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Molecular detection of neuron-specific ELAV-like-positive cells in the peripheral blood of patients with small-cell lung cancer

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Abstract. *Background:* n-ELAV (neuronal-Embryonic Lethal, Abnormal Vision)-like genes belong to a family codifying for onconeural RNA-binding proteins. Anti-Hu-antibodies (anti-Hu-Ab) are typically associated with paraneoplastic encephalomyelitis/sensory neuropathy (PEM/PSN), and low titres of anti-Hu-Ab, were found in newly diagnosed Small Cell Lung Cancer (SCLC). The aim of this study is to develop a sensitive and quantitative molecular real-time PCR assay to detect SCLC cells in peripheral blood (PB) through nELAV-like transcripts quantification.

Methods: Peripheral blood samples from 25 SCLC untreated patients and 12 healthy blood donors were investigated by real-time PCR. mRNA levels for HuB (ELAV2), HuC (ELAV3) and HuD (ELAV4) were measured in peripheral blood samples with an absolute quantification method using plasmid dilutions as calibration curves.

Results: A statistically significant increase in mRNA expression level was detected for HuB and HuD in SCLC patients as compared with samples from healthy blood donors. After establishing cut off values based on the level of expression in control samples, 28% of the SCLC samples were positive for HuD expression. Overall 60% of the SCLC displayed increased level of HuD or HuB transcripts.

Conclusion: Our preliminary results suggest that neuron-ELAV mRNA are detectable in peripheral blood of SCLC patients using real-time quantitative PCR.

Keywords: ELAV-like, plasmid dilutions, real-time quantitative PCR, SCLC

Abbreviations

n-ELAV:	Neuronal-Embryonic Lethal, Abnormal Vision
PEM/PSN:	Paraneoplastic encephalomyelitis/sensory neuropathy
anti-Hu-Ab:	Anti-Hu-antibodies
SCLC:	Small cell lung cancer
PB:	Peripheral blood
RRM:	RNA recognition motif
ELISA:	Enzyme linked immunosorbent assay
qRT-PCR:	Real-time quantitative real-time PCR
VALSG:	Veterans' Affairs Lung Study Group
GAPDH:	Glyceraldehyde phosphate dehydrogenase
MRD:	Minimal residual disease

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1. Introduction

ELAV-like proteins, also called Hu antigens, are a small family of RNA-binding proteins involved in the development and maintenance of nervous system [3, 19,29]. Four ELAV-like proteins have been identified in the genomes of vertebrates, named *ELAVL1* (*HuR*), *ELAVL2* (*HuB*), *ELAVL3* (*HuC*) and *ELAVL4* (*HuD*), [16,17,22,27]. HuR is expressed in all tissues examined [28], whereas *HuB*, *HuC* and *HuD* are normally expressed only on terminally differentiated neurons [5, 17,19]. These proteins are highly homologous in sequence and highly evolutionarily conserved. All four members of the family encode for RNA-binding proteins endowed with three RNA-interacting domains known as RRM (RNA recognition motif) [6].

The human members of ELAV family are of particular interest since they are tumour antigens associated predominantly with SCLC. During the course of their disease, SCLC affected patients expresses some tissue-specific Hu antigens and develops antibodies against these proteins. The antibodies developed against these tumour antigens reach the brain were they induce an autoimmune inflammatory response resulting in paraneoplastic encephalomyelitis/sensory neuropathy (PEM/PSN) [2,10]. Previous studies reported that low titres of anti-Hu-Ab on HuD, were also detected in 16–25% of newly diagnosed SCLC without PEM/PSN [8,12,20,21]. SCLC of patients Hu-positive grow more indolently, suggesting that the expression of ELAV like proteins might be associated with a better disease prognosis [12].

Several prospective studies have suggested that the detection of tumour cells in the bone marrow or peripheral blood of cancer affected patients may contribute for the determination of minimal residual disease and as prognostic marker for the development of distant metastases and overall survival [1,13,14].

To date, Hu-positive SCLC patients are identified by serological identification of Hu proteins by using several techniques, immunohistochemistry, immunoblotting or enzyme linked immunosorbent assay (ELISA) of these proteins [7,9,11,18]. Recently, a potential application of ELAVL4 real-time quantitative real-time PCR (qRT-PCR) for detection of disseminated neuroblastoma cells are described [26]. The use of this technique offers several advantages over conventional PCR in terms of time and reduction of contamination by using a closed amplification and detection system. The aim of this study was to develop and validate a quantitative RT PCR approach for the detection of HuB, HuC and HuD transcripts in peripheral blood (PB) of patients with small-cell lung cancer. Expression of *nELAV* gene was analyzed in 25 PB samples from patients with SCLC without PEM/PSN, collected at diagnosis. PB samples from 12 normal controls were included as negative controls.

2. Materials and methods

2.1. Patients and samples

A group of 13 male untreated SCLC patients with limited disease and 11 male and 1 female untreated patients with extensive disease SCLC, recruited at Internal Medicine Division of "Casa Sollievo della Sofferenza" Hospital, were enrolled in this study. All SCLC patients were diagnosed and staged according with the two stage system, introduced by the Veterans' Affairs Lung Study Group (VALSG) [4]. Peripheral blood samples taken at diagnosis were analysed.

Negative controls were blood samples of never smoking male healthy volunteers ($n = 12$) recruited at Transfusional Centre of "Casa Sollievo della Sofferenza" Hospital, who had no evidence of any clinically detectable disease at the time of blood withdrawal. For all individuals enrolled in this study a written informed consent was obtained.

For each patient 2.5 ml of peripheral blood were collected on PAX-gene™ Blood RNA Tubes (PreAnalytiX), stored at room temperature for a minimum of 2 hours, then at -20°C for a minimum of 24 h, before processing or storing at -80°C.

2.2. Sample processing, RNA extraction and cDNA synthesis

Total RNA was extracted using PAXgene Blood RNA kit (PreAnalytix). RNA was eluted in RNAse free-water and stored at -80°C until used. RNA quality and concentration were measured by using 2100 Expert Analyzer (Agilent Technology) and only RNA with RIN (RNA Integrity Number) $\geqslant 7.0$ was processed. After heating at 65°C for 5 min in order to denature RNA and to inactivate RNases, 500 ng of total RNA was subjected to reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen). cDNA was synthesized according to the manufacturer's instructions.

Table 1
nELAV-like primer sequences used in real-time RT-PCR experiments

Marker		Primer sequence
HuD	Forward	5′-ACACATACACGAAAGAGAGAGAAACAA-3′
	Reverse	5′-AACACTGGCTTATAAAGTCCATGGT-3′
HuB	Forward	5′-TTCTTTTTACATAACTGCCTTGAACCT-3′
	Reverse	5′-AAGACACAACCAACTGAAGAATTACAA-3′
HuC	Forward	5′-CCCAGGCTGGGCTGTTC-3′
	Reverse	5′-CTCTGCATTCTTTTTAGCCGAAA-3′

2.3. Taqman primers design

The set of primers designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA) were located in the 3′UTR of *HuB*, *HuC* and *HuD* genes respectively and are listed in Table 1. The primers were designed to differentiate between the highly homologous *n-ELAV-like* genes (Suppl. Fig. 1: http://www.qub.ac.uk/isco/JCO). Glyceraldehyde phosphate dehydrogenase (*GAPDH*) was chosen as housekeeping gene, and commercially available primers were used (see the User Bulletin #2, Applied Biosystems, for the primers sequences).

2.4. Plasmids construction, amplification and purification

PCR fragments for all three *n-ELAV-like* genes and *GAPDH* as control gene were generated by using primers listed in Table 1, were cloned in the pCR® 4-TOPO® Vector (Invitrogen) and introduced in *Escherichia coli* DH5α™. Plasmid DNA from the selected transformant cells was isolated by using the QIAprep® Spin Miniprep Kit (Qiagen). Recombinant vectors, linearised with Not I, were serially diluted. Five plasmid dilutions of pCR® 4-TOPO®-HuB, pCR® 4-TOPO®-HuC, pCR® 4-TOPO®-HuD, pCR® 4-TOPO®-GAPDH (in the range of 1×10^6 copies to ten copies) were used to construct the standard curves for real-time PCR.

2.5. Taqman quantitative real-time PCR (qRT-PCR) conditions

SYBR Green amplification mixture (10 μl) contained 2.5× QuantiTect™ SYBR Green PCR Master Mix (Qiagen), 250 nM of each forward and reverse primers and 1 μl of template cDNA or plasmid product (serial dilutions). Reactions were run on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Cycling conditions were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 s and 60°C for 60 s. Each assay was carried out in triplicate and the transcription level was normalized using GAPDH as reference gene. A standard curve (used as calculation method) with five plasmid dilutions of pCR® 4-TOPO®-HuB, pCR® 4-TOPO®-HuC, pCR® 4-TOPO®-HuD, pCR® 4-TOPO®-GAPDH was included in each respective PCR run. Calibration curves were constructed by plotting the threshold cycle versus logarithm of the relative copy number.

2.6. qRT-PCR data analysis

Crossing points (beginning of the PCR exponential phase) were assessed by the second derivate maximum algorithm and plotted against the concentrations of the standards. Sample concentration was calculated using the plasmid standard curve, resulting in plasmid concentrations expressed as copy number of corresponding standard molecules. The relative sample amount was expressed as ratio marker (n-ELAV-like/GAPDH).

2.7. Statistics

nELAV mRNA expression in healthy blood donors and cancer patients were compared by using the Mann–Whitney U-test (for unpaired non-normally distributed groups) followed by a normal approximation with continuity correction. Values of $P < 0.05$ were considered statistically significant.

2.8. Sensitivity of the qPCR assays

To establish quantitative range and main detection limit, 10-fold serial dilutions of plasmid containing HuD, HuC or HuB insert were analyzed in triplicate by qRT-PCR (Suppl. Fig. 2: http://www.qub.ac.uk/isco/JCO). For all three target genes we were able to detect as low as ten plasmid copies, although accurate quantification requires at least 100 plasmid copies. The dynamic range for quantification using plasmid calibration curves was six orders of magnitude for all three markers.

3. Results

3.1. Expression of nELAV markers in healthy volunteers

HuB, *HuC* and *HuD* expression was evaluated in PB samples from 12 healthy blood donors (HBD). For *HuD* and *HuB* a median target genes/*GAPDH* copy number ratio of 0 (95% confidence interval, CI 0–30) and 0 (95% CI 0–3, HuB) were detected respectively. Whereas for *HuC* a median target genes/*GAPDH* copy number ratio of 26 (95% CI 0–73) was found.

3.2. Expression of nELAV markers in SCLC patients

To determine *HuB*, *HuC* and *HuD* expression in SCLC patients, 25 peripheral blood samples from affected individuals were analyzed. A statistically significant difference in target genes/*GAPDH* copy number ratio was found for *HuD* (median 1; 95% CI 0–25) and *HuB* (median 4; 95% CI 0–25) as compared with healthy blood donors ($P \leqslant 0.00034$ and $P \leqslant 0.00001$, respectively). For *HuC* the median target genes/*GAPDH* copy number ratio was 2 (95% CI 0–26) and no statistically significant differences were detected as compared with levels of mRNA expression in healthy blood donors ($P = 0.24257$).

Since low levels of nELAV expression were detected for all three markers in samples from healthy blood donors, a cut-off value for the target genes/*GAPDH* copy number ratio was introduced to distinguish between normal and pathological samples. These cut-off values were calculated as the upper limit of the 95% CI of the distribution of the target gene/*GAPDH* copy number ratios in blood samples from healthy blood donors and were set as follows *HuD* $>$ 30, *HuB* $>$ 3, *HuC* $>$ 73 (Fig. 1). Six out of the twenty-five

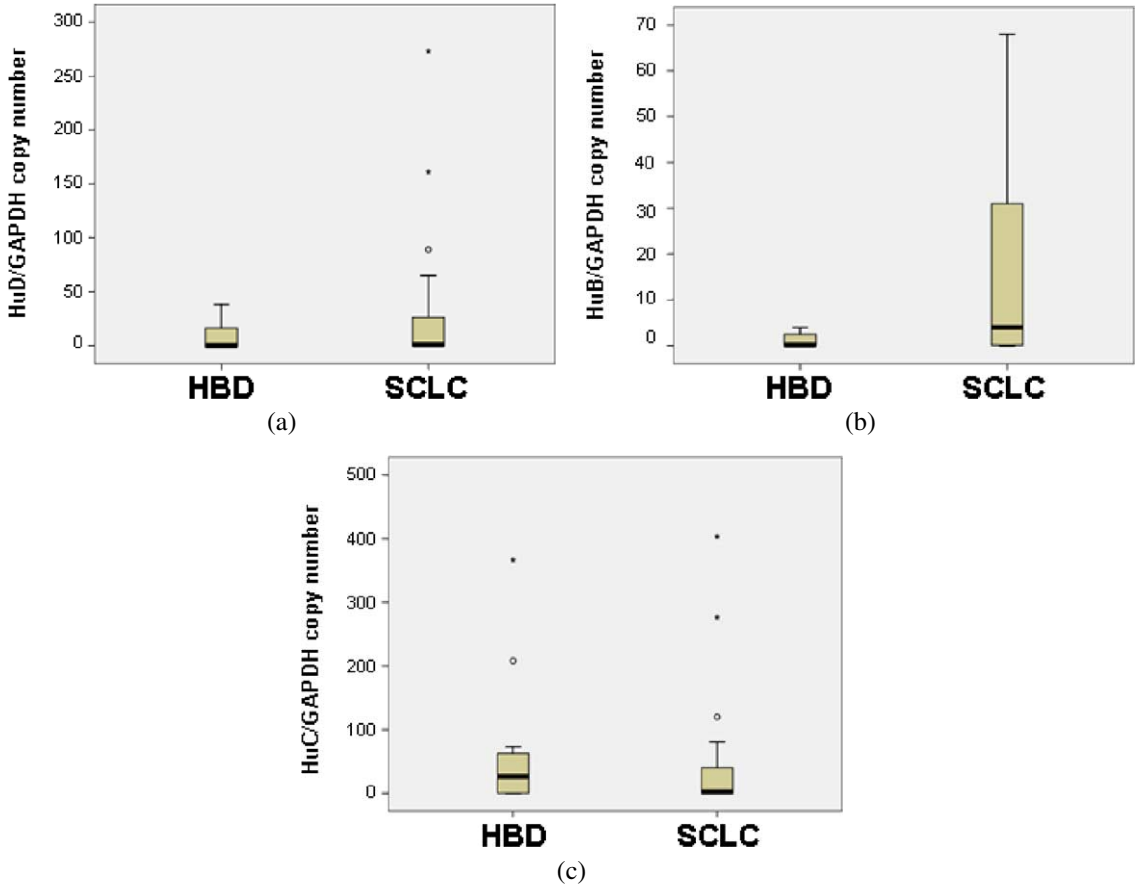

Fig. 1. Box plots transcripts in peripheral blood samples from healthy blood donors (HBD) and SCLC patients (SCLC) for HuD (a), HuB (b) and HuC (c). The boxes mark the interquartile range (interval between the 25th and 75th percentile). The lines inside the boxes denote median values. The whiskers represent the interval between the 10th and 90th percentiles. The empty circle indicate the outliners values between 1.5 and 3 length upper or down from the interquartile range. '*' indicates the extreme cases with more than three boxes length upper or down from the interquartile range.

Table 2
Correlation between patient characteristics and HuD, HuB and HuC positiveness of SCLC patients ($n = 25$)

Samples	HuD	HuB	HuC	Metastasis	Status
SCLC-1	+	−	+	Bone, liver	ED
SCLC-2	−	+	−	Brain	ED
SCLC-3	−	−	−	Bone, liver	ED
SCLC-4	−	−	−	Bone, liver	ED
SCLC-5	−	+	−	Non-detected	LD
SCLC-6	−	−	−	Non-detected	LD
SCLC-7	−	−	−	Non-detected	LD
SCLC-8	−	−	−	Bone	ED
SCLC-9	+	+	−	Liver	ED
SCLC-10	−	−	−	Brain, adrenal gland	ED
SCLC-11	−	−	−	Omolateral lung, liver	ED
SCLC-12	−	−	−	Bone	ED
SCLC-13	−	+	−	Non-detected	LD
SCLC-14	−	−	−	Non-detected	LD
SCLC-15	−	−	−	Non-detected	LD
SCLC-16	+	+	+	Non-detected	LD
SCLC-17	+	+	+	Non-detected	LD
SCLC-18	−	+	−	Non-detected	LD
SCLC-19	+	+	+	Brain, controlateral lung	ED
SCLC-20	−	+	−	Controlateral lung	ED
SCLC-21	−	+	−	Non-detected	LD
SCLC-22	+	+	−	Non-detected	LD
SCLC-23	−	+	−	Non-detected	LD
SCLC-24	−	−	−	Controlateral lung	ED
SCLC-25	−	+	−	Omolateral lung	LD

ED = Extensive Disease; LD = Limited Disease.

SCLC patients were positive for *HuD* expression (24% (6/25), 13 were positive for *HuB* expression (52%) and only 4 showed *HuC* expression (16%) (Table 2). Concomitant positiveness of *HuD* and *HuB* was detected in 5 of the 25 pathological samples (20%). In total 14 out of the 25 SCLC peripheral blood samples (56%) displayed positive expression of *HuD*, *HuB* or both. Among these 70% (9/13) of patients showed limited-disease stage in comparison with 34% (4/12) with extensive disease. Only one HuD and HuB-positive patients had brain metastases at the time of diagnosis (Table 2).

4. Discussion

The high sensitivity of qRT-PCR assays for detecting mRNA expression of tumour associated antigens has led, in the last few years, to a large number of studies aimed to use these technique for the definition of minimal residual disease and as prognostic indicator.

The main advantage of real-time techniques is the ability of detecting one tumour cell in one million normal peripheral blood cells, which is at least 10 times more sensitive than immunohistochemistry [23] with a detection limit of one tumour cell/10^6–10^8 normal cells [24–26]. A further advantage of quantitative PCR is the possibility to take into account variations in RNA and/or cDNA amount by quantifying housekeeping genes and subsequent normalization of marker concentration to that of the housekeeping gene.

In this study, a quantitative qRT-PCR method was applied to detect SCLC cells in peripheral blood of patients with SCLC by using *nELAV* genes as tumour markers. nELAV genes play an essential role in the development of nervous system and is highly expressed in neuroendocrine tumours, such as SCLC [7]. In addition auto-antibodies against Hu-proteins could be detected in serum of patients affected by this tumour type [8,12,20].

Recently, the detection of *HuD* transcript by qRT-PCR was reported as molecular marker for the definition of MRD (minimal residual disease) in neuroblastomas patients [26]. To the best of our knowledge the use of this technique to investigate on *HuB* and *HuC* and *HuD* expression in SCLC has not been previously reported.

We first assessed *nELAV* expression in the peripheral blood samples of a group of 12 healthy blood donors.

Since low level of expression was detected in this group of individuals for all three genes, cut off value was established to distinguish normal and pathological samples. These value was set at the upper limit of the 95% CI of the median of control group, thus a SCLC affected patients analyzed was considered positive for the expression of the *nELAV* mRNA when exceeding this value. Twenty-four percent, 52% and 16% of samples scored positive for *HuD*, *HuB* and *HuC* respectively, whereas the 20% of samples show concordant results for both *HuD* and *HuB* markers.

Overall 56% of the tumour analyzed showed *HuD* or *HuB* transcript above the cut-off values.

The rate of HuB positiveness patients in limited disease (70%) seems to be different from those with extensive disease (34%), but a prospective study of disease evolution in patients with limited disease is needed to confirm if the HuB and/or HuD expression in peripheral blood may reflect a specific level of diffusion and aggressive form in this tumour type. The detection and quantification of *nELAV* transcripts in peripheral blood is assured over a wide concentration range down to 10 standard molecules and qRT-PCR as-

say for the three *nELAV* mRNA showed a sensitivity of detecting one tumour cell among 10^6 normal peripheral blood mononuclear cells, which is in accordance with previous reports. Moreover, the use of plasmid dilutions for quantification assure a more reproducible and accurate quantifications of gene expression than dilutions based on cell lines [15] since the variation between different PCR runs is extremely low and absolute copy numbers can be calculated based on concentration measurements.

In summary, our qRT-PCR experiments indicate that the combined detection of *HuB* and *HuD* transcript by absolute quantification is able to detect more than half of the patients affected by SCLC. The high sensitivity of this technique could be particularly useful for monitoring the disease after treatment and for the detection of minimal residual disease in SCLC patients with an apparent complete response to therapy. However, additional studies with larger patients groups are required to confirm these experimental observations.

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References

[1] J. Aerts, W. Wynendaele, R. Paridaens, M.R. Christiaens, W. van den Bogaert, A.T. van Oosterom and F. Vandekerckhove, A real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to detect breast carcinoma cells in peripheral blood, *Ann. Oncol.* **12** (2001), 39–46.

[2] N.E. Anderson, J.M. Cunningham and J.B. Posner, Autoimmune pathogenesis of paraneoplastic neurological syndromes, *Crit. Rev. Neurobiol.* **3**(3) (1987), 245–299.

[3] D. Antic and J.D. Keene, Embryonic lethal abnormal visual RNA-binding proteins involved in growth, differentiation, and posttranscriptional gene expression, *Am. J. Hum. Genet.* **61**(2) (1997), 273–278.

[4] A. Argiris and J.R. Murren, Staging and clinical prognostic factors for small-cell lung cancer, *Cancer J.* **7**(5) (2001), 437–447.

[5] K. Barami, K. Iversen, H. Furneaux and S. Goldman, Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain, *J. Neurobiol.* **28** (1995), 82–101.

[6] E. Birney, S. Kumar and A.R. Krainer, Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors, *Nucleic Acids Res.* **21** (1993), 5803–5816.

[7] J. Dalmau, H.M. Furneaux, C. Cordon-Cardo and J.B. Posner, The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues, *Am. J. Pathol.* **141** (1992), 881–886.

[8] J. Dalmau, H.M. Furneaux, R.J. Gralla, M.G. Kris and J.B. Posner, Detection of anti-Hu antibody in the serum of patients with small cell lung cancer: a quantitative Western blot analysis, *Ann. Neurol.* **27** (1990), 544–552.

[9] J. Dalmau and J.B. Posner, Neurologic paraneoplastic antibodies (anti-Yo; anti-Hu; anti-Ri): the case for a nomenclature based on antibody and antigen specificity, *Neurology* **44** (1994), 2241–2246.

[10] R.B. Darnell, Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity, and the brain, *Proc. Natl. Acad. Sci. USA* **93** (1996), 4529–4536.

[11] E.J. Dropcho and P.H. King, Autoantibodies against the Hel-N1 RNA-binding protein among patients with lung carcinoma: an association with type I anti-neuronal nuclear antibodies, *Ann. Neurol.* **36** (1994), 200–205.

[12] F. Graus, J. Dalmau, R. Rene, M. Tora, N. Malats, J.J. Verschuuren, F. Cardenal, N. Vinolas, J. Garcia del Muro, C. Vadell, W.P. Mason, R. Rosell et al., Anti-Hu antibodies in patients with small-cell lung cancer: association with complete response to therapy and improved survival, *J. Clin. Oncol.* **15** (1997), 2866–2872.

[13] A. Hochhaus, A. Weisser, P. La Rosee, M. Emig, M.C. Müller, S. Saußele, A. Reiter, C. Kuhn, U. Berger, R. Hehlmann and N.C.P. Cross, Detection and quantification of residual disease in chronic myelogenous leukemia, *Leukemia* **14** (2000), 998–1005.

[14] M. Ismail, W. Wynendaele, J. Aerts, R. Paridaens, L. Van Mellaert, J. Anné, R. Gaafar, N. Shakankiry, H.M. Khaled, M.R. Christiaens, S. Omar, P. Vandekerckhove and A.T. van Oosterom, Quantification of CK19 mRNA in peripheral blood (PB) and bone marrow (BM) from primary operable breast cancer (BC) patients pre- and postoperatively to investigate possible shedding of CK19+ cells during the operation. Proceedings of ECCO-11, *Eur. J. Cancer* **37**(Suppl. 6) (2001), S117.

[15] M.S. Ismail, W. Wynendaele, J.L. Aerts, R. Paridaens, L. Van Mellaert, J. Anné, R. Gaafar, N. Shakankiry, H.M. Khaled, M.R. Christiaens, S. Omar, P. Vandekerckhove and van A.T. Oosterom, Real-time quantitative RT-PCR and detection of tumour cell dissemination in breast cancer patients: plasmid versus cell line dilutions, *Ann. Oncol.* **14**(8) (2003), 1241–1245.

[16] P.H. King, T.D. Levine, R.T. Fremeau and J.D. Keene, Mammalian homologs of Drosophila ELAV localized to a neuronal subset can bind *in vitro* to the 3′UTR of mRNA encoding the Id transcriptional repressor, *J. Neurosci.* **14** (1994), 1943–1952.

[17] W.-J. Ma, S. Cheng, C. Campbell, A. Wright and H. Furneaux, Cloning and characterization of HuR, a ubiquitously expressed ELAV-like protein, *J. Biol. Chem.* **271** (1996), 8144–8151.

[18] G.T. Manley, P.S. Smitt, J. Dalmau and J.B. Posner, Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites, *Ann. Neurol.* **38** (1995), 102–110.

[19] M.F. Marusich, H.M. Furneaux, P.D. Henion and J.A. Weston, Hu neuronal proteins are expressed in proliferating neurogenic cells, *J. Neurobiol.* **25**(2) (1994), 143–155.

[20] W.P. Mason, J. Verschuuren, F. Graus et al., Anti-Hu antibodies in patients with small cell lung cancer, but no paraneoplastic disorder (Abstract), in: *Proceedings of the 120th Annual Meeting of the American Neurological Association*, Vol. 83, Washington, DC, October 22–25, 1995.

[21] D.M. Park, L.E. Abrey, J.O. Dalmau, M. Bisogna, L.M. Krug, M.G. Kris, V.A. Miller, K.K. Ng, H.T. Thaler and J.B. Posner, Prevalence and significance of anti-Hu in small cell lung cancer (Abstract), in: *ASCO Annual Meeting*, San Francisco, CA, 2001.

[22] K. Sakai, M. Gofuku, Y. Kitagawa, T. Ogasawara, G. Hirose, M. Yamazaki, C.S. Koh, N. Yanagisawa and L. Steinman, A hippocampal protein associated with paraneoplastic neurologic syndrome and small cell lung carcinoma, *Biochem. Biophys. Res. Commun.* **199** (1994), 1200–1208.

[23] A. Schoenfeld, K.H. Kruger, J. Gomm, H.D. Sinnett, J.-C. Gazet, N. Sacks, H.G. Bender, Y. Luqmani and R.C. Coombes, The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using immunohistochemistry and reverse transcriptase polymerase chain reaction for keratin 19, *Eur. J. Cancer* **33** (1997), 854–861.

[24] R. Schuster, N. Max, B. Mann, K. Thilo, F. Heufelder, J. Gröne, F. Rokos, H.-J. Buhr, E. Thiel and U. Keilholz, Quantitative real-time RT-PCR for detection of disseminated tumor cells in peripheral blood of patients with colorectal cancer using different mRNA markers, *Int. J. Cancer* **108**(2) (2004), 219–227.

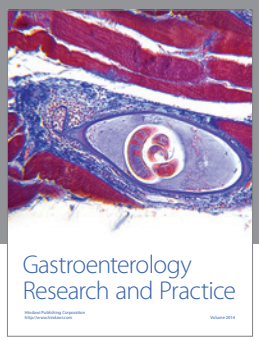
[25] A. Stathopoulou, I. Vlachonikolis, D. Mavroudis, M. Perraki, Ch. Kouroussis, S. Apostolaki, N. Malamos, S. Kakolyris, A. Kotsakis, N. Xenidis, D. Reppa and V. Georgoulias, Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance, *J. Clin. Oncol.* **20**(16) (2002), 3404–3412.

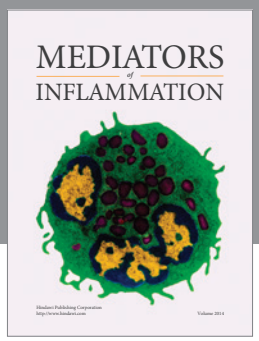
[26] K. Swerts, B. De Moerloose, C. Dhooge, J. Vandesompele, C. Hoyoux, K. Beiske, Y. Benoit, G. Laureys and J. Philippe, Potential application of ELAVL4 real-time quantitative reverse transcription-PCR for detection of disseminated neuroblastoma cells, *Clin. Chem.* **52** (2006), 438–445.

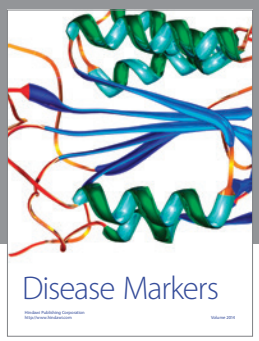
[27] A. Szabo, J. Dalmau, G. Manley, M. Rosenfeld, E. Wong, J. Henson, J.B. Posner and H.M. Furneaux, HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and sex-lethal, *Cell* **67** (1991), 325–333.

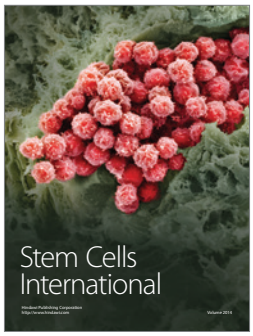
[28] Y. Wakamatsu and J.A. Weston, Sequential expression and role of Hu RNA-binding proteins during neurogenesis, *Development* **124**(17) (1997), 3449–3460.

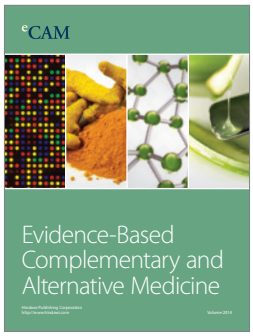
[29] K.M. Yao, M.L. Samson, R. Reeves and K. White, Gene ELAV of Drosophila melanogaster: a prototype for neuronal-specific RNA binding protein gene family that is conserved in flies and humans, *J. Neurobiol.* **24** (1993), 723–739.

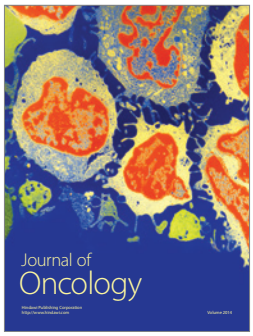


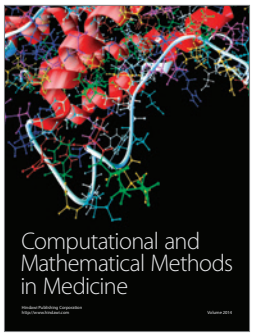


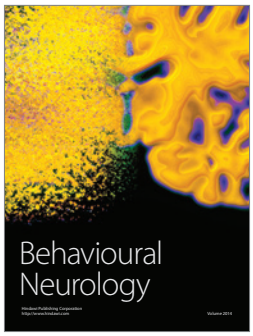


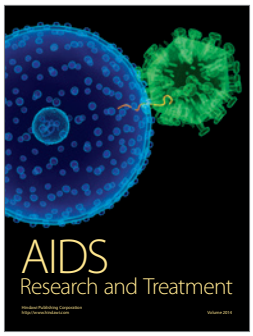


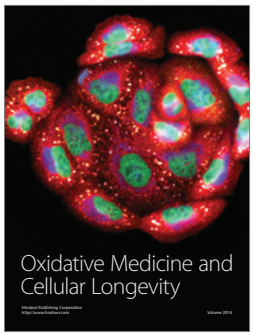












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