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Specific Expression of Potential Tumour Marker Proteins, Similar to No On or Off Transient A and HIRA-interacting Protein 5, in Mouse N1E-115 Neuroblastoma Cell Line

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Abstract. *Background: Improved cancer detection involving suitable biomarkers with easy applicability is a challenge in our fight against cancer. Biomarkers that provide significant insight into the behaviour of neuroblastoma would greatly aid in identifying patients at risk of disease progression, those whose disease has progressed sub-clinically and those who would benefit from currently available systemic therapies. Materials and Methods: Matrix-assisted laser desorption and ionization-time-of-flight (MALDI-TOF-TOF) is an evolving proteomic technology for improving biomarker discovery, that allows for rapid and sensitive analysis of complex protein mixtures generated from body fluids, cells and/or tissues. MALDI-based profiling identifies unique, differentially-expressed proteins relating to specific cancer-related disease states. A proteomic map of the murine neuroblastoma N1E-115 cell line was generated and MALDI-TOF-TOF utilized in a search for tumour marker candidates. Results: The analytical tool identified and characterized similar to no on or off transient A [fragment] and HIRA-interacting protein 5 proteins that have never been described in any normal or tumour cell lines. Conclusion: These findings suggest that the proteins may serve as candidate markers for screening, staging and drug selection for the management of neuroblastoma, owing to their tumour-specific expression.*

Tumours of the nervous system, especially the brain, the eye and the sympathetic nervous system, are the second most common category of childhood cancers, after leukaemia and lymphoma (1). Most neurogenic tumours of childhood are clinically and histologically distinct neoplasms. Some are composed of primitive or undifferentiated cells, a fact that is consistent with their origin during early life when the nervous system is still developing. These include the primitive neuroectodermal tumours of the brain, notably medulloblastoma of the cerebellum, retinoblastoma and neuroblastoma (1).

Neuroblastoma is a neuroblastic tumour of the primordial neural crest, that stands out as the most common extracranial solid tumour of childhood, comprising between 8 and 10% of all childhood cancers. (2-4). It is an enigmatic tumour demonstrating diverse clinical and biological characteristics and behaviour (5-7). Tumours may regress spontaneously, reflecting induction of apoptosis or differentiation, or they may prove extremely aggressive tumours, unresponsive to multi-modality treatment and accounting for most of the paediatric cancer mortalities under 5 years of age (8-10). The spectrum of paediatric neuro-ectodermal tumours ranges from undifferentiated, truly malignant neuroblastomas, *via* ganglioneuroblastomas to well-differentiated, mostly benign ganglioneuromas. Within the group of malignant neuroblastomas, different risk categories can be identified: patients with high, intermediate or low risk tumours (10).

A number of genetic and biological features have been investigated in recent years in an effort to improve the understanding of the behaviour of neuroblastoma and to identify tumour markers that would improve cure rates by facilitating the screening, diagnosis, prognosis, or monitoring of patients. In particular, many prognostic studies have identified a number of tumour markers associated with overall or disease-free survival, including amplification of *MYCN*, gain of chromosome 17p, loss of chromosome 1p, neuronal-specific enolase and lactate dehydrogenase. However, uncertainty overshadows the clinical usefulness of these markers, owing to their non-specificity, the small size of many studies and poor

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Key Words: Neuroblastoma cell line, no on or off transient A, HIRA-interacting protein 5, proteomics, biomarker.

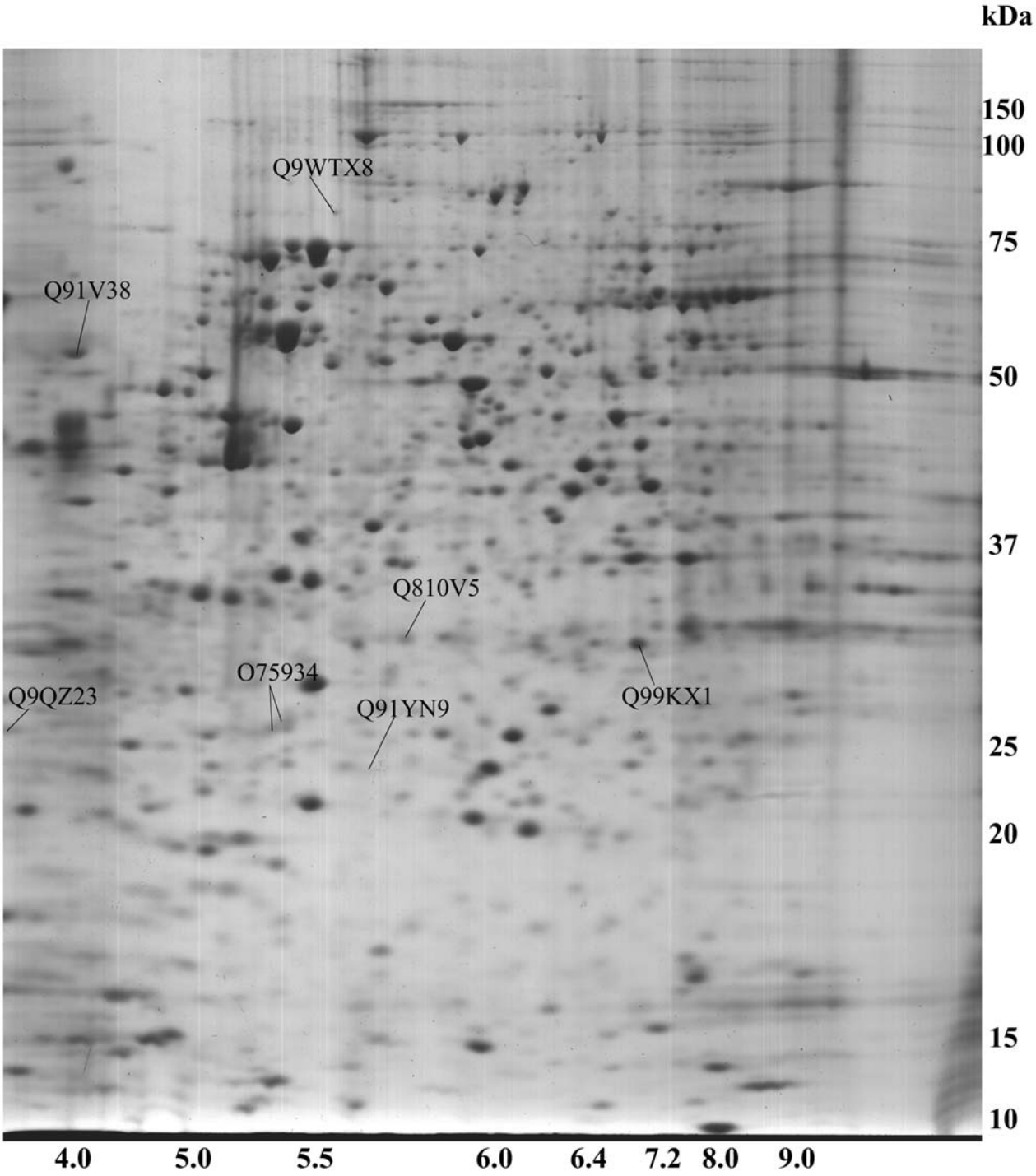


Figure 1. *2-DE gel image of identified tumour-related proteins: Proteins were extracted and separated on an immobilized pH 3-10 non-linear gradient strip followed by separation on a 9-16% gradient polyacrylamide gel. Gels were stained with Coomassie blue and spots were analysed by MALDI-MS and MS/MS.*

statistical reporting (9). There is, therefore, a great need to define markers useful for tumour diagnosis as well as molecular targets for designing novel therapeutic strategies.

The molecular basis of neuroblastoma is now rapidly being unveiled with the current comprehensive genomic and proteomic approaches. Cell lines have been extensively used as experimental models in this regard. The aim of the

Table I. *Identification of tumour-related proteins in N1E-115 neuroblastoma cell line.*

Accession number	Name	MW (kDa)		pI		Score	Peptides matched	Sequence coverage	Expect.
		*Th	*Ob	*Th	*Ob				
Q810V5	Similar to no on or off transient A [Fragment]	30.31	31	5.46	5.75	117	18	47%	1.4e-007
Q9QZ23	HIRA-interacting protein 5 (histone cell cycle regulation defective interacting protein 5)	22.14	26	4.23	3.6	117	10	33 %	1.4e-007
Q91V38	Tumour rejection antigen gp96	92.49	55	4.74	4.2	113	25	26 %	3.4e-007
Q99KX1	Myeloid leukaemia factor 2	28.06	31	6.46	6.9	67	13	37 %	0.013
Q91YN9	Bcl2-associated athanogene 2	23.47	23	6.01	5.65	93	12	45 %	3.6e-005
O75934	Putative spliceosome-associated protein (breast carcinoma amplified sequence 2)	26.13	26	5.48	5.4	104	17	64 %	2.7e-006
			25.5		5.35				
Q9WTX8	Mitotic checkpoint protein isoform MAD1a	83.54	82	5.52	5.6	63	17	31 %	0.035

*Th: Theoretical, Ob: Observed

present study was to systematically hunt for tumour markers for neuroblastoma by making use of the mouse neuroblastoma cell line, N1E-115. To this end, protein profiling of the N1E-115 mouse neuroblastoma cell line was performed by two-dimensional gel electrophoresis (2-DE) coupled to matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy (MS), followed by tandem mass spectroscopy (MS/MS). Interestingly, this high-throughput analytical technique identified and characterized, among others, two possible new marker proteins for neuroblastoma; *similar to no on or off transient A and HIRA-interacting protein 5* (HIRIP5) (HIRA, named after yeast *HIR* genes; HIR is an acronym for 'histone regulator') that have never been described in any normal or tumour cell lines.

Materials and Methods

Cell culture. N1E-115 mouse neuroblastoma cells were obtained from the American Type Culture Collection (ATCC) (ATCC no. CRL 2263). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 4,500 mg/L glucose, L-glutamine, without pyruvate and with 10% fetal bovine serum, 60 μg/ml penicillin and 100 μg/ml streptomycin, and incubated in a humidified incubator with 5% CO_2 at 37˚C.

Sample preparation. N1E-115 cells were washed three times in 10 ml phosphate-buffered saline (Gibco BRL), centrifuged for 10 min at 800 x g at room temperature and subsequently homogenized with 1.0 ml of sample buffer consisting of 7 M urea (Merck, 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), 1 mM phenyl-methylsulfonyl fluoride, 0.5% carrier ampholytes and protease inhibitor complete (Roche, Switzerland). After homogenization, samples were left at room temperature for 1 h and centrifuged at 150,000 x g for 60 min and the supernatant was transferred into an Ultrafree-4 centrifugal filter unit (Millipore, Bedford, MA, USA) for desalting and concentrating proteins. The protein content of the supernatant was quantified by the Bradford protein assay system (11). The standard curve was generated using bovine serum albumin and absorbance was measured at 595 nm.

Two-dimensional gel electrophoresis (2-DE). Samples were subjected to 2-DE, as described elsewhere (12, 13). 0.8 mg protein was applied to immobilized, pH 3-10, non-linear gradient strips in sample cups at their basic and acidic ends. Focusing was started at 200 V and the voltage was gradually increased to 8000 V at 4 V/min and kept constant for a further 3 h (approximately 150,000 Vh in total). After the first dimension, strips (13 cm) were equilibrated for 15 min in a buffer containing 6 M urea, 20% glycerol, 2% sodium dodecyl sulfate and 2% 1,4-dithioerythritol, and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of 1,4-dithioerythritol. After equilibration, the strips were loaded onto 9-16% gradient sodium dodecylsulfate polyacrylamide gels for second-dimensional separation and the gels (180x200x1.5 mm) were run at 40 mA per gel. Immediately after the second dimension run, the gels were fixed for 12 h in 50% methanol containing 10% acetic acid and stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 12 h on a rocking shaker. The molecular mass was determined by running standard protein markers (Bio-Rad Laboratories, Hercules, CA, USA), covering the range 10-250 kDa. pI values were used as

Table II. *Amino acid sequence of tumour-related proteins with peptide matched.*

Acc. No.	Protein name	Protein sequence
Q810V5	Similar to no on or off transient A [fragment]	**1** VEPLEQLDDE DGLPEKLAQK NPMYQKERET PPR**FAQHGTF EYEYSQRWKS** **51** LDEMEKQQRE QVEKNMKDAK **DKLESEMEDA YHEHQANLLR** QDLMRRQEEL **101** RR**MEELHSQE MQKR**KEMQLR QEEERRRREE EMMIRQREME EQMRRQREES **151** YSRMGYMDPR ERDMR**MGGGG TMNMGDPYGS GGQKFPPLGG GGGIGYEANP** **201 GVPPATMSGS MMGSDMR**TER **FGQGGAGPVG GQGPRGMGPG TPAGYGR**GRE **251** EYEGPNKKPR F
Q9QZ23	HIRA-interacting protein 5 (histone cell cycle regulation defective interacting protein 5)	**1 MFIQTQDTPN PNSLKFIPGK PVLETRTMDF PTPAAAFR**SP LARQLFRIEG **51** VK**SVFFGPDF ITVTK**ENEEL DWNLLKPDIY ATIMDFFASG LPLVTEETPP **101** PPGEAGSEED DEVVAMIKEL LDTR**IRPTVQ EDGGDVIYR**G FEDGIVRLKL **151** QGSCTSCPSS IITLKSGIQN MLQFYIPEVE GVEQVMDDDE SDEKEANSS
Q91V38	Tumour rejection antigen gp96	**1** MRVLWVLGLC CVLLTFGFVR ADDEVDVDGT VEEDLGKSRE GSR**TDDEVVQ** **51 REEEAIQLDG LNASQIR**ELR EKSEK**FAFQA EVNR**MMK**LII NSLYK**NKEIF **101** LRELISNASD ALDK**IRLISL TDENALAGNE ELTVK**IKCDK EK**NLLHVTDT** **151 GVGMTR**EELV KNLGTIAK**SG TSEFLNK**MTE AQEDGQSTSE LIGQFGVGFY **201** SAFLVADKVI VTSKHNNDTQ HIWESDSNEF SVIADPRGNT LGR**GTTITLV** **251 LKEEASDYLE LDTIK**NLVRK **YSQFINFPIY VWSSK**TETVE EPLEEDEAAK **301** EEKEESDDEA AVEEEEEEKK PKTKK**VEKTV WDWELMNDIK PIWQRPSK**EV **351** EEDEYKAFYK SFSK**ESDDPM AYIHFTAEGE VTFK**SILFVP TSAPRGLFDE **401** YGSKKSDYIK LYVR**RVFITD DFHDMMPKYL NFVKGVVDSD DLPLNVSR**ET **451** LQQHKLLKVI RKKLVRKTLD MIKK**IADEKY NDTFWK**EFGT NIKLGVIEDH **501** SNRTRLAKLL RFQSSHHSTD ITSLDQYVER MKEKQDKIYF MAGSSRKEAE **551** SSPFVERLLK KGYEVIYLTE PVDEYCIQAL PEFDGKRFQN VAKEGVKFDE **601** SEKTKESREA TEKEFEPLLN WMKDKALKDK IEKAVVSQRL TESPCALVAS **651** QYGWSGNMER IMKAQAYQTG KDISTNYYAS QKKTFEINPR HPLIRDMLRR **701** IKEDEDDKTV MDLAVVLFET ATLRSGYLLP DTKAYGDRIE RMLRLSLNID **751** PEAQVEEEPE EEPEDTSEEA EDSEQDEGEE MDAGTEEEEE ETEKESTEKD **801** EL
Q99KX1	Myeloid leukemia factor 2	**1** MFRFMR**DVEP EDPMFLMDPF AIHR**QHMSRM LSGGFGYSPF LSITDGNMPA **51** TRPASRRMQA GAVSPFGMLG MSGGFMDMFG MMNDMIGNME HMAAGGNCQT **101** FSSSTVISYS NTGDGAPK**VY QETSEMR**SAP GGIRETRRTV R**DSDSGLEQM** **151 SIGHHIR**DRA HILQRSRNHR TGDQEER**QDY INLDESEAAA FDDEWRR**ETS **201** RYR**QQRPLEF RRHEASVGGG RR**AEGPPR**LA IQGPEDSPSR** QSRRYDW
Q91YN9	Bcl2-associated athanogene 2	**1** MAQAKISAKA HEGRFCRSSS MADRSSR**LLE SLDQLELR**VE ALRDAATAVE **51** QEK**EILLEMI HSIQNSQDMR QISDGEREEL NLTANRLMGR TLTVEVSVET** **101 IR**NPQQEESL KHATRIIDEV VSKFLDDLGN AK**SHLMSLYS ACSSEVPPGP** **151 VDQKFQSIVI GCALEDQK**KI KRRLETLLRN IDNSDKAIKL LEHAKGAGSK **201** SLQNTDGKFN
O75934	Putative spliceosome associated protein (breast carcinoma amplified sequence 2)	**1** MAGTGLVAGE VVVDALPYFD QGYEAPGVR**E AAAALVEEET RR**YRPTK**NYL** **51 SYLTAPDYSA FETDIMR**NEF ERLAARQPIE LLSMK**RYELP APSSGQKNDI** **101 TAWQECVNNS MAQLEHQAVR IENLELMSQH GCNAWKVYNE NLVHMIEHAQ** **151 K**ELQKLRK**HI QDLNWQRK**NM QLTAGSKLR**E MESNWVSLVS K**NYEIER**TIV** **201 QLENEIYQIK QQHGEANKEN IR**QDF
Q9WTX8	Mitotic checkpoint protein isoform MAD1a	**1 MEDLGENTTV LSSLRSLNNF ISQRMEGTSG LDVSTSASGS LQKQYEYHMQ** **51 LEER**AEQIRS KSYLIQVER**E KMQMELSHKR** ARVELERAAS TNARNYEREV **101** DRNQELLARI RQLQECEATA EEKMREQLER HRLCKQNLDA VSQQLREQED **151** SLASAREMIS SLKGRVSELQ LSAMDQKVQV KRLESEKQEL KEQLELQQRK **201 WQEANQKIQE LQASQDER**AE HEQKIK**DLEQ KLCLQEQDAA VVKSMKSELM** **251 R**MPRMERELK R**LHEENTHLR EMK**ETNGLLT EELEGLQRKL SR**QEKMQEAL** **301 VDLELEK**EKL LAKLQSWENL DQTMGLNLRT PEDLSRFVVE LQQRELTLK**E** **351 KNNSITSSAR** GLEKVQQQLQ DEVRQANAQL LEERKKRETH EALARRLQKR **401** NALLTKERDG MRAILGSYDS ELTQTEYSTQ LTQRLWEAED MVQK**VHAHSS** **451 EMEAQLSQAL EELGVQK**QRA DTLEMELKML KAQTSSAESS FSFCKEEVDA **501** LRLKVEELEG ERSR**LEQEKQ VLEMQMEK**LT LQGDYNQSRT KVLHMSLNPI

Table II. *continued.*

Acc. No.	Protein name	Protein sequence
		551 SMARQRQHED HDRLQEECER LRGLVHALER GGPIPADLEA ASSLPSSKEV **601** AELRKQVESA ELKNQRLK**EV FQTKIQEFRK VCYTLTGYQI DVTTESQYR**L **651** TSRYAEHQTD CLIFKATGPS GSK**MQLLETE FSR**SVPELIE LHLLQQDSIP **701** AFLSALTIEL FSRQTSI

*Bold letters: matched peptide

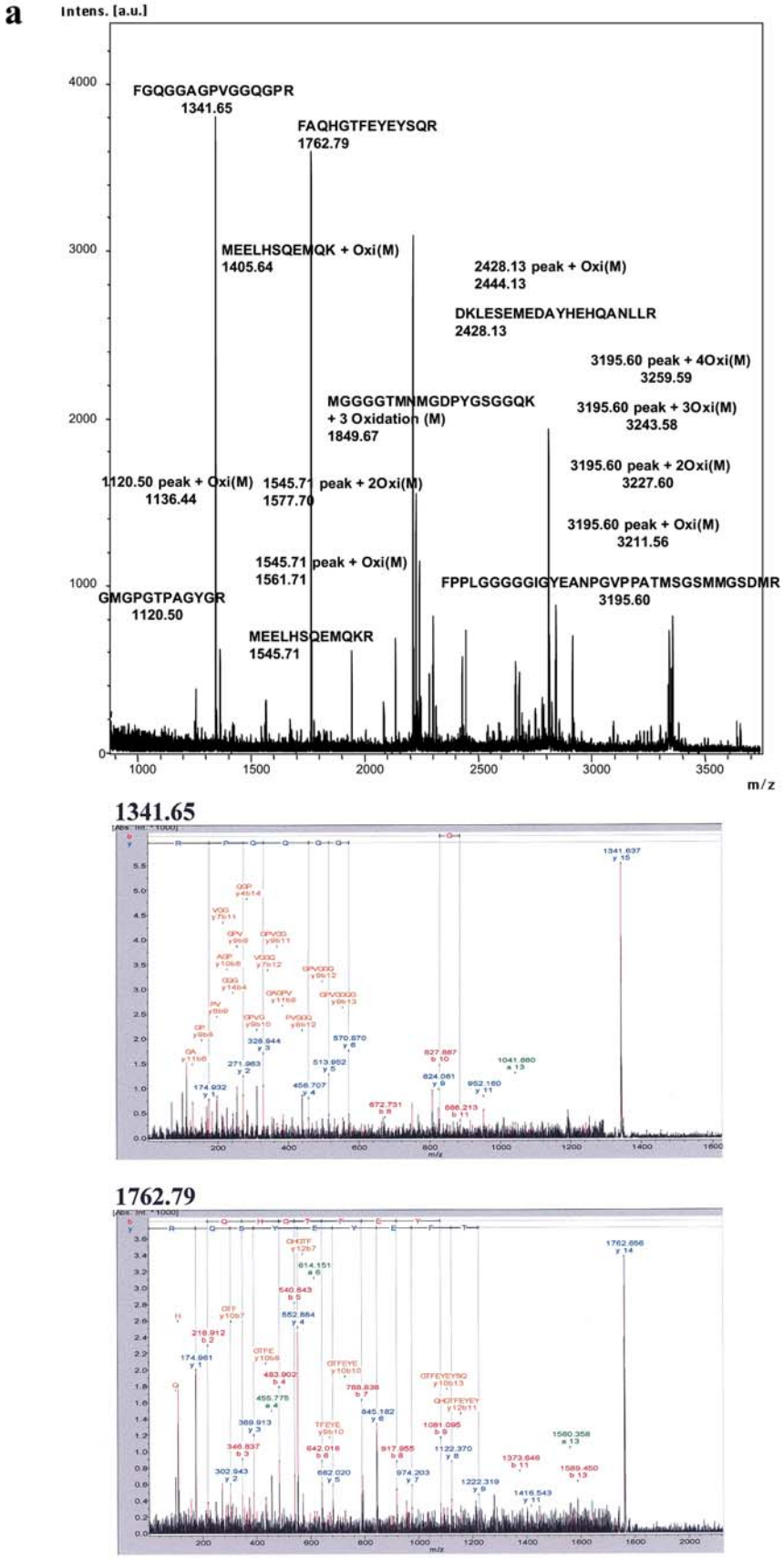
given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess dye was washed out from the gels with distilled water, which were then scanned with Imagescanner (Amersham Bioscience). Electronic images of the gels were recorded using Photoshop (Adobe) and PowerPoint (Microsoft) software.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Spots were excised with a spot picker (PROTEINEER sp™, Bruker Daltonics, Germany), placed into 96-well microtitre plates. In-gel digestion and sample preparation for MALDI analysis were performed by an automated procedure (PROTEINEER dp™, Bruker Daltonics). Briefly, the spots were excised and washed seven times 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 re-swollen with 40 ng/μl trypsin (Promega, Madison, WI, USA) in enzyme buffer (consisting of 5 mM octyl β-D-glucopyranoside) and 10 mM ammonium bicarbonate) and incubated for 4 h at 30˚C. Peptide extraction was performed with 10 μl of 1% trifluoroacetic acid in 5 mM octyl β-D-glucopyranoside and directly applied onto a target (AnchorChip™, Bruker Daltonics) that was spotted with α-cyano-4-hydroxycinnamic acid (Sigma) matrix thinlayer. The mass spectrometer used in this work was an Ultraflex™ TOF/TOF (Bruker Daltonics) operated in the reflector mode. An accelerating voltage of 25 kV was used. 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 50–200 consecutive laser shots. Those samples, which were analysed by peptide mass fingerprinting (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). For protein search, a mass tolerance of 100 ppm and one missing cleavage site were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as criteria for correct identification.

Results

Protein expression was assessed in a mouse neuroblastoma cell line cultured under standard conditions. A total of five spots, representing tumour-related proteins, were detected (Figure 1). The spots seemed to distribute in the pH 3-6 interval, with a molecular weight range of 25-90 kDa. Table I shows the list of identified proteins with the name of the protein, accession number, molecular size and pI. The one spot-one protein aspect was applicable for all the proteins identified. In general, there was a good agreement between the theoretically-predicted and experimentally-found pI and Mr values, except for a large deviation in the case of the tumour rejection antigen gp96, probably indicating that the spot represents a truncated form of gp96, as it has been described that the protein exhibits size-based heterogeneity (14). The proteins of interest were isolated by excision from the 2-DE gel, digested, extracted and identified by MALDI-MS/MS analysis. MS identifications, including score, number of matching peptides and sequence coverage for all the assigned proteins, are reported in Tables I and II. MS/MS data were provided to *similar to no on or off transient A* [fragment] and HIRIP5, since these two proteins reflect the major proteins of interest in the present study.

Following identification by peptide mass fingerprint (PMF), the workflow control software automatically selected two and three peaks for *similar to no on or off transient A* and HIRIP5, respectively, from the MS spectrum to generate an MS/MS spectrum, as MS/MS analysis of one or two available peptides provides unambiguous identification of a protein in question. Indeed, the generated LIFT-TOF/TOF spectra of peaks (m/z 1341.65 and m/z 1762.79) were nicely matched to the peptide sequences (FGQGGAGPVGGQGPR) and (FAQH GTFEYEYSQR), respectively, confirming unambiguous assignment of the spot to *similar to no on or off transient A* [fragment] (Figure 2a). Likewise, peaks (m/z 1324.67, m/z


a
Intens. [a.u.]
FGQGGAGPVGGQGPR
1341.65
FAQHGTFEYEYSQR
1762.79
MEELHSQEMQK + Oxi(M)
1405.64
2428.13 peak + Oxi(M)
2444.13
DKLESEMEDAYHEHQANLLR
2428.13
3195.60 peak + 4Oxi(M)
3259.59
MGGGGTMNMGDPYGSGGQK
+ 3 Oxidation (M)
1849.67
3195.60 peak + 3Oxi(M)
3243.58
3195.60 peak + 2Oxi(M)
3227.60
1120.50 peak + Oxi(M)
1136.44
1545.71 peak + 2Oxi(M)
1577.70
3195.60 peak + Oxi(M)
3211.56
1545.71 peak + Oxi(M)
1561.71
FPPLGGGGGIGYEANPGVPPATMSGSMMGSDMR
3195.60
GMGPGTPAGYGR
1120.50
MEELHSQEMQKR
1545.71
m/z
1341.65
1762.79

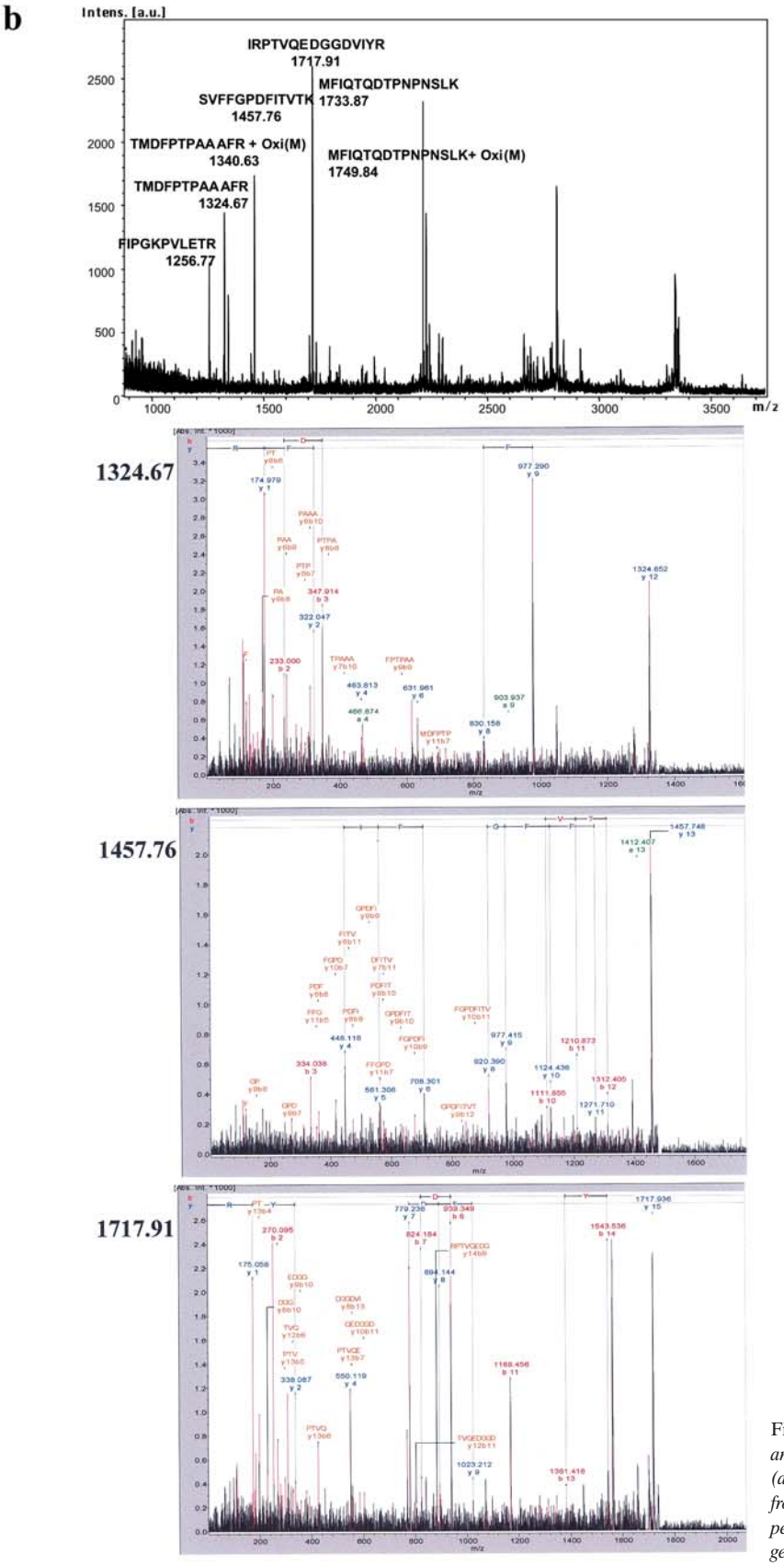


Figure 2. *Peptide mass fingerprint (PMF) and MS/MS analysis of similar to no on or off transient A [fragment) (a) and HIRIP5 (b): Picks were automatically selected from the MS spectra by the computer software and the peptide sequences were matched with the spectra generated by MS/MS analysis.*

1457.76 and m/z 1717.91) matched with the peptide sequences (TMDFPTPAAAFR), (SVFFGPDFITVTK) and (IRPTVQEDGGDVIYR), respectively, unequivocally assigning the spot to HIRIP5 (Figure 2b).

A literature review revealed that *similar to no on or off transient A* protein [fragment] and HIRIP5 have never been described in any normal or tumour cell lines and are, thus, specific for the mouse N1E-115 neuroblastoma cell line. Moreover, to the best of our knowledge, *similar to no on or off transient A* protein [fragment] is here also described at the protein level for the first time. Since MS and MS/MS data did not provide evidence for the presence of post-translational modifications, possible sites were assessed by mining of the databases using the ExPasy proteomics tools (http: //www.expasy.org/). This search had shown that the protein could have one potential O-glycosylation and seventeen phosphorylation sites. A similar run for HIRIP5 produced two potential O-glycosylation and ten phosphorylation sites.

Discussion

Neuroblastoma is an embryonic tumour of neuroectodermal cells derived from the neural crest and destined for the adrenal medulla and sympathetic nervous system. The clinical hallmark of neuroblastoma is its variability. The biological hallmark is the complexity of the genetic abnormalities acquired by the tumour cells, some of these abnormalities being powerful prognostic markers independent of the clinical features. This fact helps in risk stratification of patients at presentation, with the most intensive treatments being reserved for high-risk cases, so that children with relatively benign tumours can be spared the deleterious effects of unnecessary chemotherapy (15). The literature is full of conflicting claims about tumour markers and there remains a need to define more precise prognostic factors to assist in treatment stratification. Our attempt to discover tumour markers uncovered two proteins, *similar to no on or off transient A* and HIRIP5, that are here described for the first time in cell lines.

Similar to no on or off transient A protein. The gene encoding the mouse *similar to no on or off transient A protein* is unknown and has never been described at the protein level. Its mRNA was first identified from the mammary tumour metastasized to lung and the sequence was deposited in the database (Strausberg R, direct submission, http: //ca.expasy.org/cgi-bin/niceprot). Blast searches revealed that the protein has 50% identity to the polypyrimidine tract binding protein associated splicing factor (PSF). The PSF protein, both in human and in mouse, is composed of two major regions (16). The highly conserved C-terminal region contains the two RNA-recognition motifs needed for interactions with RNA and for the localization of the protein in speckles. The N-terminal region is unusually rich in proline, glycine and glutamine residues, and is believed to play a role in interactions and recruiting other molecules. In *Drosophila*, the *no-on-transientA* (*nonA*) gene encodes a putative RNA-binding protein, but its function at the biochemical level is unknown. However, the *nonA* gene has been shown to confer certain behavioural characteristics, including visual and courtship song (17, 18). The similarity to PSF and *Drosophila nonA* gene product suggests that the *similar to no on or off transient A* protein could be an RNA-binding protein probably involved in transcription, splicing or translation. Several RNA-binding proteins, including eukaryotic intiation factor 4E and neuron-specific RNA-binding proteins, have been identified as biomarkers for tumours in general and neuroblastoma in particular (19, 20). Thus, the neuroblastoma cell line-specific expression of *similar to no on or off transient A* protein makes the protein a likely candidate biomarker for neuroblastoma.

HIRIP5. HIRIP5 belongs to a phylogenetically conserved family of proteins which are largely uncharacterised. A HIRIP5 homologue exists in *Drosophila*, *C. elegans*, plants and both budding and fission yeast. Prokaryotes also synthesize proteins highly similar to HIRIP5. Human HIRIP5 was discovered through a two-hybrid screen using a fragment of HIRA as the bait, and the mouse homologue through a database mining approach starting from the human HIRIP5 cDNA sequence (21). The *HIRA* gene, first reported as *TUPLE1* for its partial similarity to the yeast general transcriptional repressor *TUP1*, was named for its sequence similarity to two yeast proteins, Hir1p and Hir2p. *In situ* hybridisation experiments have demonstrated high levels of transcripts in various structures, including the neural crest of murine embryos (22) and in the neural crest-derived regions of chicken embryos (23). The expression of HIRA is regulated during embryogenesis and the presence of seven contiguous WD40 repeats would probably make HIRA serve as a scaffold for protein–protein interactions (24). It appears that the chromatin-related function of HIRA may well depend on a HIRA-containing multiprotein complex that could also contain HIRIP5. Taken together, all these findings, coupled to the cell line-specific expression of HIRIP5, make the protein emerge as a potential biomarker for neuroblastoma.

Although HIRIP5 appears to interact with HIRA, a nuclear protein, indirect evidence shows that HIRIP5 is mitochondrial. The gene encoding for the *S. cerevisiae* protein, Nfu1p, a protein found to be most similar to HIRIP5, appears to localize to the mitochondrial matrix and Nfu1p has been postulated to be implicated in the metabolism of iron in mitochondria (25, 26). If this

assumption is true, it does not rule out the possibility of HIRIP5 as a tumour marker, since deletion of mitochondrial genes and mitochondrial-encoded proteins are associated with oncocytic transformation (27).

Other tumour-related proteins. The proteins that are discussed under this heading include tumour-related proteins that have been described in cancer-related literature. Tumour rejection antigen peptide gp96 (GRP94) is a heat-shock protein that is located in the endoplasmic reticulum, where it exerts protective functions during cellular stress and plays an important role in the maintenance of protein homeostasis. The housekeeping activity of this protein is ascribed to its ability to chaperone nascent or aberrantly-folded proteins. Studies have also shown that gp96 confers a tumour protective effect in a number of experimental cancers, including melanoma, prostate and colon carcinoma (28, 29). The basis for the immunological activity of this protein could stem from its ability to chaperone tumour-derived immunogenic peptides and deliver them to antigen-presenting cells for specific T cell stimulation, and its activity as a natural adjuvant, promoting maturation and activation of antigen-presenting cells and eliciting secretion of proinflammatory cytokines and chemokines (29).

Mitotic spindle-assembly check point protein MAD1 (mitotic arrest deficient-like protein 1, MAD1L1) has been characterized as an essential component of the mitotic spindle-assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate (30). The induction of *MAD1L1* transcripts by p53 (31) and the abrogation of MAD1L1 function by the human T-cell leukemia virus type 1 oncoprotein, Tax, suggest that MAD1L1 has critical roles in cell cycle control and tumour suppression (30, 32).

A fundamental mechanism of genetic alteration is amplification of entire gene sequences that result in over-expression of a gene product or protein. If the amplified gene is a member of the oncogene family and/or a regulator of DNA replication or cell cycle progression, over-expression of this oncoprotein may result in enhanced growth advantages for these cells. Breast cancer amplified sequence 2 (BCAS2) is one such gene, initially identified to be amplified and up-regulated in the breast cancer cell lines MCF-7 and BT-20 (33). Later, BCAS2 was purified from both *Schizosaccharomyces pombe* and HeLa cell nuclear extract as a component of spliceosome, the machinery that removes introns from mRNA precursors during RNA processing (34, 35). BCAS2 was also identified as an oestrogen receptor alpha-interacting protein, suggesting that BCAS2 might play an important role in breast cancer development by increasing the oestrogen receptor's function (36).

Methodologically, the proteomic approach used is well-accepted in the scientific community and unambiguously identifies spots by a protein chemical rather than an immunochemical technique, independent of antibody availability and specificity (37, 38). The next steps would be to validate these proteins as markers in neuroblastoma by testing other neuroblastoma cell lines and tissues as well as non-malignant cells and tissues. This challenging question is now being addressed in our laboratory.

To sum up, carcinogenesis proceeds through a very long pre-clinical period. Our collective hope is that multiple opportunities exist for chemoprevention to arrest or reverse the progression towards malignancy. In the hope of faster progress with fewer subjects and lower total cost, much effort is being expended on the search for reliable biomarkers to predict the likelihood of developing cancer and/or to signal the effectiveness of chemopreventive therapy (39). The present results form the basis for the possible use of *similar to no on or off transient A* [fragment] and HIRIP5 proteins as biomarkers for neuroblastoma detection.

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