17,309 TS: 144 PM: 16	-	-	-	+ OMW: 17,309 TS 220 PM 27	+ OMW: 17,309 TS: 190 PM: 24
Q60518	ORF1; putative (synonymous: fibroblast growth factor-5)	4,338	10.3	5.8	-	+ OMW: 4392 TS: 63 PM: 4	-	-	-	-	-	-	-	-
Q8WUM4	Programmed cell death 6-interacting protein	96,023	6.1	6.7 6.6 6.5 6.8	-	+ OMW: 96,563 TS: 111 PM: 21	+ OMW: 96,563 TS: 214 PM: 38	+ OMW: 96,646 TS: 193 PM: 31	-	-	+ OMW: 96,646 TS: 247 PM: 41	-	+ OMW: 96,563 TS: 160 PM: 32	-

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Table II *continued*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
Q9UQ80	Proliferation-associated protein 2G4	43,787	6.1	6.6 6.8 7.1	-	-	-	+ OMW: 41,679 TS: 108 PM: 24	-	+ OMW: 44,101 TS: 109 PM: 21	+ OMW: 44,101 TS: 261 PM: 37	-	+ OMW: 44,101 TS: 214 PM: 32	+ OMW: 44,101 TS: 108 PM: 24
P30086	Phosphatidylethanolamine-binding protein	20,926	7.4	7.4	-	-	-	-	-	-	+ OMW: 20,443 TS: 92 PM: 11	-	-	-
Q07161	Protein phosphatase type 1 catalytic subunit Similar to isoform of P2136 (96% identity)	38,631	6.2	6.6	-	-	-	-	-	-	-	-	+ OMW: 39,405 TS: 115 PM: 15	-
P31150	Rab GDP dissociation inhibitor alpha	50,583	5.0	4.2	-	+ OMW: 51,117 TS: 123 PM: 16	-	-	-	-	-	-	-	-
P50395	Rab GDP dissociation inhibitor beta	50,663	6.1	6.2 6.3 6.6	-	-	-	-	+ OMW: 51,088 TS: 263 PM: 37	-	-	-	++ OMW: 51,088 TS: 444 PM: 48	-
P43487	Ran-specific GTPase activating protein	23,310	5.2	5.3 5.1 4.5	-	-	-	-	-	+ OMW: 23,396 TS: 76 2M: 12	-	+ OMW: 23,396 TS: 76 PM: 13	-	+ OMW: 23,467 TS: 91 PM: 14
Q13283	Ras-GTPase-activating protein binding protein 1	52,164	6.4	5.4 5.2 5.5 5.6	-	+ OMW: 52,189 TS: 84 PM: 19	-	-	+ OMW: 52,189 TS: 165 PM: 18	-	+ OMW: 52,189 TS: 101 PM: 15	-	+ OMW: 52,189 TS: 131 PM: 20	+ OMW: 52,189 TS: 103 PM: 20
O14908	RGS19-interacting protein 1	36,049	5.9	6.1 6.0	-	+ OMW: 28,399 TS: 122	-	-	-	-	-	-	-	+ OMW: 36,141 TS: 123

continued on the next page

Table II *continued*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
						PM: 18								PM: 11
Q15293	Reticulocalbin 1 (precursor)	38,890	4.9	7.0	-	-	-	-	-	-	-	+ OMW: 38,866 TS: 91 PM: 14	-	-
Q96D15	Reticulocalbin 3 [Precursor]	37,493	4.7	4.5	-	-	-	+ OMW: 37,470 TS: 114 PM: 18	-	-	-	-	-	-
P52565	Rho GDP-dissociation inhibitor 1	23,207	5.3	4.8 4.9 4.7 5.1	+ OMW: 20,571 TS: 67 PM: 7	-	-	-	+ OMW: 20,571 TS: 146 PM: 18	+ OMW: 19,475 TS: 64 PM: 21	-	-	-	+ OMW: 20,571 TS: 80 PM: 9
P52566	Rho GDP dissociation inhibitor 2	22,988	5.1	5.1	-	-	-	-	-	+ OMW: 23,031 TS: 143 PM: 20	-	-	-	-
P49903	Selenide,water dikinase 1	42,911	5.7	6.2 6.0 5.8 5.7	-	-	-	-	-	+ OMW : 43,396 TS: 93 PM: 25	+ OMW: 46,512 TS: 88 PM: 15	+ OMW: 43,396 TS: 79 PM: 22	+ OMW : 43,396 TS 128 PM 30	-
Q15019	Septin 2	41,487	6.2	6.7 6.6 7.1	-	+ OMW : 41,689 TS : 151 PM: 26	-	-	-	-	+ OMW: 41,689 TS: 150 PM: 19	+ OMW: 41,689 TS: 128 PM: 24	-	+ OMW: 41,689 TS: 241 PM: 33
Q92599	Septin 8	55,756	5.9	6.2 6.0	-	+ OMW: 58,318 TS: 77 PM: 16	-	-	-	-	-	-	+ OMW: 58,318 TS: 178 PM: 26	-

continued on the next page

Table II *continued*

Acces. no	Protein name	TMW[1]	TIP[2]	OIP[3]	Saos-2	SK-N-SH	HCT 116	CaOva3	A-549	HL-60	A-375	A-673	MCF-7	Hela
Q9NVA2	Septin 11	49,398	6.4	7.0 7.1 6.9 6.2	+ OMW: 49,652 TS: 133 PM: 21	++ OMW: 49,652 TS: 93 PM: 14	+ OMW: 49,652 TS: 114 PM: 16	-	-	-	+ OMW: 34,179 TS: 66 PM: 11	-	++ OMW : 58,318 TS: 178 PM: 26 OMW: 49,652 TS: 180 PM: 24	-
Q13177	Serine/threonine-protein kinase PAK2	58,005	5.7	5.5 6.5 6.0	-	+ OMW: 55,166 TS: 85 PM: 15	-	-	-	-	-	-	+ OMW: 55,166 TS: 77 PM:17	+ OMW: 55,166 TS: 133 PM: 23
P62136	Serine/threonine protein phosphatase PP1-alpha catalytic subunit	37,512	5.9	6.0 6.1 6.5	-	+ OMW: 38,229 TS: 167 PM: 23	-	+ OMW: 35,844 TS: 127 PM: 18	-	-	-	+ OMW: 38,229 TS: 178 PM: 23	-	-
P30153	Serine/threonine protein phosphatase 2A, 65 kDa regulatory subunit A, alpha subunit	65,092	5.0	4.6 4.8	-	+ OMW: 65,934 TS: 181 PM: 30	-	-	+ OMW: 65,849 TS: 202 PM: 34	-	-	-	-	-
P36873	Serine/threonine protein phosphatase PP1-gamma catalytic subunit	36,984	6.1	6.4	-	-	-	-	-	-	-	-	+ OMW: 37,701 TS: 162 PM: 18	-
P62140	Serine/threonine protein phosphatase PP1-beta catalytic subunit	37,187	5.8	6.1 5.9	-	+ OMW: 37,961 TS: 122 PM: 16	-	-	-	-	-	-	+ OMW: 37,961 TS: 207 PM: 26	-

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this group of proteins also displayed cell line-specific and cell line-associated patterns of expression. Among the five identified signaling enzyme-interacting proteins, there were two proteins with SH3 domains: *growth factor receptor bound protein (GRB)-2 and SH3 domain GRB2-like protein B2 (syn: Endophilin B2)* that exhibited cell line-specific expression,

Table II *continued*

Q9NR46	SH3 domain GRB2-like protein B2	43,974	5.7 5.8		-	-	-	-	-	-	-	-	+ OMW: 44,175 TS: 72 PM: 12	-
P16949	Stathmin	17,171	5.8	5.7 5.5 5.9	-	-	-	-	+ OMW: 17,161 TS: 149 PM: 13	+ OMW: 17,292 TS: 91 PM: 11	-	+ OMW: 17,161 TS: 66 PM: 8	+ OMW: 17,161 TS: 145 PM: 14	-
P46459	Vesicle fusing ATPase	82,654	6.4	6.9	-	-	-	-	-	-	-	-	+ OMW: 83,113 TS: 161 PM: 29	-
P21796	Voltage-dependent anion-selective channel protein 1	30,641	8.6	9.2 7.4 7.7	-	+ OMW: 30,737 TS: 150 PM: 16	-	-	+ OMW: 30,737 TS: 169 PM: 21	+ OMW: 30,737 TS: 69 PM: 10	-	-	+ OMW: 30,737 TS 178 PM 21	+ OMW: 30,737 TS: 176 PM: 22
P45880	Voltage-dependent anion-selective channel protein 2	38,093	6.3	7.4 7.3	-	-	+ OMW: 32,060 TS: 75 PM: 9	-	-	-	-	-	-	+ OMW: 32,088 TS: 122 PM: 29
Q15942	Zyxin	61,277	6.2	6.7	-	+ OMW: 62,436 TS: 126 PM: 23	-	-	-	-	-	-	-	-

Saos-2: **bone osteosarcoma**, SK_N_SH: **brain neuroblastoma**, HCT 116: **adenocarcinoma of colon**, CaOva-3: **adenocarcinoma of ovary**, **A-549:** alveolarcarcinoma of lung, HL-60: **peripheral blood; promyelocyte leukemia**, A_375: **skin malignant melanoma**, A_673: **muscle rhabdomyosarcoma**, MCF-7: **breast cancer** , Hela cervix: **cervix adenocarcinoma**

[1]Theoretical molecular weight; [2]Theoretical isoelectric point; [3]Observed isoelectric point; Mascot-search-MS-results: *[1]Observed molecular weight; *[2]Total score; *[3]Number of matched peptides

with *GRB-2* being expressed only in the Hela cell line and GRB2-like protein B2 in the MCF-7 cell line (Figure 9). Adenyl cyclase-associated protein and A-kinase anchoring protein (AKAP)-9 were expressed in several cell lines. AKAPs are proteins that bind to regulatory subunits of cAMP-dependent protein kinase A to direct the kinase to discrete intracellular locations (15).

DNA-dependent protein kinase catalytic subunit is the only member of the kinase family expressed in a cell line-specific manner (A-673). The other kinases such as *nucleoside diphosphate kinase (NDK-A), Ser/Thr protein kinase PAK (p21-activated kinase)-2, dual specificity mitogen-activated protein kinase kinase 2 (MAPKK) and A6-related protein,* also known as protein tyrosine kinase 9-like, were identified in several cell lines, with NDK-A being expressed in six individual cell lines.

Unlike most kinases, several protein phosphatases (PPs) did show cell line-specific expression. PP1 *gamma-catalytic subunit* (Figure 9), *Cdc25B phosphatase* (Figure 7) and PP1 *catalytic subunit* were expressed in a single cell line . As

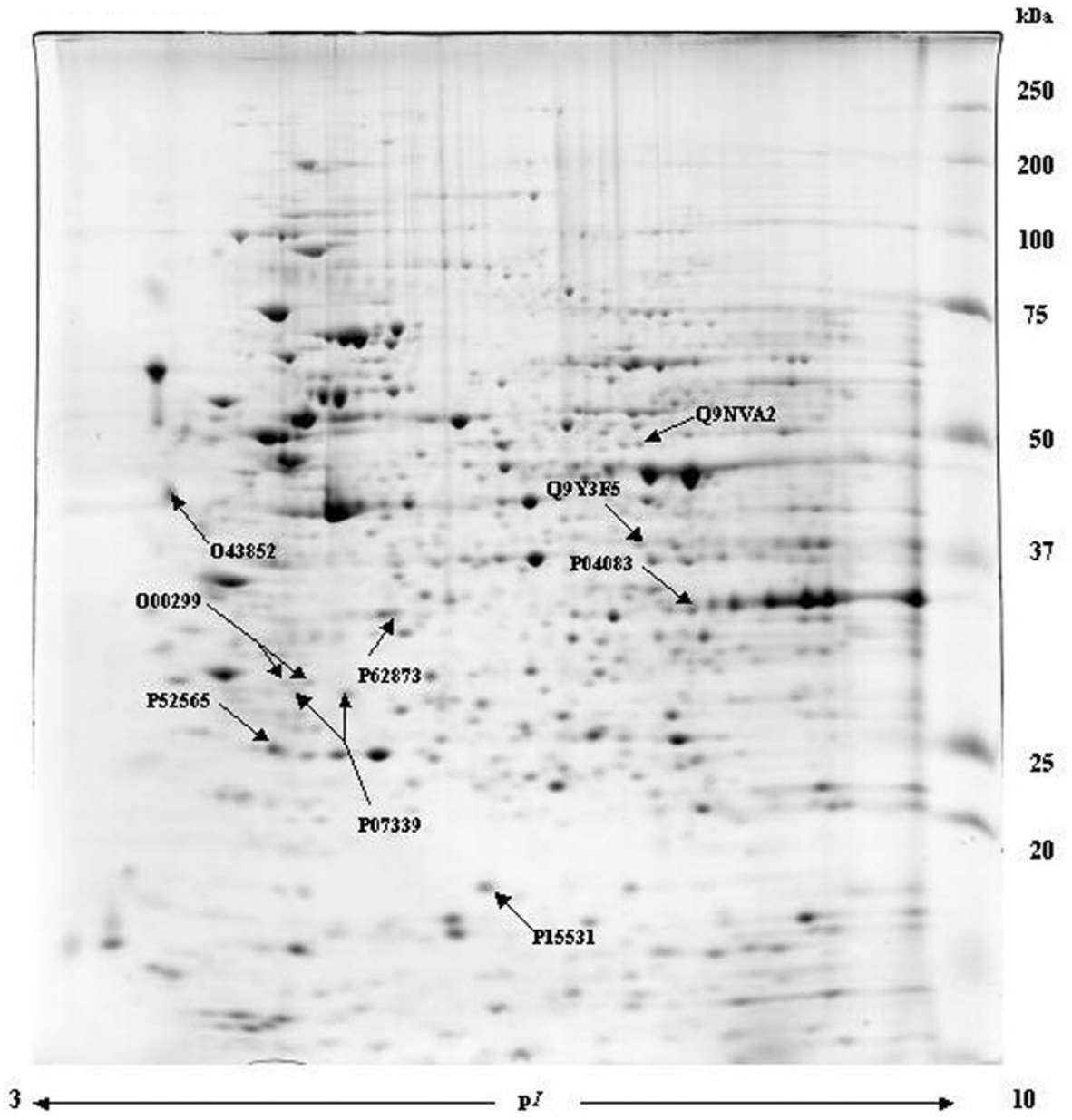

Figure 1. *Identified signaling proteins of Saos-2 cell line.*

shown by blast-search sequence alignments, the *PP1* catalytic subunit is an isoform of the *Ser/Thr PP1 alpha-catalytic subunit* with 96% sequence-identity and the alpha isoform of regulatory subunit A, protein phosphatase is also an isoform of Ser/Thr PP 2A with 99% identity. Both of these isoforms are expressed specifically in MCF-7 (Figure 9).

The serine protease, maspin and a cysteine protease cathepsin D were expressed in two and three cell lines, respectively. By contrast, A-375 cell line-specific expression was noted for the protease *calpain-3* (Figure 7) as well as the lipase, *cytosolic phospholipase A2 gamma* (Figure 4) and *1-phosphatidylinositol-4,5 biphosphate phophodiesterase gamma 2* (Figure 7). On the other hand, MCF-7 cell line-specific expression was observed for the *vesicle fusing ATPase* (Figure 9). Both proteins of the amidohydrolase family, *dihydropyrimidinase-related protein-2* and -3, showed cell-line specific expression.

Calcium binding proteins. The proteins involved in regulating intracellular calcium concentration by binding to the cation are EF-hand motif proteins that are present either in the

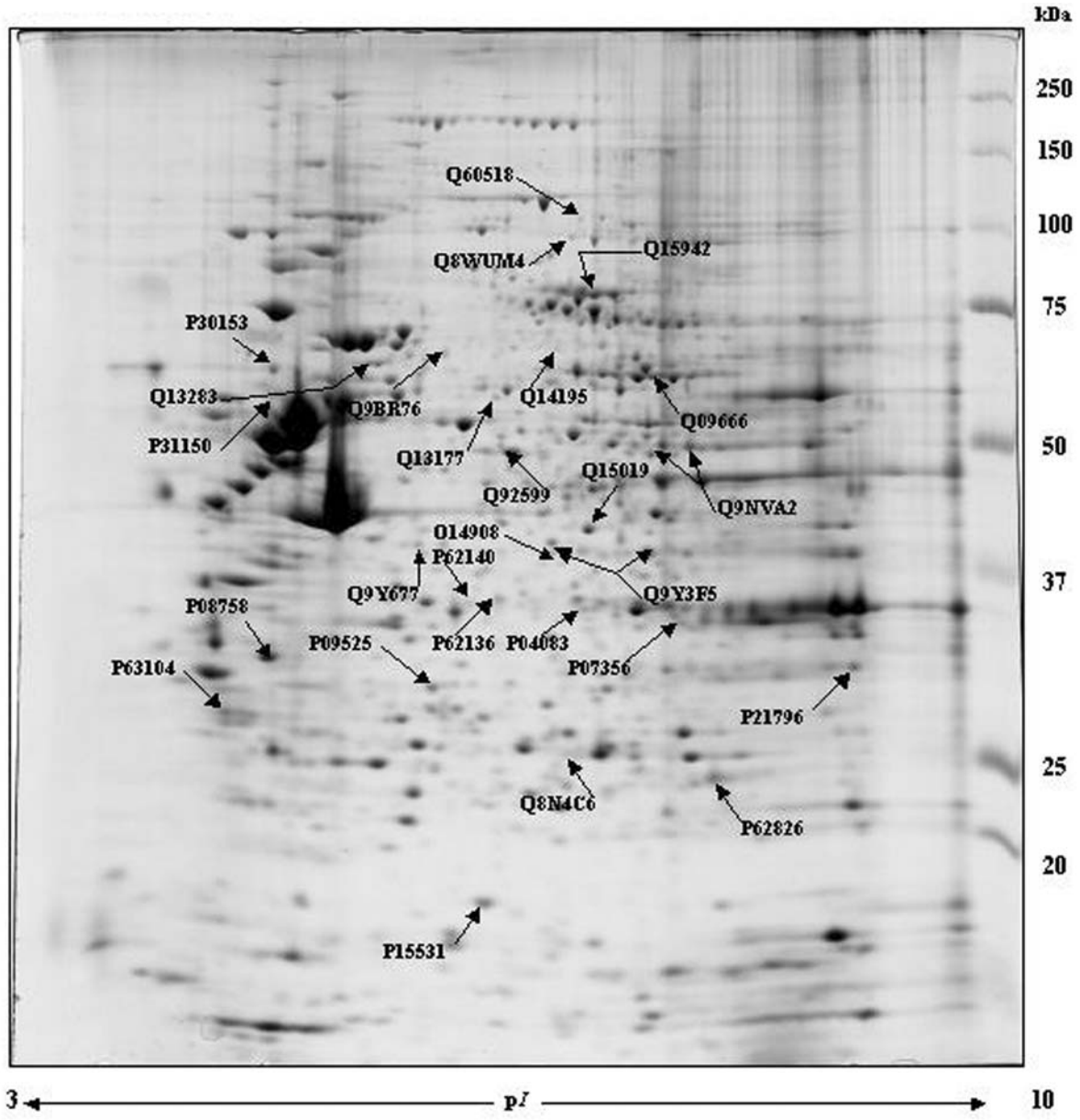

Figure 2. *Identified signaling proteins of Sk-N-SH cell line.*

cytosol (*e.g.*, calmodulin), localised in the endoplasmic reticulum lumen (*e.g.*, calnexin) or present in the secretory pathways of mammalian cells (*e.g.*, calumenin). The latter group of proteins are a growing family of calcium binding proteins that belong to the CREC (Cab45, reticulocalbin, *endoplasmic reticulum calcium binding protein, calumenin*) family. Members of this family, including *calumenin* and *reticulocalbin,* were identified. *Calumenin precursor* was identified in three cell lines, whereas *reticulocalbin-3* and *reticulcalbin-1 precursor* were detected only in the CaOva3 (Figure 4) and A-673 (Figure 8) cell lines, respectively. In addition, one protein, identified as *calcium binding transporter (fragment),* also displayed restricted expression in the MCF-7 cell line with two isoforms. Another group of calcium binding proteins identified were annexins. There are at least 10 annexins that form a multigene family of soluble calcium binding proteins and interact with phospholipids and celullar membranes in a calcium-dependent manner. Annexins, including *annexin I-V & VII,* were found to be expressed by different cell lines, on the other hand annexin A4 and A5 were expressed only by the HL-60 and A-673 cell lines, respectively.

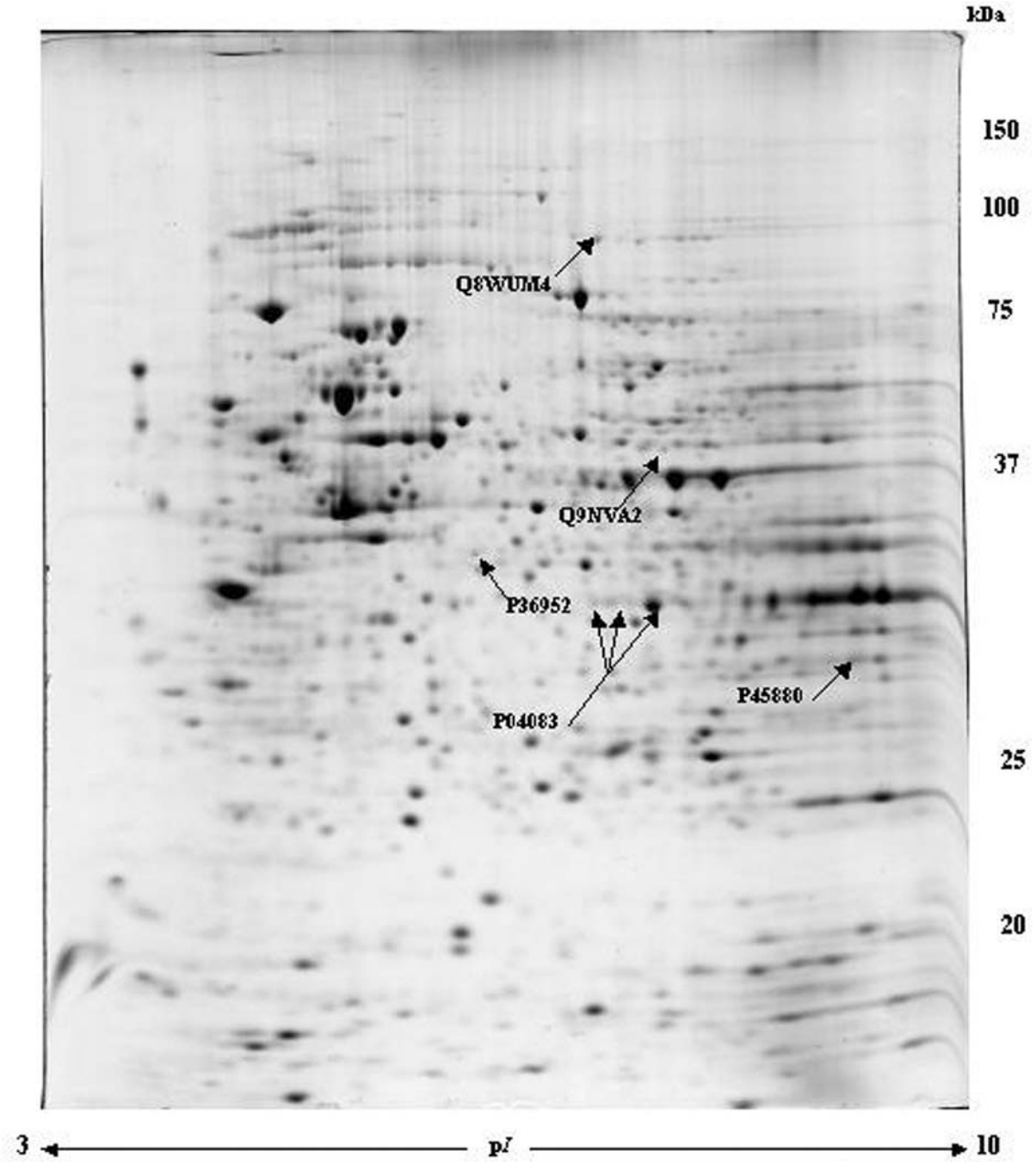

Figure 3. *Identified signaling proteins of HCT116 cell line.*

14-3-3 proteins. 14-3-3 proteins constitute a family of abundant, highly conserved and ubiqitous polypeptides that are involved in various cellular processes. There are seven different isoforms of *14-3-3 proteins* that are expressed in mammalian cells and three of these isoforms were identified in different cell lines. Except for *14-3-3 protein gamma* (Figure 8, Figure 10) expressed in two cell lines, the 14-3-3 isoforms were representing cell line-specific expressional patterns. Accordingly, *14-3-3 zeta/delta* (Figure 2) and *sigma* (Figure 10) were identified only in the SK-N-SH and Hela cell lines, respectively.

Channel proteins and adhesion proteins. Proteins that serve as ion channels either in the cytosol or mitochondria were also

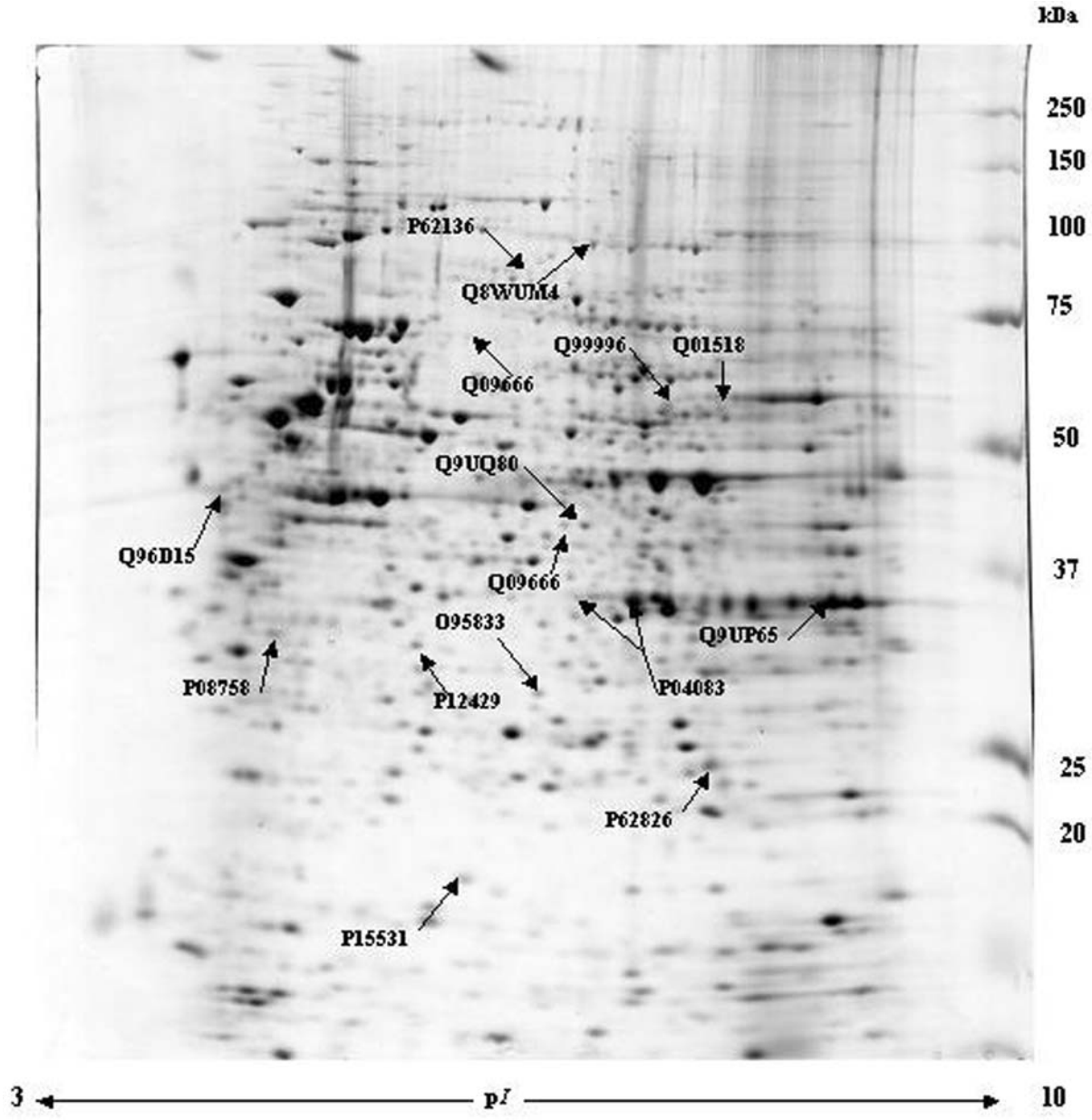

Figure 4. *Identified signaling proteins of Caov3 cell line.*

identified by proteomics. These proteins include two types of ion channels with two different isoforms that displayed isoform-dependent expression in tumor cell lines. In the first type, *chloride intracellular channel protein 1* and *3* were identified that had an expression in six cell lines and one cell line (CaOva3) (Figure 4), respectively. The second type of ion channels were the VDAC (voltage-dependent anion channel) proteins. *VDAC protein isoform 1* and *isoform 2* were expressed in several cell types. Cell spreading, proliferation and survival are modulated by focal adhesion molecules linking extracellular matrix proteins, integrins and the cytoskeleton. One protein, known as *zyxin,* that functions as an adhesion molecule, was identified and this protein was detected only in the SK-N-SH tumor cell line (Figure 2).

Miscellaneous proteins. This group includes a host of proteins involved in different signal transduction pathways. Those proteins with cell line-specific expression include (cell line-expressed is given in parenthesis) *Aryl hydrocarbon-receptor interacting protein* (A-375), a protein that plays a positive role in

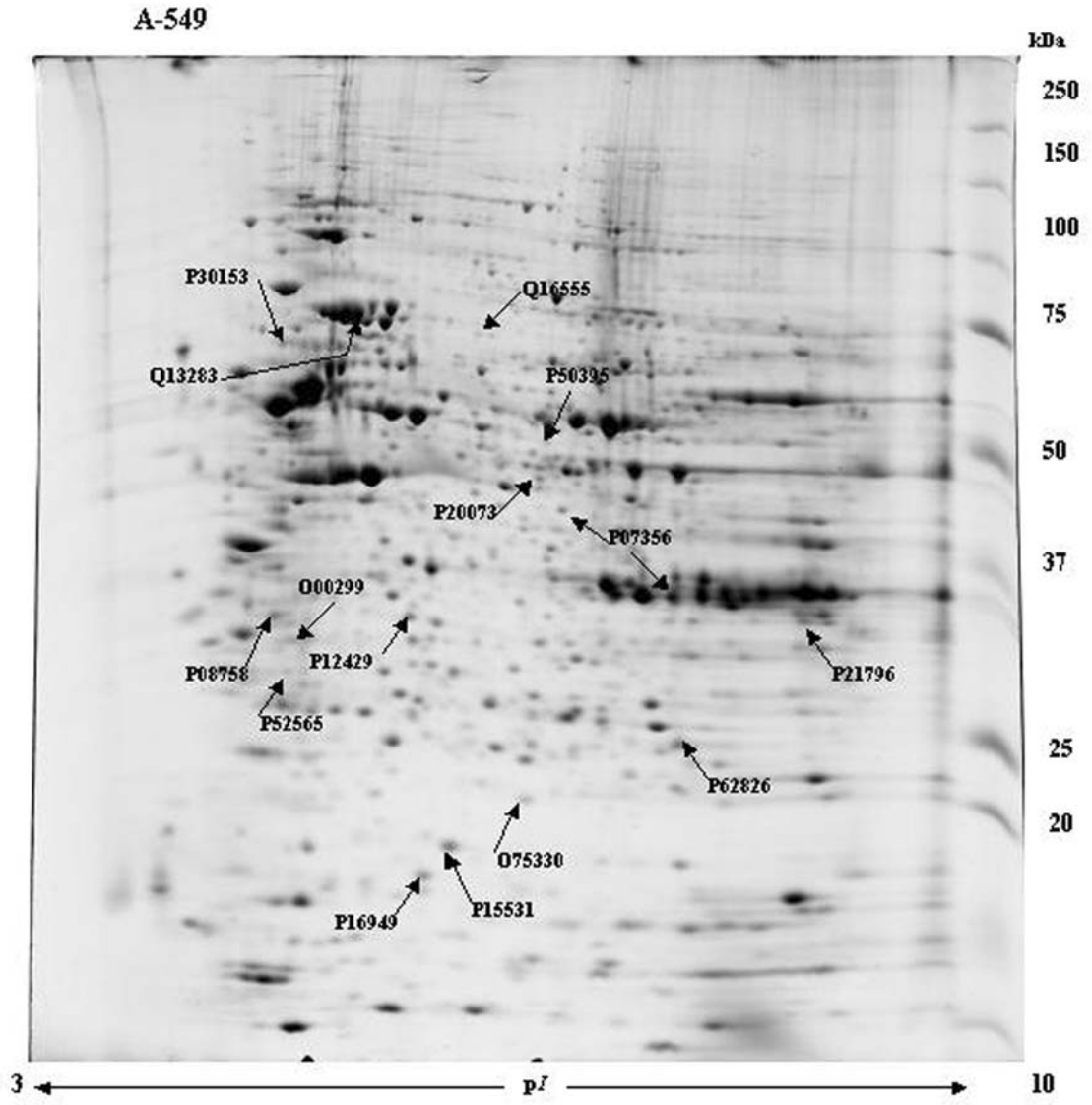

Figure 5. *Identified signaling proteins of A-549 cell line.*

aromatic hydrocarbon receptor-mediated signaling and as a negative regulator of hepatitis B virus X protein; *DAAM (disheveled associated activator of morphogenesis)-1* (Hela), *putative ORF1 (syn: fibroblast growth factor 5)* (SK-N-SH), *AMSH (syn: Stam binding protein)* (MCF-7) (Figure 9) and *cullin homolog 3* (MCF-7) (Figure 9). The rest of the proteins, including *Stathmin; COP9 complex subunit 4*, part of the eight-subunit complex of COP9 signalosome that regulates multiple signaling and cell cycle pathways (16); *Programmed cell death 6 interacting protein*, a protein that may play a role in regulation of both cell proliferation and apoptosis; *Proliferation associated protein 2G4*, a novel mitogenic-inducible protein associated with the cell cycle; *Neuroblast differentiation associated protein AHNAK fragment*, a phosphoprotein that activates phospholipase gamma and is found in the cytoplasm of squamous cell carcinoma and melanoma (17); *RHAMM (receptor for hyaluronic acid mediated motility)*, a ubiquitous receptor for hyaluronic acid, whose expression enhances tumor progression and dissemination in endometrial carcinoma (18) and brain tumor (19); and *Ninein*, a centrosomal protein required for chromosomal segregation (20) showed multiple cell line expression, ranging from two to five cell lines.

Discussion

The present work attempted to generate comprehensive proteome maps of proteins that may play significant roles in different signal transduction pathways, using the

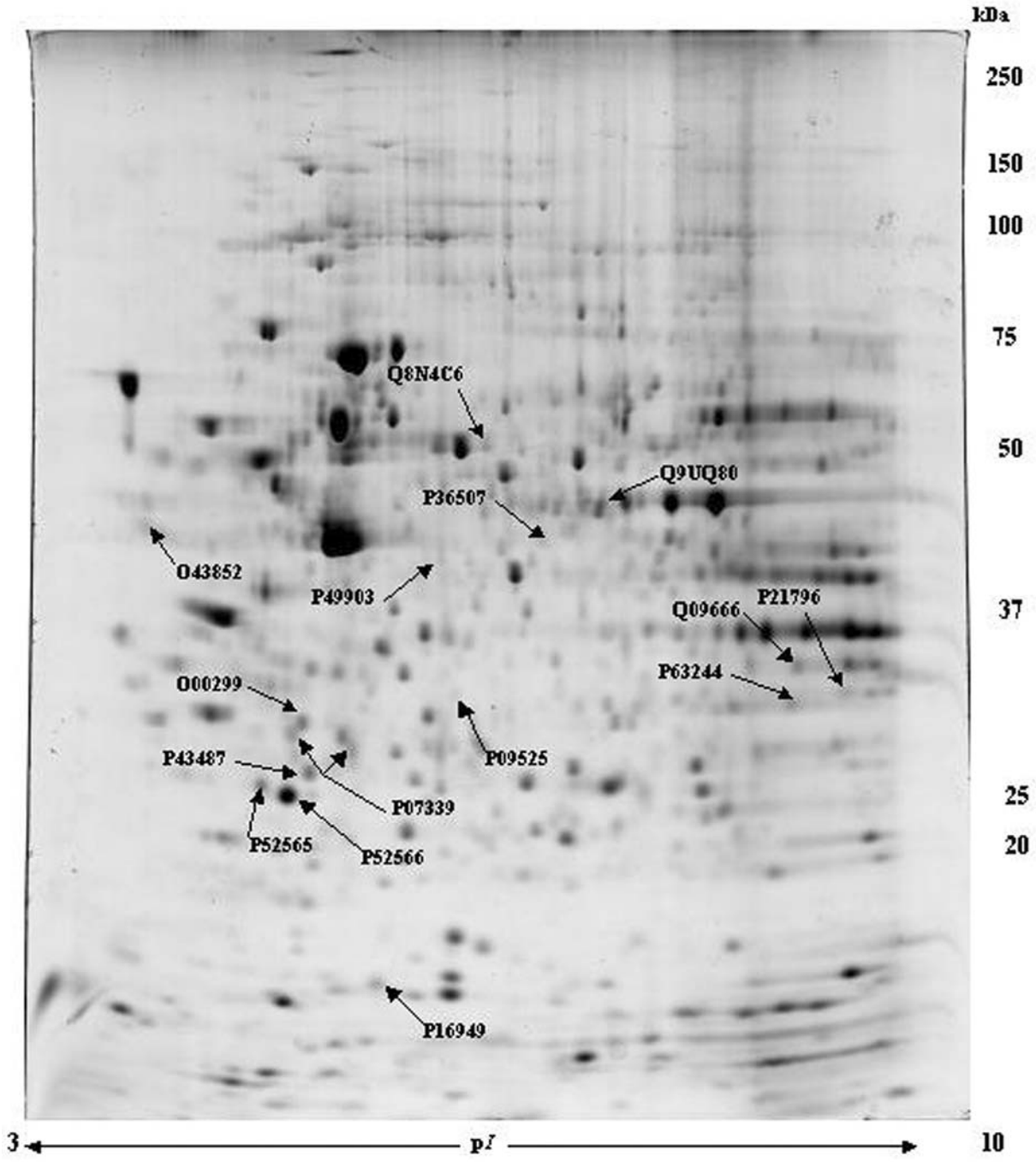

Figure 6. *Identified signaling proteins of HL-60 cell line.*

proteomics approach in ten individual tumor cell lines. The biological meaning of this finding may be that cells could activate different signaling pathways in the signal cascade leading to tumorigenesis.

G-proteins. G-proteins act as binary molecular switches, cycling between an inactive GDP-bound form and an active GTP-bound form at the membrane and, thereby, transduce and regulate signals from cell surface receptors into the cytoplasm *via* specific effector pathways that regulate different cellular processes. G-protein signaling depends on the action of GEFs (guanine nucleotide exchange factors), which markedly accelerate the rate of GDP dissociation, thereby enhancing activation, and GAPs (GTPase activating

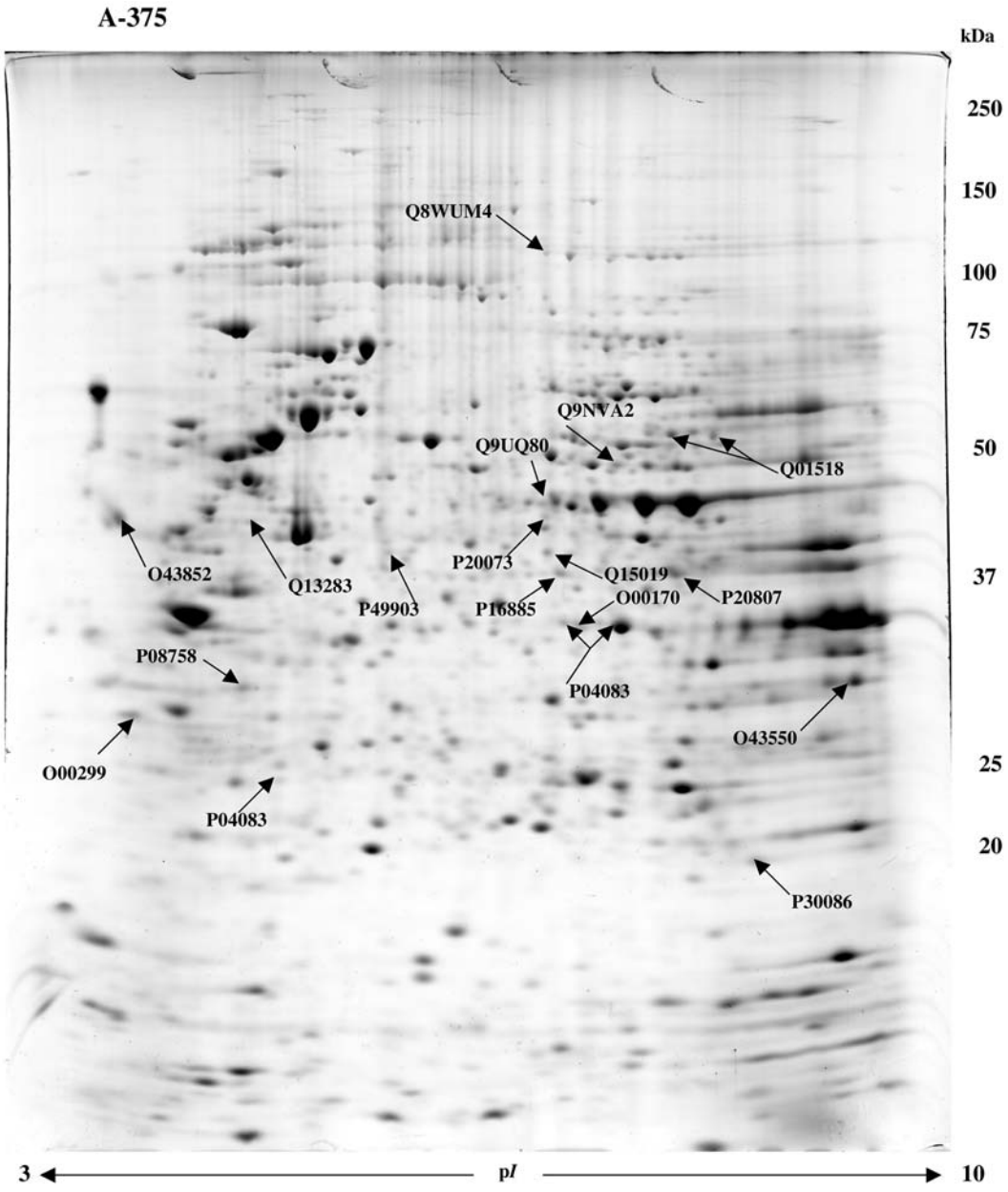

Figure 7. *Identified signaling proteins of A-375 cell line.*

factors), which significantly enhance the intrinsic GTPase activity, thereby accelerating deactivation (4). A heterotrimer G-protein is composed of different *alpha, beta* and *gamma* subunits, which in turn are classified into different subtypes, coupled to different effectors depending on the type of the subunit and the cell involved. Given this mere fact, expression of *guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1* by the Saos-2 and MCF-7 cell lines, but not by others, is not surprising. Although neither GAPs nor GEFs were detected in the present work, two GAP-interacting proteins were identified, *Ras-GTPase activating protein binding protein-1 and RGS19-interacting protein-1*. Ras-GTPase activating protein binding protein-1 is a protein that physically associates with Ras-GTPase activating protein, a GAP protein essential for Ras signaling, through the SH3 domain. This binding is shown to occur when cells are in a proliferating state and may allow Ras to remain in its active confirmation (21). Moreover, it is also shown to bind to human ubiqutin specific protease and inhibit its ability to disassemble ubiqutin chains (22).

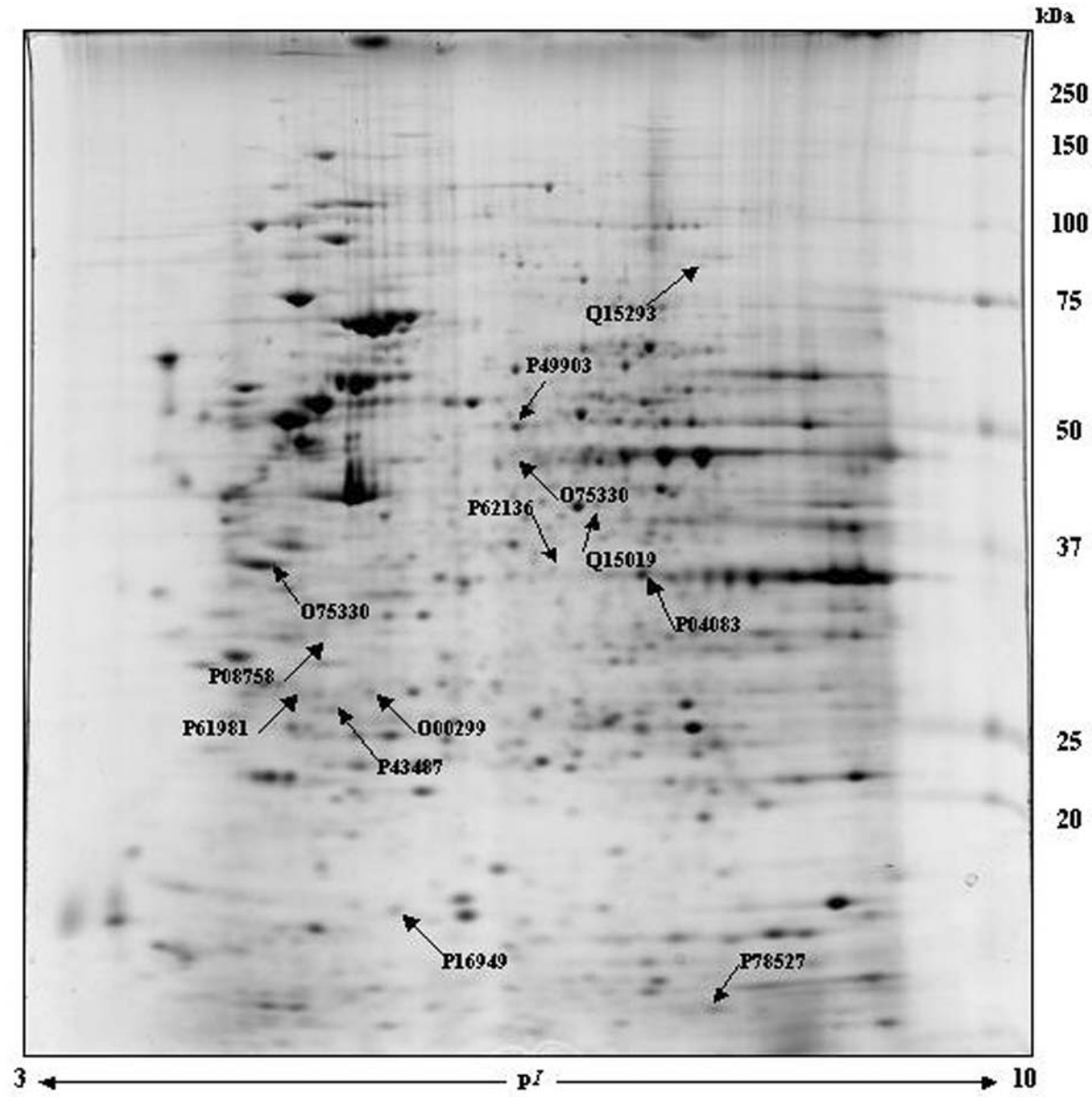

Figure 8. *Identified signaling proteins of A-673 cell line.*

These findings suggest that cell lines expressing *Ras-GTPase activating protein binding protein-1* would have enhanced oncogenic activity of Ras as a result of complexing of proteins either that shut off Ras activation or that increase its turnover. *RGS19-interacting protein-1* interacts with the C-terminus of *RGS-19*, a GAP protein for G-alpha-i, and the complex formed is believed to serve as component of a G-protein-coupled signaling complex involved in regulation of vesicular trafficking (23). Thus, differential expression of GAP-interacting proteins not only just abrogate GAPs function, but also confer additional activity depending upon the type of protein encoded. The monomeric small G-proteins superfamily consists of the Ras, Rab, Rho, Sar1, Arf and Ran families. The Ras family regulates gene expression at least through the MAPK (mitogen activated protein kinase) cascade; the Rab, Arf and Sar1 families regulate intracellular vesicle trafficking; the Rho family mainly regulates cytoskeletal reorganisation and cell adhesion; and the Ran family regulates nucleocytoplasmic transport (3, 24-26). Apart from GAPs and GEFs that regulate G-protein signaling, the small G-proteins are also regulated by GDIs. The present work identified four GDIs; *Rab GDI-alpha, Rab GDI-beta, Rho GDI-1* and *Rho GDI-2,* which is in agreement with the literature (26). GDIs generally work by stabilising

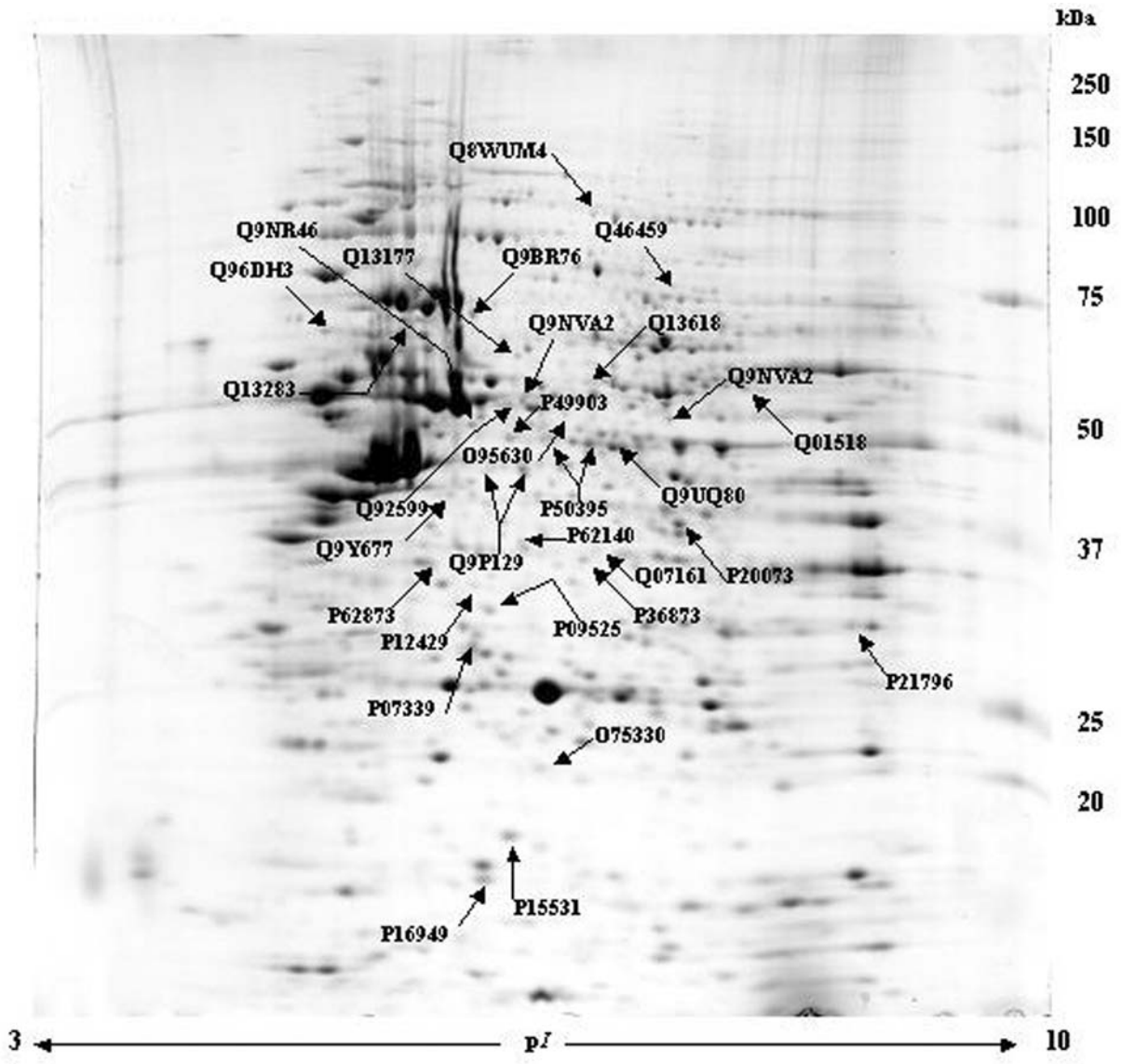

Figure 9. *Identified signaling proteins of MCF-7 cell line.*

the GDP-bound conformation. There are three isoforms of *Rho GDI* (alpha, beta and gamma) that have different expressional patterns. Whereas the *alpha* isoform is ubiquitously expressed, the *beta* and *gamma* isoforms display a unique tissue expression, with *beta* restricted to hematopioetic tissues and *gamma* in brain, kidney, testis and pancreas (26). Rho GDIs bind to a subset of Rho proteins, including Rho, Rac and Cdc42, inhibit nucleotide exchange and sequester these proteins away from the membrane, where normally they would be active, thereby playing an important role in controlling cellular responses mediated by these proteins (24). For example, expression of Rho GDIs has been shown to be inversely related to apoptosis in lung (27) and colorectal carcinoma (28). The detection of *Rho GDIbeta-2* in HL-60 cells is in line with the expression pattern of this isoform and may be used as a good biological marker and potential anticancer target in leukemia. Moreover, since the Rho family-Rho GDI system exerts dynamic control of the actin cytoskeleton, restricted expression of Rho GDI to the HL-60 cell line might indicate that this protein is dispensable in other cell lines. Not unlike the Rho GDIs, there are three different isoforms of the Rab GDI, with the *beta* and GDI2 subunits displaying ubiquitous expression and that of *alpha* largely restricted to the brain (29, 30), which is in agreement with the expression profile observed in this work. The Rab GDIs interact with prenylated forms of Rab, an essential post-translational modification required for membrane association, and playing

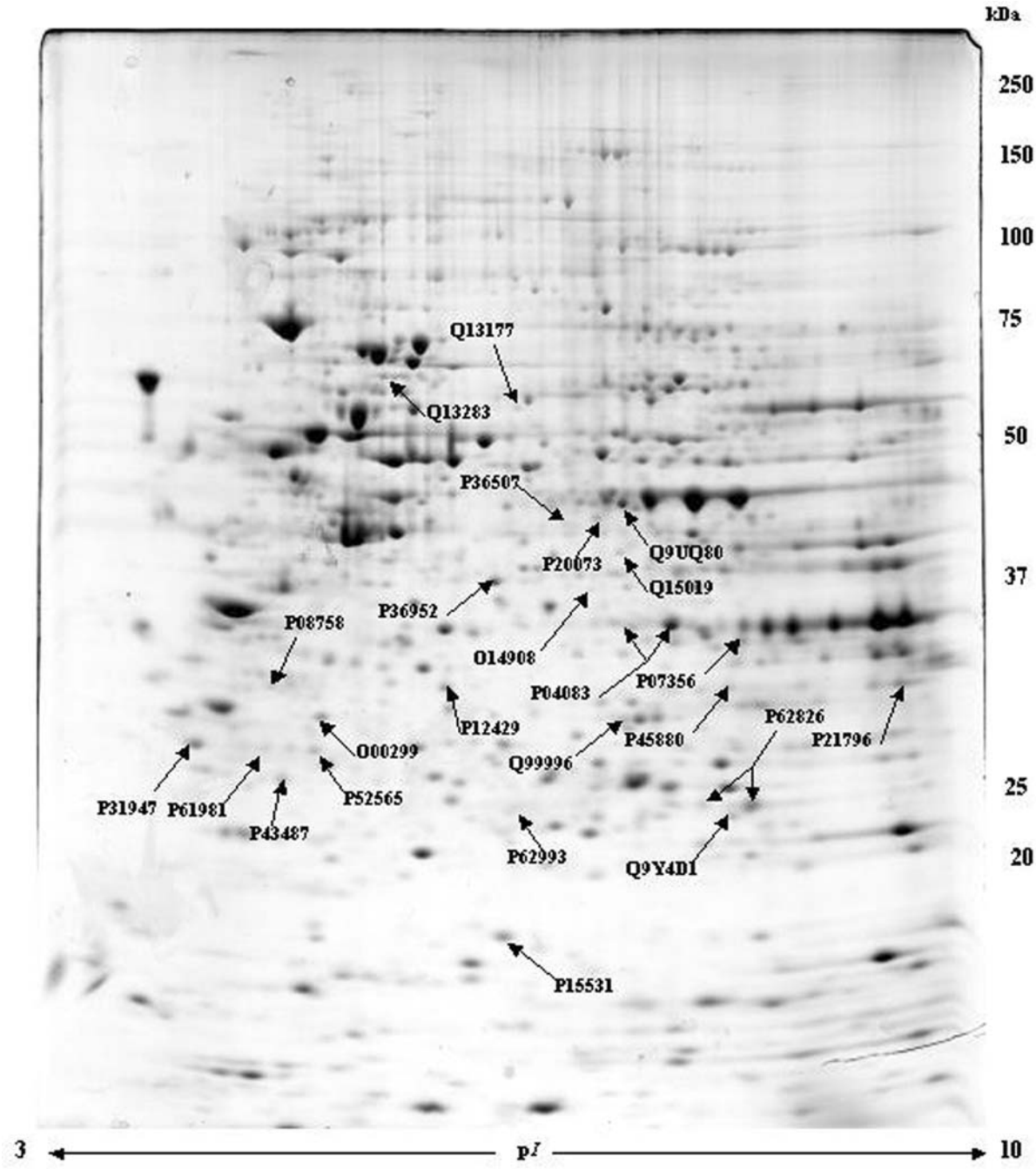

Figure 10. *Identified signaling proteins of HeLa-cervix cell line.*

an important role in extracting the targeted protein to the cytosol and chaperoning the Rab protein throughout its cycle between the cytosol and membranes. *GDI-alpha* was specifically expressed in the SK-N-SH neuroblastoma cell line and vesicle fusing *ATPase* in the MCF-7 cell line. This may suggest that *GDI-alpha* may have other roles in the neuroblastoma cell line apart from a neurosecretory function. This is further corroborated by the finding of *Rab GDI-beta* in the A-549 and MCF-7 cell lines. The malfunctioning of the vesicular traffic system might be a cause of the abnormal biological behavior of cancerous cells. For example, a defect in transport of molecules, such as E-cadherin, which prevents cellular adhesion and encourages dissemination, can facilitate cancer development. Hence,

Rab GDIs can be of use as prognostic markers for cancers associated with vesicular malfunction, including liver cancer and gastric adenocarcinoma (31, 32). *Ran-specific GTPase activating protein* was identified as one of the immunogenic antigens in invasive breast carcinoma (33) and its detection in three cell lines could possibly indicate the use of this protein as a target for immunotherapy.

Signaling enzymes and their interacting proteins. Phosphorylation of structural, effector and regulatory proteins is a major intracellular control mechanism in eukaryotes. The phosphorylation state of a protein is a dynamic process controlled by both protein kinases and phosphatases and plays a crucial role in a wide range of events, including cellular growth and differentiation, cell division, apoptosis and carcinogenesis (34). The Ser/Thr MAPKs are activated in response to upstream receptor tyrosine kinase and/or cytokine receptor that associate with hetrotrimeric G-proteins to trigger downstream pathways. The Ras-Raf-MEK-ERK (ERK, extracellular signal regulated kinase; MEK, MAP/ERK kinase), the p38 kinase and JNK (Jun-amino terminal kinase) signaling pathways, in the MAP kinase cascade, are key regulators of embryogenesis, cell proliferation and differentiation, apoptosis and inflammation (35). The *dual specificity MAPKK* is a kinase that activates p38 kinase and JNK, thereby regulating gene transcription. This protein was identified in the HL-60 and Hela cell lines, indicating that this signaling pathway is operative in these cell lines. Overexpression of *phosphatidylethanolamine-binding protein* (Raf-1 kinase inhibitor protein) interferes with the activation of MEK and ERK, and transformation elicited by an oncogenically activated Raf-1 kinase (36). This protein thus represents a class of protein kinase inhibitors that regulate the activity of the Raf-MEK/ERK module. The PAK family of Ser/Thr kinases are downstream effectors of the Rho family of G-proteins, including Rho, cdc42 and Rac. Interaction of PAKs with these proteins stimulates PAK kinase activity, resulting in the accumulation of F-actin and formation of membrane ruffles, lamellipodia and microspikes (37). The expression of *PAK2* in SK-N-SH, MCF-7 and Hela cells thus could indicate the invasive potential of these tumors. *DNA-dependent protein kinase* participates in the cellular response to and the repair of chromosomal DNA double-strand breaks and is currently identified as a potential treatment target for cancer (38). The identification of this protein in the A673 cell line would be important for soft tissue cancer.

Another kinase of importance is *NDK*, which is encoded by the human nm23 gene that generates nucleoside triphosphates from the corresponding diphosphates and maintains the intracellular nucleotide pool. *NDK* has an important role in inhibition of tumor metastasis and differentiation in leukemic cells (39), and changes in its expression have also been reported to be associated with colon carcinogenesis (40). Moreover, it is shown to regulate vesicular internalisation by supplying GTP to dynamin-mediated activities, which may enable tumor suppressor activity by facilitating endocytosis of growth factor receptors (41). Protein phosphatases, which functionally counteract protein kinases, can have tumor suppressor or tumor promoting roles. The *phosphoserine/phosphothreonine protein phosphatases*, such as *PP1* and *PP2A,* are shown to suppress growth of cancerous cells in leukemia (42) and ovarian carcinoma (43), respectively, whereas the dual specificity protein phosphatases, such as *Cdc25B,* cooperate with other oncogenes in oncogenic transformation by dephosphorylating cyclin dependent kinase (cdk)-1 at the G2/M check point and driving the cell cycle (44). Different groups and/or isoforms of PPs were identified in different cell lines, with *PP1-gamma catalytic subunit* being specific for the MCF-7 cell line. Likewise, *Cdc25B* was also specific to the A-375 cell line. This specific expression could, therefore, provide a means for evaluation of prognosis and developing therapeutic strategies.

The present analysis also revealed differential expression of other enzymes involved in signaling processes. The ubiquitous lysosomal aspartyl protease *cathepsin D* plays a role in metastatic spread by promoting the destruction of normal tissue architecture and in tumor growth by influencing growth factor receptor-mediated signaling. *Cathepsin D* expression shows a variable expression in different carcinomas and has a controversial prognostic value (45-47).

A novel serpin, *maspin*, a member of a serine protease inhibitor family, has demonstrated a robust tumor-suppressing effect by acting at the level of tumor invasion and metastasis in different cancers (48, 49). It is suggested that maspin's protective effect may emanate from inhibition of cell motility by regulating the Rac1 and subsequently PAK1 activity, and promoting of cell adhesion *via* the phosphoinositide-3 kinase/ERK pathways (50). Since *maspin* is undetectable in tumor-free tissue, the co-expression of maspin and PAK2 in the Hela cell line might be a better predictor of outcome than each protein alone. *Calpain* was suggested to play an important role in the invasion of human prostatic cancer and can be targeted to reduce tumor migration, as calpain-regulated rear detachment enables forward locomotion for cell migration initiated by growth factors and adhesion receptors (51). *Calpain 3* targeting may be of value in melanoma as it is specifically expressed in the A-375 tumor cell line. The lipase *phospholipase A2 gamma*, specifically expressed in the A-375 tumor cell line, releases arachidonic acid and the second messenger calcium. Expression of *phospholipase A2 gamma* demonstrates the potential of lipases as biomarkers in the respective cell lines indentified.

Adaptor proteins, such as *Grb2,* play a significant role in coupling signals from a receptor at the cell surface to intracellular effectors by protein-protein interaction using a

defined protein motif. *Grb2* is an SH2 and SH3 domain-containing protein that transmits receptor tyrosine kinase signals to the mitogenic Ras by binding to Shc *via* the SH2 domain and to the nucleotide exchange factor Sos through the SH3 domain. Blocking of *Grb2* binding to Sos by high affinity molecules abrogates activation of Ras and uncouples *Grb2* from its downstream signaling system in chronic mylogenous leukemia (52) and dominant negative Grb2 mutants are shown to reduce the invasive potential of ligand-independent oncogenic signaling (53). One could, therefore, suggest that specific expression of *Grb-2* in the Hela cell line could make the protein a potential biomarker and treatment target.

Cyclase-associated proteins are multifunctional molecules that contain domains involved in actin-binding, *adenyl cyclase association*, SH3 binding and oligomerisation. Many studies have shed light on how these proteins could bridge the knowledge gap in linking signals generated in specific pathways to elements of cytoskeleton, to control cell migration, adhesion, invasion and cytokinesis (54), important elements in carcinogenesis.

Calcium binding proteins. The calcium concentration should be stringently regulated as it plays a fundamental role in cellular processes, as a second messenger, ranging from endocytosis to signal transduction. This is achieved by proteins that have EF-hand domains, consisting of a short calcium binding loop flanked on both sides by helical domains. Recently, a new role emerged for calcium in regulating oncogenic Ras signaling through GEFs and GAPs (4) and evidence has been obtained implicating some CREC family members in malignant transformation (55). Differential expression of reticulocalbin in a colorectal tumor cell line (56) and hepatoma (57) has been reported. Here, we found cell line-specific expression of *reticulocalbin 3* in a colon-adenocarcinoma cell line and *reticulocalbin 1 precursor* in a rhabdomyosarcoma cell line, as well as *calumenin precursor* in osteosarcoma, malignant melanoma and promyelocytic leukemia. These findings reinforce the role of CREC family members in tumor biology and suggest that different members may be required in different tumor cell lines. The genuine function of annexins are not well established, however, they are thought to play important roles in maintaining calcium homeostasis and regulating the cytoskeleton and cell motility. Loss or translocation of annexins from the cell surface has been shown to contribute to the development and progression of glioblastoma, prostate cancer and squamous cell carcinoma, and they are considered to be strong predictors of outcome (58-61). Consistent with the role of annexins in tumor biology, all tumor cell lines expressed at least one type of annexin, indicating that in some cell lines they may have redundant function but in others, particulary in those where a single member is expressed, may serve a specific function.

Septins and 14-3-3 proteins. Septins are a family of highly-related GTPases that participate in diverse aspects of cell biology, ranging from cytokinesis through vesicle trafficking to oncogenesis. In mammals, several septin expression forms have been described (62). Cell cycle-dependent expression of Nedd 5 was observed in brain tumors (63) and some septins, including septin 6, septin 9 and septin 5 are fusion partners for the mixed lineage leukemia (MLL) gene and contribute to *de novo* leukemia in children as well as therapy-related leukemia (64-66). Detection of septin 6-like proteins in a limited number of cell lines and wider expressiosn of septin 2 not only lends support for their role in tumor biology but also for the use of some septins in differentiating soft tissue tumors from solid tumors.

14-3-3 proteins are an important family of proteins as they regulate numerous signaling circuits that are implicated in cancer development, with *14-3-3-sigma protein* being the isoform usually implicated. This isoform is frequently down-regulated in several human cancers (9), as it regulates the oncogenic kinase Raf-1, binds and blocks *Cdc25B* that is needed for cell cycle progression, and positively regulates the tumor suppressor p53 (8, 67). Although *14-3-3-sigma* is considered the isoform that makes the cancer connection, recent studies show that other isoforms, such as 1*4-3-3-beta,* could also have a role, since forced expression of antisense *14-3-3-beta* mRNA diminished growth in rat hepatoma cells (68). The three 14-3-3 protein isoforms identified in the present work showed selective expression towards a particular cell line not seen by other investigated signaling proteins. This could probably be exploited for developing a potential biomarker and therapeutic avenue in the quest for developing new anticancer agents.

Channel proteins and adhesion proteins. VDAC is the most abundant protein of the outer mitochondrial membrane that provides a major channel for the movement of ions, ADP/ATP and metabolites in and out of mitochondria. It is a core component of the permeability transition pore and opening of the latter causes release of cytochrome c, probably through the VDAC channel to initiate the apoptosis cascade (69, 70). Two isoforms of VDAC showed mutually exclusive expression, with *VDAC-2* expression being observed in the HCT-116 and Hela cervix cell lines. This probably indicates that cells may use specific isoforms of VDAC proteins to modulate the cell suicide program, as it is the major determinant in survival of the genetically altered cancer cells. Adherence of cells to the extracellular matrix or to each other occurs at specialised structures of the plasma membrane called focal adhesions. These membrane sites are important links of the cell cytoskeleton to the extracellular matrix and are associated with signal transduction pathways that regulate cell proliferation and survival. *Zyxin* is a focal adhesion-associated phosphoprotein, with one domain involved in the control of

actin assembly and three protein-protein adaptor domains implicated in the regulation of cell growth and differentiation. *Zyxin* is found to be up-regulated in melanoma cells and its expression was directly correlated with cell spreading and proliferation (71). Zyxin was specifically expressed in the SK-N-SH tumor cell line and therapies directed at this particular protein may abrogate neuroblastoma cell growth.

Miscellaneous proteins. Putative ORF1 is a secreted form of the FGF family of growth factors that is overexpressed in different cancers (72, 73). It may participate in autocrine and paracrine pathways to promote cancer cell growth and enable cancer cells to resist apoptosis and desensitize them to chemotherapy by constitutively activating nuclear factor kappaB (74). P*utative ORF1* was identified only in the SK-N-SH tumor cell line and this may have significance in using the protein as an immunotherapy target in neuroblastoma, since it was identified as a tumor-associated antigen recognised by T cells (72). *AMSH* is an adaptor protein shown to be involved in TGF-beta/BMP (transforming growth factor-beta/bone morphogentic protein) signaling, that is important in regulation of developmental cell growth and differentiation (75).

AMSH expression in the MCF-7 tumor cell line indicates that there is a relatively pronounced activation of the TGF-beta/BMP signaling pathway compared to other cell lines and this can be used to restrain epithelial cell proliferation in breast cancer. *DAAM-1* is a disheveled-binding protein transducing Wnt signals to the PCP (planar cell polarity) pathway, which is required for cytoskeletal changes and reorganisation of the cell surface for activities such as cell migration (76, 77). This finding suggests that the non-cannonical pathway of Wnt signaling may be operative only in Hela cells, where *DAAM-1* expression was observed and it also points to the invasive potential of cervical tumors. *Stathmin* is a p53-regulated protein known to influence microtubule dynamics. A growing body of evidence links *stathmin* with microtubule drug resistance in breast cancer, owing to altered drug binding and growth arrest at the G2/M boundary (77). *Stathmin* expression could, thus, be taken into consideration before initiating anticancer agents acting on microtubules (78).

Collectively, although not all structures could be discussed in this work, the present data demonstrate the differential expression of a series of signaling proteins in individual cell lines that may tentatively explain why a certain signaling pathway is not operative in a particular tumor. Moreover, cell-line specific expression of proteins was observed that could pave the way for the development of potential biomarkers and targets for new anticancer agents.

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