Neuropathology and Applied Neurobiology (2015), **41**, 319–332 doi: 10.1111/nan.12127

The protein expression profile of meningioma cells is associated with distinct cytogenetic tumour subgroups

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Aims: Limited information exists about the impact of cytogenetic alterations on the protein expression profiles of individual meningioma cells and their association with the clinicohistopathological characteristics of the disease. The aim of this study is to investigate the potential association between the immunophenotypic profile of single meningioma cells and the most relevant features of the tumour. **Methods:** Multiparameter flow cytometry (MFC) was used to evaluate the immunophenotypic profile of tumour cells ($n = 51$ patients) and the Affymetrix U133A chip was applied for the analysis of the gene expression profile ($n = 40$) of meningioma samples, cytogenetically characterized by interphase fluorescence *in situ* hybridization. **Results:** Overall, a close association between the pattern of protein expression and the cytogenetic profile of tumour cells was found. Thus, diploid tumours displayed higher levels of expression of the CD55 complement regulatory protein, tumours carrying isolated monosomy 22/del(22q) showed greater levels of bcl2 and PDGFRβ and meningiomas carrying complex karyotypes displayed a greater proliferation index and decreased expression of the CD13 ectoenzyme, the CD9 and CD81 tetraspanins, and the Her2/neu growth factor receptor. From the clinical point of view, higher expression of CD53 and CD44 was associated with a poorer outcome. **Conclusions:** Here we show that the protein expression profile of individual meningioma cells is closely associated with tumour cytogenetics, which may reflect the involvement of different signalling pathways in the distinct cytogenetic subgroups of meningiomas, with specific immunophenotypic profiles also translating into a different tumour clinical behaviour.

Keywords: CD44, CD53, iFISH, meningioma, multiparameter flow cytometry, protein expression profile

Introduction

Meningiomas consist of a heterogeneous group of central nervous system (CNS) tumours both on histopathological and on genetic/molecular grounds [1–3]. Most meningiomas show a benign clinical behaviour and

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patients are cured after complete surgical resection of the tumour. However, up to 20% of the cases will show tumour recurrence, which leads to an increased morbidity and mortality [1,4]. For decades now, it is well established that grade II (e.g. atypical) and grade III (e.g. anaplastic) meningiomas show higher recurrence rates and a poorer prognosis, compared with grade I tumours [1,2]. Despite this, in absolute numbers, the majority of recurrences observed among meningioma patients still occur in histologically benign/grade I tumours [4].

In recent years, evidence has accumulated which shows an association among histologically benign/grade I meningiomas, between complex tumour karyotypes (≥2 genetic alterations), particularly those that include monosomy 14, and a shorter patient relapse-free survival (RFS) [3,5–7]. Although distinct cytogenetic subtypes of meningiomas are associated with specific histopathological subtypes and unique gene expression profiles (GEP), to the best of our knowledge, no study has been reported so far in which the pattern of expression of a broad panel of proteins has been analysed in meningiomas to determine whether the immunophenotypic profile of single cells from individual tumours is associated with the most relevant features of the disease, including tumour histopathology and cytogenetics, as well as patient outcome. In this regard, we have recently shown that multiparameter flow cytometry (MFC) immunophenotyping is a well-suited technique for the evaluation of the pattern of (quantitative) expression of relatively large numbers of tumour-associated proteins in individual tumour cells, when an appropriate marker combination is used for exclusion of other types of non-neoplastic cells (e.g. inflammatory cells) infiltrating the tumour [8].

In this study, we analysed the pattern of expression of a large panel of markers by MFC, in 51 meningiomas. Our ultimate goal was to determine the potential association between the immunophenotypic profile of individual tumour cells and the clinical, histopathological and cytogenetic features of the disease, as well as patient outcome. Overall, our results show that a close association exists in meningiomas between the pattern of protein expression and the cytogenetic profile of tumour cells, pointing out the involvement of different pathogenetic mechanisms associated with unique protein expression profiles, in different cytogenetic subgroups of meningiomas.

Materials and methods

Patients and samples

A total of 75 patients (20 males and 55 females; mean age of 60 ± 14 years; range: 23 to 84 years) diagnosed with meningioma at the Neurosurgery Service of the University Hospital of Salamanca (Salamanca, Spain), and who gave their informed consent to participate, according to the guidelines of the local Ethics Committee and the Declaration of Helsinki, were included in this study. Overall, 78 freshly frozen (liquid nitrogen) tumour samples from the 75 patients, were studied. According to their localization, tumours were distributed as follows: parasagittal, 9 cases (12%); convexity, 21 (27%); parasagittal and convexity, 8 (10%); tentorial, 2 (3%); cranial base, 25 (32%); spinal tumours, 12 (15%); and one case (1%) corresponded to an intraosseous tumour. According to the extension of brain oedema, 11 (15%) patients were classified as having light oedema (smaller or equal to the volume of the tumour), 16 (21%) moderate (doubling the volume of the tumour) and 10 (13%) as showing severe oedema (more than twice the volume of the tumour); the remaining 38 (51%) patients showed no oedema. From the histopathological point of view, 64 (82%) tumours were benign/grade I meningiomas, 11 (14%) were grade II tumours and 3 (4%) were grade III meningiomas [1]. All but three cases underwent complete tumour resection at diagnostic surgery. Adjuvant radiotherapy was given after surgery in four WHO grade II/III and two grade I tumours.

From all tumours, 29/78 (37%) showed a diploid interphase fluorescence *in situ* hybridization (iFISH) cytogenetic profile, 26/78 cases (33%) had isolated monosomy 22/del(22q) and 22/78 cases (28%) displayed a complex iFISH karyotype, as defined by the presence of cytogenetic alterations (losses and/or gains) of ≥2 chromosomes [6], as specified for individual tumours in Supplementary Table S1; the remaining case showed an isolated loss of chromosome 1p. More detailed information about those samples that have also been previously reported in other studies is provided in Supplementary Table S2. Ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood (PB) samples were also obtained from each patient and processed in parallel. At the moment of closing this study 6/75 patients had relapsed (8%) and one showed tumour regrowth (after partial tumour resection) after a median follow-up of 48 months (range: 1 to 238 months).

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Multiparameter flow cytometry (MFC) immunophenotypic studies

Immunophenotypic analysis of the cells present in the tumour was performed in a subset of 51 freshly frozen meningioma samples, using a FACSCanto II flow cytometer – Becton/Dickinson Biosciences (BD), San Jose, CA, USA – equipped with the FACSDiva™ 6.0 software (BD). After thawed, tumour samples were dissociated into single cell suspensions through conventional mechanical disaggregation procedures [8,9]. Single cell suspensions were then stained for 30 min at 4°C in the darkness, with four-colour combinations of monoclonal antibodies (MAb) conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE). For the identification of all nucleated cells and the discrimination between neoplastic meningioma cells and infiltrating immune cells, PE- and FITC-conjugated MAb were systematically combined with the DRAQ5 DNA dye (Cytognos SL, Salamanca, Spain) and CD45-Pacific Blue (PacB) (DAKO, Glostrup, Denmark), as previously described [8]. The following FITC- and PE-conjugated MAb were used: CD2-FITC, CD13-PE, CD14-PE, CD58-PE, HER2/neu-PE and HLA-DR-FITC, purchased from BD; CD53-PE, CD55-FITC, CD81-PE, CD99-PE, epidermal growth factor receptor (EGFR)-PE, insulin-like growth factor receptor (IGFR)-PE and platelet derived growth factor receptor β (PDGFRβ)-PE, purchased from BD Pharmigen (San Diego, CA, USA); CD9-FITC, CD63-FITC and HLA-I-FITC obtained from Beckman/Coulter (Fullerton, CA, USA); CD44-PE and CD59-FITC from Immunostep SL (Salamanca, Spain); bcl2-FITC and CD38-FITC were from DAKO and Cytognos SL respectively (Supplementary Table S3). For sample preparation, a stain-and-then-wash method was used, as previously described [8]. For cytoplasmic (Cy) markers (Cybcl2), cells were permeabilized (1 h at −20°C) prior to intracellular staining, as described elsewhere [10]. Staining with DRAQ5 was systematically performed by adding this reagent 5 min prior to the measurement in the flow cytometer; an aliquot of each tumour sample stained only for DRAQ5 was also prepared in parallel for each tumour sample, to assess control baseline autofluorescence levels [11]. The proliferation index (PI) of tumour cells was calculated as the percentage of cells showing a higher DNA content than that of G0/G1 cells, after excluding debris and cell doublets in a FSC-Area *vs.* SSC-Area and a DRAQ5-Area *vs.* DRAQ5-Width bivariate dot plots respectively [11]. Data analysis was performed using the INFINICYT software (Cytognos SL), and both the percentage of positive cells and the amount of protein expressed per cell – mean fluorescence intensity (MFI) expressed in arbitrary units scaled from 0 to 262 144 – were calculated for each individual marker analysed within the tumour cell population from each sample.

Gene expression profiling (GEP)

GEP analysis was performed in a subset of 40 (freshly frozen) meningioma samples, using the Human Genome 133A Affymetrix array (Affymetrix Inc., Santa Clara, CA, USA), as reported elsewhere [6,12]. Total RNA was isolated from thawed tumour samples using TRIzol (Invitrogen, Carlsbad, CA, USA) and the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) and its integrity/purity was determined using a microfluidic electrophoretic system (Agilent 2100 Bioanalyser; Agilent Technologies, Palo Alto, CA, USA). GEPs were then determined according to the manufacturer's instructions, using the one-cycle cDNA synthesis kit and the Poly-A RNA gene chip control kit (Affymetrix Inc.). Data files with data on the gene expression levels were normalized – Robust microarray normalization (RMA) – and analysed by specific software tools – R (version 2.7.0; http://www.r-project.org) and Bioconductor software (http://www.bioconductor.org). For the investigation of the functional impact of specific GEPs, the Ingenuity Pathway Analysis (IPA) software was used (Ingenuity Systems Inc., Redwood City, CA, USA).

Statistical methods and hierarchical clustering

For continuous variables, median, mean and standard deviation (SD) values, as well as range and the 10th, 25th, 75th and 90th percentiles, were calculated; for categorical variables, frequencies were reported. Statistical significance (P-value < 0.05; with a FDR correction for multiple comparisons of <10%) of differences observed between groups was determined through the nonparametric Kruskal–Wallis and Mann–Whitney U tests (for continuous variables), or the Pearson's Chi-square test (for categorical variables). The Spearman's correlation was used to explore the degree of correlation between different variables. The Kaplan–Meier method was used to construct RFS curves, and the log-rank test was applied to compare RFS curves. For multivariate analysis of patient RFS, the Cox regression model was used (SPSS 15.0 software, SPSS, Chicago, IL, USA).

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Unsupervised hierarchical clustering analysis was performed using the Pearson correlation and the average linkage clustering method (Cluster 3.0 and Tree View software; Stanford University, Stanford, CA, USA), after logarithmic (base 2) transformation of normalized data sets against the median value of all samples analysed.

Results

Immunophenotypic profile of meningioma cells and its association with the clinicobiological and cytogenetic features of the disease

Overall, tumour cells systematically displayed high reactivity for the CD9, CD63 and CD81 tetraspanin molecules, the CD55/CD59 complement regulatory proteins, HLA-I and the CD13 ectoenzyme (Supplementary Table S4). The other markers investigated showed more variable patterns of expression. Some were detected in most cells from the majority of cases – PDGFRβ (77 ± 28% of PDGFRβ$^+$ cells from 96% of cases), IGFR (73 ± 25% of IGFR$^+$ cells from 96% of cases), HER2/neu (73 ± 26% positive cells from 96% of cases), EGFR (69 ± 24% of EGFR$^+$ cells from 78% of cases), CD38 (66 ± 27% of CD38$^+$ cells from 86% cases), bcl-2 (65 ± 24% positive cells from 86% of cases), CD14 (76 ± 19% positive cells from 76% of cases) and HLA-DR (69 ± 23% positive cells from 80% of cases), while other proteins were present in a lower percentage of cases (62 ± 24%, 61 ± 22%, 60 ± 21% and 51 ± 21% of CD53, CD58, CD99 and CD2 positive cells in 63%, 59%, 47% and 22% of cases respectively). Additionally, meningioma cells showed a mean (± one standard deviation) MFC PI of 10% ± 6%, with a greater variability among different cases (wider range of percentages) than when assessed in parallel, as percentage of MIB-1$^+$ cells by immunohistochemistry, in a subset of 10 cases (data not shown).

From the clinical point of view, female meningiomas and convexity/parasagittal tumours showed greater expression of Cybcl2 ($P = 0.03$ and $P = 0.01$ respectively). Parasagittal tumours also showed higher expression of CD63 *vs.* all other meningiomas ($P \leq 0.04$), whereas spinal tumours presented lower reactivity ($P \leq 0.03$ *vs.* intracranial tumours) for the IGFR growth factor receptor. Additionally, fibroblastic meningiomas showed higher bcl2 levels/tumour cell *vs.* other histological subtypes ($P = 0.002$), while CD99 expression was greater in transitional *vs.* meningothelial meningiomas ($P = 0.004$) and the reactivity for PDGFRβ was significantly lower among high grade meningiomas ($P = 0.04$). Presence of moderate to severe oedema was associated with higher CD38 expression ($P = 0.02$) and lower reactivity for HLA-DR ($P = 0.02$).

Despite all the above associations, protein expression profiles of tumour cells from meningiomas were most strongly associated with tumour cytogenetics (Table 1). Thus, meningiomas with complex karyotypes showed decreased expression of the CD55 complement regulatory protein ($P = 0.01$ *vs.* diploid tumours), the CD9 ($P < 0.001$ *vs.* all other groups) and CD81 ($P < 0.03$ *vs.* all other groups) tetraspanins, the CD13 ectoenzyme ($P < 0.04$ *vs.* all other groups) and the HER2/neu growth factor receptor ($P < 0.02$ *vs.* all other groups) (Table 1 and Figure 1**A**). In turn, cases with isolated monosomy 22/del(22q) displayed a higher reactivity for the PDGFRβ receptor ($P < 0.01$ *vs.* diploid and complex tumours; Table 1 and Figure 1**B**) and bcl2 ($P < 0.005$ *vs.* diploid and complex tumours; Table 1 and Figure 1**C**). In addition, a progressively higher PI was found from diploid tumours, to cases with isolated −22/22q$^-$ and meningiomas with complex karyotypes ($P < 0.003$).

Unsupervised hierarchical clustering analysis based on the immunophenotypic features of tumour cells, revealed two clearly distinct subgroups of meningiomas (Figure 2). Interestingly, while one group included mostly (21/25 cases, 84%) patients with diploid meningiomas (11/17 diploid cases; 65%) or tumours carrying an isolated cytogenetic alteration (10/17 tumours with monosomy22/del(22q); 60%), the other group comprised almost all patients with a complex karyotype (12/16 cases, 75%). Of note, the majority of grade II/III meningioma samples (4/5 cases) were included in this latter group. The former group was characterized by a low PI together with a higher reactivity for CD9, CD55, CD81, CD13, PDGFRβ and HER2/neu, while the latter group displayed higher PI and lower levels of expression of the above referred markers (Figure 2).

Concerning patient outcome, although recurrent tumours showed higher levels of expression of HLA-DR ($P = 0.02$), CD44 ($P = 0.01$) and CD53 ($P = 0.006$), only the two latter markers retained a significant adverse impact on RFS ($P = 0.01$ and $P = 0.04$ respectively; Figure 3), in addition to tumour grade ($P < 0.001$) and cytogenetics ($P = 0.003$; Supplementary Figure S1). Multivariate analysis for RFS, including those variables which showed prognostic impact in the univariate analysis,

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Table 1. Association between the clinicobiological and cytogenetic characteristics of meningioma patients and both the immunophenotype and proliferation index of tumour cells

		Immunophenotypic markers													
	PI	*HLA-DR*	*CD55*	*CD9*	*CD53*	*CD63*	*CD81*	*CD44*	*CD99*	*CD13*	*CD38*	*Cybcl2*	*HER2/neu*	*IGFR*	*PDGFRβ*
Age															
<50 ($n = 13$)	8 ± 6	3181 ± 2626	4580 ± 2141	29268 ± 23183	485 ± 406	3964 ± 1800	20900 ± 12571	5332 ± 6785	362 ± 288	24018 ± 26393	2169 ± 2312	1128 ± 760	2375 ± 1912	1283 ± 693	2663 ± 2833
>50 ($n = 38$)	11 ± 6	2404 ± 2974	5189 ± 2392	24705 ± 15454	564 ± 950	3334 ± 1587	15903 ± 14881	6631 ± 9990	256 ± 219	16771 ± 23806	1707 ± 1905	831 ± 496	1743 ± 1599	1244 ± 1011	2167 ± 2081
Sex												†			
Female ($n = 37$)	9 ± 6	2479 ± 1999	4967 ± 1911	25168 ± 10492	488 ± 497	3454 ± 1675	16580 ± 11889	5764 ± 7887	267 ± 199	18954 ± 24673	1757 ± 1726	987 ± 606	1995 ± 1796	1253 ± 936	2544 ± 2548
Male ($n = 14$)	12 ± 6	2928 ± 4556	5208 ± 3260	27673 ± 20586	692 ± 1418	3602 ± 1633	18754 ± 19991	7718 ± 12351	325 ± 331	17733 ± 24660	2003 ± 2670	695 ± 466	1664 ± 1390	1258 ± 964	1640 ± 1143
Tumour localization						†#						†§		†‡	
Convexity/ parasagittal ($n = 31$)	12 ± 6	2999 ± 3414	4819 ± 2358	24220 ± 17369	628 ± 1009	3994 ± 1848	18348 ± 15947	6016 ± 8072	312 ± 272	14697 ± 18985	1917 ± 2314	1047 ± 611	1663 ± 1520	1194 ± 789	2650 ± 2457
Cranial base/ tentorial/ intraosseous ($n = 17$)	8 ± 5	1934 ± 1501	5532 ± 2418	30239 ± 18705	472 ± 496	2852 ± 853	16696 ± 11823	7556 ± 11775	239 ± 178	27638 ± 32384	1790 ± 1511	675 ± 481	2559 ± 1928	1542 ± 1127	1547 ± 1819
Spinal ($n = 3$)	7 ± 3	2284 ± 2940	4427 ± 1251	18121 ± 11182	78 ± 19	1980 ± 378	7795 ± 8811	2122 ± 1817	230 ± 211	8036 ± 4918	1062 ± 803	770 ± 459	683 ± 171	254 ± 160	1989 ± 1298
Oedema		†									†				
No/light ($n = 30$)	10 ± 5	3321 ± 3398	4920 ± 2242	27952 ± 18972	621 ± 1017	3339 ± 1624	18920 ± 16117	5210 ± 7352	323 ± 281	16688 ± 19546	1239 ± 1180	1006 ± 652	2048 ± 1689	1255 ± 948	2269 ± 2233
Moderate/severe ($n = 21$)	11 ± 7	1575 ± 1470	5196 ± 2485	22890 ± 15383	434 ± 502	3718 ± 1699	14687 ± 11352	7857 ± 11417	225 ± 154	21377 ± 30402	2661 ± 2599	766 ± 438	1699 ± 1705	1254 ± 936	2325 ± 2377
Tumour grade															†
Grade I ($n = 46$)	9 ± 6	2545 ± 2922	5043 ± 2154	26711 ± 18074	536 ± 872	3332 ± 1488	17420 ± 14862	5836 ± 9203	295 ± 246	19610 ± 25354	1761 ± 1914	920 ± 605	2032 ± 1719	1269 ± 975	2465 ± 2324
Grade II/III ($n = 5$)	15 ± 8	3123 ± 2740	4951 ± 3915	18109 ± 10645	612 ± 545	4995 ± 2445	14941 ± 9548	10571 ± 9333	175 ± 153	9499 ± 10574	2410 ± 2904	788 ± 298	727 ± 661	1116 ± 423	854 ± 1081
Tumour histopathology									†‡			†#			†§
Meningothelial ($n = 14$)	11 ± 7	1955 ± 1536	4878 ± 2355	26617 ± 17020	437 ± 485	3328 ± 2003	11197 ± 7620	6565 ± 9419	154 ± 91	11801 ± 13390	2172 ± 2948	759 ± 383	1548 ± 1381	1359 ± 1006	1663 ± 2005
Transitional ($n = 16$)	10 ± 5	2960 ± 4201	5084 ± 2402	30896 ± 21952	778 ± 1361	3608 ± 1052	22973 ± 19520	8363 ± 12310	402 ± 312	29518 ± 35208	1582 ± 1310	783 ± 513	2427 ± 1963	1528 ± 1187	2641 ± 2171
Psammomatous ($n = 7$)	8 ± 4	2464 ± 2373	5163 ± 2196	23469 ± 14163	274 ± 256	2849 ± 1021	13987 ± 11078	2230 ± 1875	314 ± 247	18830 ± 23615	850 ± 519	796 ± 443	1953 ± 2039	794 ± 590	3276 ± 2843
Fibroblastic ($n = 7$)	10 ± 5	3278 ± 2553	5117 ± 1860	24484 ± 15990	540 ± 444	3395 ± 1852	22399 ± 14160	3341 ± 3834	358 ± 199	12446 ± 12402	2114 ± 1237	1688 ± 848	2246 ± 1634	1095 ± 649	3466 ± 2783
Other ($n = 7$)**	12 ± 9	2539 ± 2455	5017 ± 3199	16658 ± 10770	496 ± 492	4315 ± 2313	13855 ± 9527	8085 ± 8725	162 ± 134	13303 ± 16463	2369 ± 2551	813 ± 309	1031 ± 1041	1041 ± 475	707 ± 929
iFISH karyotype*	†††¶		†#	†§			†§			††§		††‡	††§		†‡
Diploid ($n = 17$)	5 ± 3	1788 ± 1178	6174 ± 2646	34809 ± 21557	434 ± 497	2906 ± 859	20467 ± 12989	8112 ± 11528	283 ± 251	36089 ± 34715	1909 ± 1727	671 ± 357	2779 ± 1838	2779 ± 1838	1571 ± 1699
Monosomy 22/del(22q) ($n = 17$)	9 ± 5	3977 ± 4174	5092 ± 1700	26848 ± 14223	724 ± 1280	3353 ± 1549	21395 ± 17872	3775 ± 6389	394 ± 280	13009 ± 10095	1353 ± 1055	1366 ± 719	2185 ± 1723	2185 ± 1723	3552 ± 2659
Complex ($n = 16$)	15 ± 6	2126 ± 2066	3913 ± 2060	16609 ± 10480	496 ± 535	4375 ± 2072	10098 ± 8276	7417 ± 9182	179 ± 114	7162 ± 7140	2313 ± 2895	696 ± 277	766 ± 492	766 ± 492	1836 ± 1998
Relapse		†			††			††							
No ($n = 47$)	10 ± 6	2145 ± 1814	5179 ± 2350	26420 ± 17807	411 ± 445	3359 ± 1514	15713 ± 12166	5587 ± 9027	263 ± 219	18852 ± 25259	1896 ± 2065	906 ± 587	1938 ± 1726	1254 ± 964	2320 ± 2349
Yes ($n = 4$)	13 ± 6	7968 ± 6816	3329 ± 1099	19379 ± 15487	2103 ± 2293	5093 ± 2548	34372 ± 27326	14684 ± 8316	512 ± 384	15875 ± 12307	980 ± 662	918 ± 587	1503 ± 1235	1256 ± 523	2010 ± 1361

Results are expressed as mean MFI ± one standard deviation (SD); markers that showed no statistical significance (HLA-I, CD59, CD2, CD58, EGFR and CD14) are not shown.

PI: proliferation index (%S + G2/M cells).

P-value (Kruskal–Wallis test): †$0.01 < P < 0.05$; ††$0.001 < P < 0.01$; †††$P < 0.001$.

Significant differences (Mann–Whitney *U* test) for:

- tumour localization: #, convexity/parasagittal *vs.* others; ‡, spinal *vs.* others; §, convexity/parasagittal *vs.* cranial base/tentorial/intraosseous;
- tumour histopathology: #, fibroblastic *vs.* all others; ‡, transitional *vs.* meningothelial; §, meningothelial/transitional/fibroblastic *vs.* other;
- iFISH karyotype: #, D *vs.* C; ‡, −22 *vs.* others; §, C *vs.* others; ¶, all groups.

*The only patient with isolated del(1p36) was excluded from the analysis.

**Includes three atypical, one secretory, one rhabdoid, one angiomatous and one papillary tumour.

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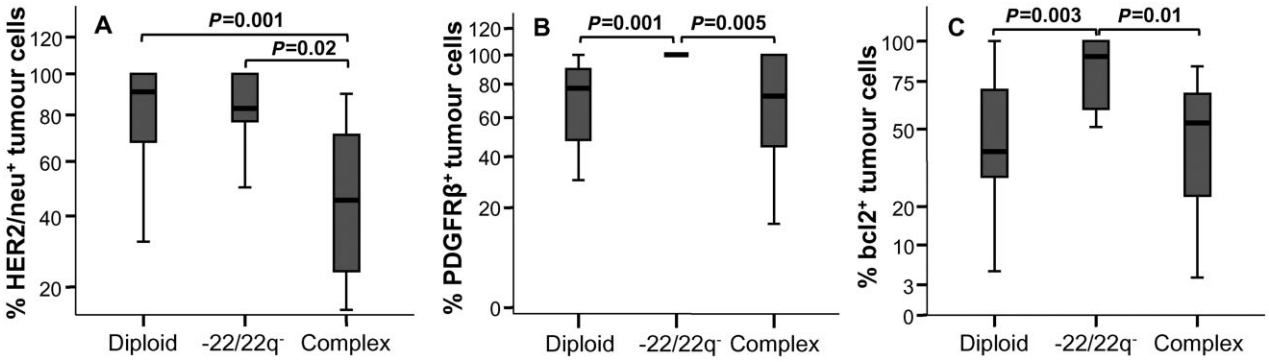

Figure 1. Distribution of Her2/neu$^+$, PDGFRβ$^+$ and bcl2$^+$ tumour cells in meningiomas grouped according to their cytogenetic profile. Expression of surface membrane Her2/neu, PDGFRβ and cytoplasmic bcl2, as percentage of all CD44$^+$CD45$^-$ tumour cells, is shown in panels **A**, **B** and **C** respectively; for all other markers investigated, no significantly different percentages of positive cells were found among the distinct cytogenetic groups of meningiomas. Notched-boxes represent 25th and 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles respectively.

showed that tumour grade was the only variable retaining an independent prognostic value ($P = 0.03$) in this group of meningiomas. Of note, co-expression of both the CD44 and CD53 markers at higher levels/tumour cell was associated with a particular adverse impact on patient RFS ($P = 0.001$; Figure 3**C**).

Relationship between mRNA and protein expression levels among the different cytogenetic subgroups of meningiomas

The relationship between mRNA and protein expression levels was investigated in a subset of 13 meningioma samples in which both sets of parameters were simultaneously analysed. Overall, a similar pattern of expression of CD13 and Cybcl2 was observed at the mRNA and protein levels among distinct cytogenetic subgroups of meningiomas (Figure 4**A** and **B**), with a high correlation coefficient between mRNA and protein expression ($r^2 = 0.9$, $P = 0.001$ and $r^2 = 0.5$, $P = 0.1$ respectively). In line with flow cytometry results, *CD13* mRNA was also decreased in tumours with a complex karyotype *vs.* diploid meningiomas ($P = 0.01$; Figure 4**A**). In turn, cases with isolated monosomy 22/del(22q) showed higher *BCL2* mRNA expression *vs.* all other tumours ($P \leq 0.03$; Figure 4**B**). Of note, despite a lower correlation coefficient, the PDGFRβ receptor also showed higher expression at both the mRNA and the protein levels among tumours carrying isolated monosomy 22/del(22q) ($P \leq 0.03$; Figure 4**C**). Conversely, an inverse correlation between both the HER2/neu and CD55 mRNA *vs.* protein levels was observed ($r^2 = -0.7$, $P = 0.006$ and $r^2 = -0.5$, $P = 0.008$ respectively), with significantly different amounts of protein/cell, but similar mRNA expression levels in the distinct cytogenetic subgroups of meningiomas (Figure 4**D** and **E** respectively). Finally, no significant correlation was found between the mRNA expression profiles and the protein levels of the CD9 and CD81 tetraspanin molecules (Figure 4**F** and **G** respectively).

Based on the differential GEP found for the three cytogenetic subgroups of meningiomas for the immunophenotypic markers here analysed, we built a schematic map representation of those intracellular pathways in which the cell surface and cytoplasmic molecules that showed unique patterns of expression in individual cytogenetic subgroups, are involved (Figure 5). High *CD13* (*ANPEP*) mRNA and protein expression was characteristic of diploid meningioma samples, while higher *PDGFRB* and *BCL2* mRNA and protein levels were usually observed in meningiomas carrying monosomy 22/del(22q); in turn, tumours with complex karyotypes typically showed higher levels of *CD44* mRNA (but not protein) expression, supporting the involvement of different signalling pathways in the distinct cytogenetic subgroups of meningiomas.

Discussion

In this study we analysed the pattern of expression of a relatively large panel of proteins in single tumour cell suspension from meningioma samples, using MFC. To the best of our knowledge, this is the first study to provide

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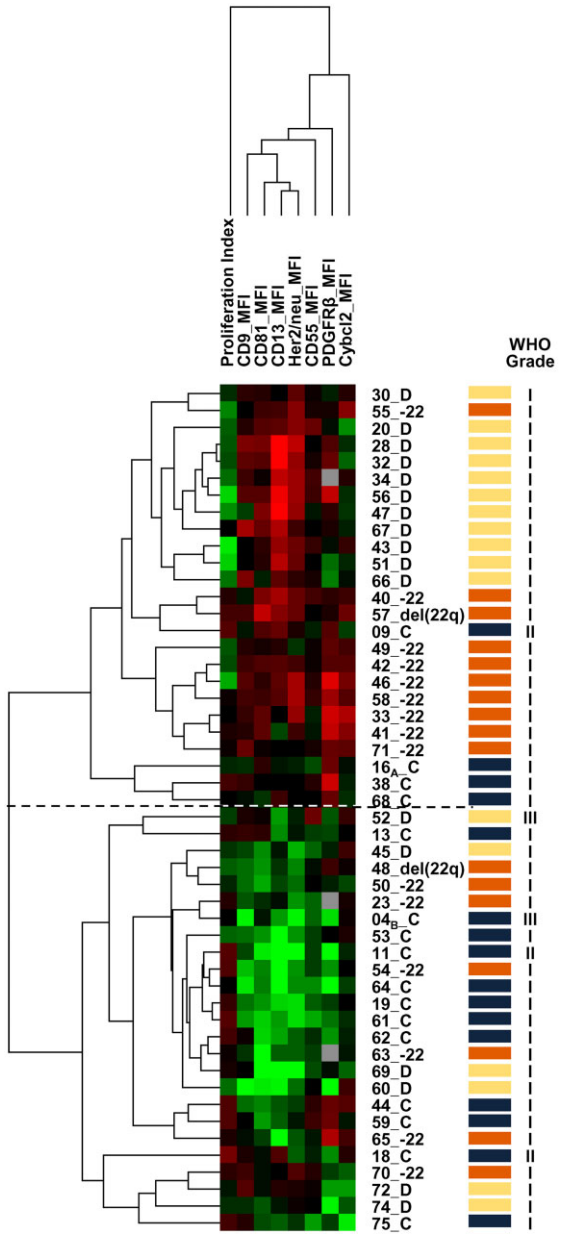

Figure 2. Hierarchical clustering analysis of meningioma samples based on the immunophenotypic characteristics and cell cycle distribution of tumour cells and its relationship with the different cytogenetic and WHO grade subgroups of the disease. Results are shown in a matrix format where each column represents a single variable and each row represents a different meningioma sample (rows identified with a 'D', '22' and 'C' correspond to diploid, isolated monosomy 22/del(22q) and complex iFISH karyotype meningiomas respectively). Normalized values are represented by a colour scale where red and green colours reflect values above and below the median values obtained for each variable respectively. On the right side of the figure, the hierarchical clustering of samples obtained is shown where most of the tumours tend to be grouped by protein expression profiles according to their iFISH cytogenetic pattern.

detailed immunophenotypic profiles of individual meningioma cells. Our results showed a clear association between the pattern of expression of several markers and tumour cytogenetics. As cytogenetically heterogeneous tumours [2,3], meningioma samples showed three major cytogenetic profiles which corresponded to diploid, isolated monosomy 22 and complex iFISH karyotypes.

Among other markers, the CD55 complement regulatory protein showed a uniquely high expression among diploid *vs.* cytogenetically altered meningiomas. Increased expression of complement regulatory proteins by tumour cells has been associated with resistance to complement-mediated cytotoxicity in several subtypes of solid tumours [13,14] and it has been proposed as a mechanism that facilitates tumour survival, leading to a poorer patient outcome [15]. Although CD55 did not show a significant association with more aggressive features of the disease, an inverse correlation was found between cell surface CD55 protein and total mRNA levels (with greater mRNA expression among cytogenetically complex tumours). Such apparent discrepancy may be due to an altered balance between protein synthesis, degradation, secretion and/or mobilization of stored CD55. In this regard, recent reports demonstrate the presence of soluble CD55 (sCD55) at the extracellular matrix level in several different tumour types [15].

Meningiomas with isolated monosomy 22/del(22q) also showed a unique protein (and mRNA) expression profile *vs.* other cytogenetic subgroups of meningiomas, consisting of significantly higher levels of Cybcl2 and PDGFRβ. These findings point to the potential relevance of PDGFRβ in this subgroup of meningiomas [16,17], where it may be associated with inhibition of apoptosis [18–20] through activation of Akt [21,22]. Finally, cytogenetically complex tumours showed uniquely low protein and mRNA expression levels of the CD13 ectoenzyme (aminopeptidase N; APN), in addition to a higher proliferation index. CD13 has been involved in a variety of cellular functions, including the control of tumour cell proliferation and invasion [23], and its relevance in meningiomas has already been reported by others. Thus, Mawrin *et al.* [24] described a significant reduction of CD13 mRNA and protein expression levels, as well as its enzymatic activity, in high-grade meningiomas [24,25]. Moreover, cytogenetically complex meningiomas also showed decreased expression of the CD9 and CD81 tetraspanins, both of which have been associated with malignant progression of solid tumours [26–29] lack

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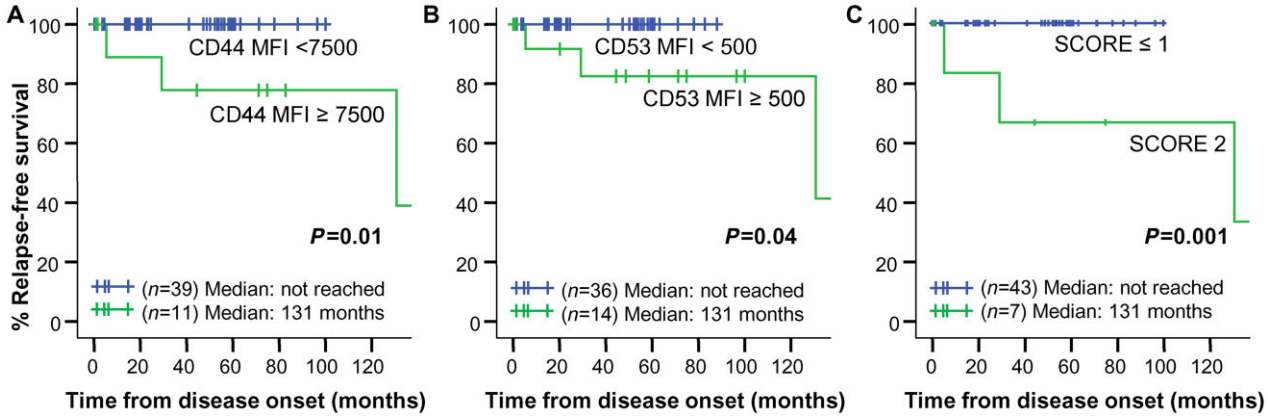

Figure 3. Impact of the pattern of protein expression of tumour cells on relapse-free survival of meningioma patients. Relapse-free survival curves of meningioma patients classified according to the levels of expression of CD44 ($n = 50$; panel **A**) and CD53 ($n = 50$; panel **B**) per tumour cell, are shown. In panel **C** the impact on relapse-free survival of a score built on the basis of the expression of both CD44 and CD53 is shown: score 0 and 1 were assigned for tumour samples expressing none or only one of the markers and score 2 was assigned to cases showing co-expression of both CD53 ≥ 500 (MFI) and CD44 ≥ 7500 (MFI). All other markers analysed did not show an impact on patient relapse-free survival. MFI: mean fluorescence intensity per tumour cell (arbitrary fluorescence units scaled from 0 to 262 144).

of expression of CD9 and CD81 being associated with lower integrin-dependent adhesion and enhanced cell growth [30]. These observations may contribute to explain, at least in part, the increased proliferation index of tumour cells from cytogenetically complex *vs.* other meningiomas. Finally, expression of the HER2/neu growth factor receptor protein was also significantly decreased among tumours with a complex karyotype *vs.* other meningiomas. Of note, expression of Her2/neu should be carefully evaluated for appropriate interpretation as this protein is expressed at the cytoplasmic membrane, but as other ErbB receptors (e.g. EGFR) it may undergo internalization and/or cleavage due to recycling of the receptor between the plasma membrane and the endosomal compartments, and because of protease-mediated cell surface cleavage in activated cells respectively [31,32]. This might contribute to explain why despite lower protein levels were found in cytogenetically complex *vs.* other meningiomas, no differences were detected at the mRNA level between the distinct cytogenetic tumour subtypes. Therefore, lower Her2/neu protein levels on the cell membrane of cytogenetically complex meningiomas may potentially reflect a higher cell activation and protein processing. Overall, these findings support previous observations [33–35] suggesting the relevance of ErbB receptors in the biology of meningiomas.

The level of expression of distinct proteins on tumour cells was also associated with other features of the disease, including patient outcome. Of note, higher expression of both CD44 and CD53 was associated with a shorter RFS. Interestingly, CD44 is involved in the regulation of cell–cell and cell–matrix adhesion, acting as a functional antagonist of the merlin protein [36,37], which is the product of the NF2 tumour suppressor gene that is frequently lost during meningioma tumorigenesis [2,36,38]. In this regard, it has been described that monosomy 22 is closely associated with the presence of coding *NF2* mutations [39,40], although not all cases with monosomy 22 carry *NF2* mutations (Supplementary Table S2) [39]. Greater CD44 expression in higher grade meningiomas has also been previously found by others [41,42]. Regarding CD53, to the best of our knowledge this is the first report in which this protein is specifically investigated in meningiomas; however, as a member of the tetraspanin family, CD53 has been associated with cell adhesion and motility [26,43] and, similarly to other tetraspanin family members (e.g. CD9/CD81), it might be related to tumour invasion [27–30].

Other clinical-phenotypic associations observed in our study included higher bcl2 levels in tumours from females with a convexity/parasagittal localization and fibroblastic histopathology, higher expression of CD63 in convexity/parasagittal tumours, lower levels of IGFR in spinal tumours and greater expression of CD99 in transitional *vs.* meningothelial meningiomas, together with higher CD38 levels among cases with moderate to severe oedema. IGF has long been shown to regulate, at least in part, meningioma growth through its effect on tumour

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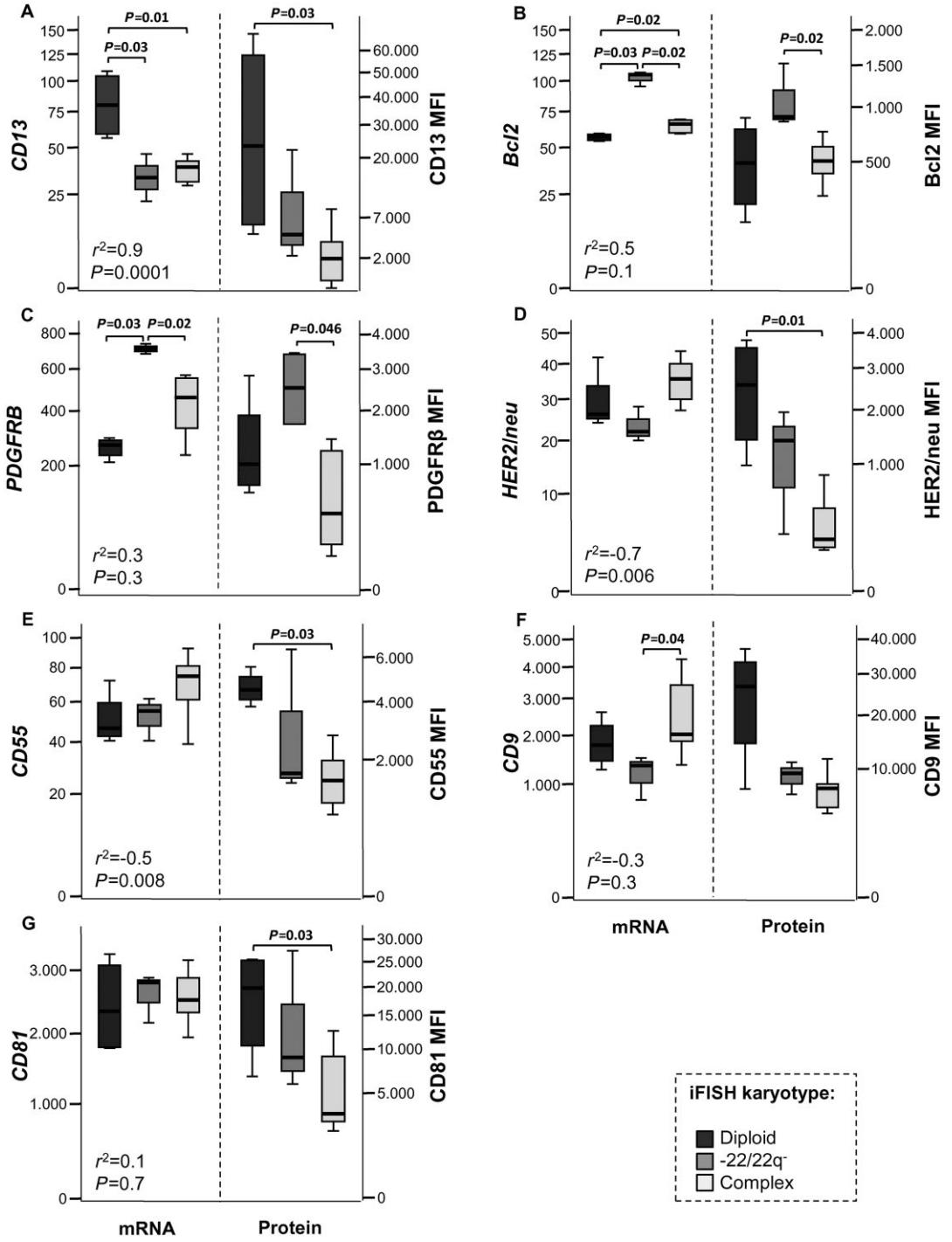

Figure 4. Relationship between the mRNA and protein expression levels of individual tumour cells in meningiomas grouped according to their iFISH cytogenetic profile. The amount of expression of CD13 (panel **A**), Cybcl2 (panel **B**), PDGFRβ (panel **C**), HER2/neu (panel **D**), CD55 (panel **E**), CD9 (panel **F**) and CD81 (panel **G**), are shown both at the mRNA (arbitrary fluorescence units) and at the protein level (MFI, mean fluorescence intensity) for cases in which both measures were performed simultaneously ($n = 13$). Notched-boxes represent 25th and 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 10th and 90th percentiles respectively. mRNA probe set numbers represented were selected from the U133A Affymetrix microarrays as follows: *CD13* 202888_s_at; *BCL2* mean of 203684_s_at/203685_at/207004_at/207005_s_at; *PDGFRB* 202273_at; *HER2/neu* 210930_s_at; *CD55* mean of 201925_s_at/201926_s_at; *CD9* 201005_at; *CD81* 200675_at.

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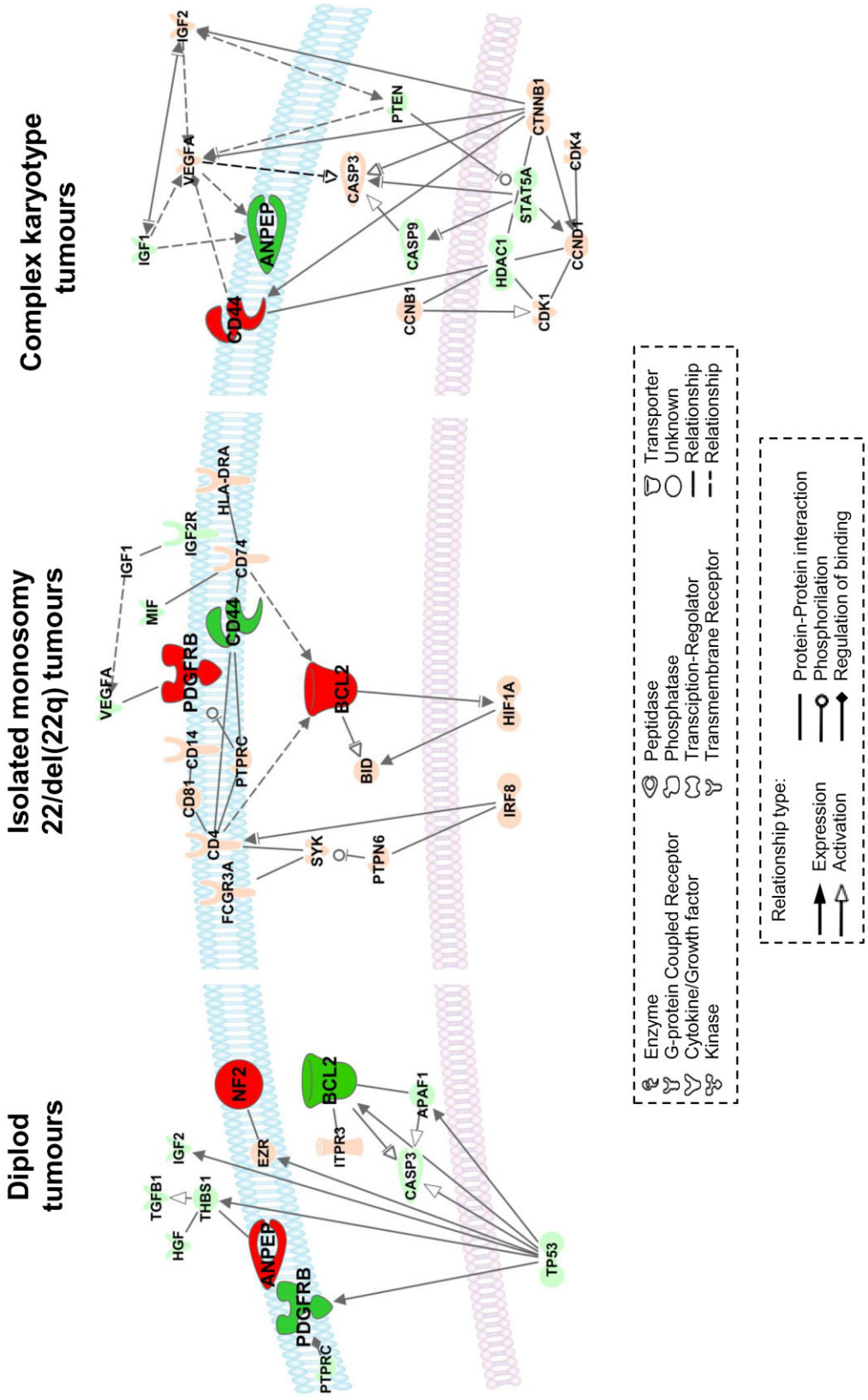

Figure 5. Schematic representation (Ingenuity Pathway Analysis software) of those signalling pathways for which distinct gene/protein expression profiles (GEP) were observed in meningioma tumour cells carrying different iFISH cytogenetic profiles ($n = 40$ tumours). Genes highlighted in red correspond to overexpressed genes and those highlighted in green are underexpressed genes in the corresponding iFISH cytogenetic subgroup of meningiomas *vs.* other cytogenetic subgroups.

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cell proliferation and survival [44,45]; however, to the best of our knowledge its expression has not been previously related in meningiomas to other disease features. Regarding CD99, this marker is used in the histological diagnostic work-up of some CNS tumours [46,47], being positive in spindle cell tumours (e.g. meningeal haemangiopericytoma and solitary fibrous tumours of the meninges) [48]; among our cases, CD99 also showed higher levels of expression among those histopathological subtypes of meningiomas which show spindle-shaped cells (fibroblastic/transitional). Of note, CD38 was the only immunophenotypic marker whose expression was significantly related with brain oedema. CD38 is a multifunctional ectoenzyme essential for the regulation of intracellular calcium. Brain oedema in meningioma patients is mainly due to an increase in the permeability of the blood–brain barrier (BBB) [49]. As regulation of extracellular and intracellular calcium levels seems to be critical in the normal functioning of the BBB [50], and calcium overload is a main cause of ischemia and brain oedema after trauma [51], our results suggest that CD38 expression by tumour cells could play a role in the genesis of oedema in these patients.

Interestingly, recent studies have focused on the mutational status of several genes other than *NF2*, which seem to be important for the biology of meningiomas; these include mutations of genes such as *AKT1*, a constituent of the PI3K which has been related to specific localizations and histological subtypes of meningiomas, and *KLF4*, a gene typically altered in secretory meningiomas [40,52]. In our series, PDGFRβ expression (a gene also related to the PI3K pathway) was associated with meningioma histology and cytogenetics.

In summary, here we show that the protein expression profile of individual meningioma cells, as evaluated by MFC immunophenotyping, is closely associated with tumour cytogenetics, which may reflect the involvement of different signalling pathways in the distinct cytogenetic subgroups of meningiomas. In addition, our data also show a close association between some of the markers investigated (e.g. CD44 and CD53) and patient RFS, suggesting that specific protein expression profiles may translate into a more aggressive *vs.* mild clinical behaviour of the tumour. Further investigations in larger series of patients, analysed with extended antibody panels and the flow cytometry techniques here described, are required to confirm this hypothesis.

Acknowledgements

This work was partially supported by grants from the Fundação para a Ciência e Tecnologia (PIC/IC/83108/2007, FCT, Portugal), Fondo de Investigaciones Sanitarias [FIS/FEDER 06/0312 and RETICC RD06/0020/0035, RD06/0020/0059 and RD12/0036/0048, Instituto de Salud Carlos III (ISCIII/FEDER), Ministerio de Sanidad y Consumo, Madrid, Spain], Caja Burgos (Spain), Fundación MMA (exp 75312010 and 87692011, Madrid, Spain), and Consejeria Sanidad Junta de Castilla y León, Gerencia Regional de Salud: GRS689/A/11 and BIOSA15/13. Patrícia Domingues is supported by grant (SFRH/BD/64799/2009) from FCT. Maria Dolores Tabernero is supported by IECSCYL (Soria, Spain).

Conflict of interest statement

The authors declare no conflict of interests.

Authorship

Patrícia Domingues performed experiments, analysed/interpreted results, made the figures and wrote the paper; Cristina Teodósio supervised the study, analysed/interpreted results and reviewed the paper; Alvaro Otero, Pablo Sousa and Jesus Maria Gonçalves provided the samples and clinical follow-up of the patients; Ana Belén Nieto performed mathematical analysis of arrays; Maria Celeste Lopes and Catarina de Oliveira supervised the study; Alberto Orfao and Maria Dolores Tabernero designed the research, supervised the study and wrote the paper.

References

1 Perry A, Louis DN, Scheithauer BW, Budka H, von Deimling A. Meningiomas. In *WHO Classification of Tumours of the Central Nervous Syste*, 4th edn. Eds Louis DN, Ohgaki H, Wiestler OD, Cavenee WK. Lyon, France: IARC Press, 2007; 164–72

2 Riemenschneider MJ, Perry A, Reifenberger G. Histological classification and molecular genetics of meningiomas. *Lancet Neurol* 2006; **5**: 1045–54

3 Domingues PH, Sousa P, Otero A, Goncalves JM, Ruiz L, Lopes MC, de Oliveira C, Orfao A, Tabernero MD. Proposal for a new risk stratification classification for meningioma based on patient age, WHO tumor grade, size, localization, and karyotype. *Neuro-oncol* 2014. doi: 10.1093/neuonc/not325 [Epub ahead of print]

© 2014 British Neuropathological Society

4 Maillo A, Orfao A, Espinosa AB, Sayagues JM, Merino M, Sousa P, Lara M, Tabernero MD. Early recurrences in histologically benign/grade I meningiomas are associated with large tumors and coexistence of monosomy 14 and del(1p36) in the ancestral tumor cell clone. *Neuro-oncol* 2007; **9**: 438–46
5 Tabernero MD, Espinosa AB, Maillo A, Sayagues JM, Alguero Mdel C, Lumbreras E, Diaz P, Goncalves JM, Onzain I, Merino M, Morales F, Orfao A. Characterization of chromosome 14 abnormalities by interphase in situ hybridization and comparative genomic hybridization in 124 meningiomas: correlation with clinical, histopathologic, and prognostic features. *Am J Clin Pathol* 2005; **123**: 744–51
6 Tabernero MD, Maillo A, Gil-Bellosta CJ, Castrillo A, Sousa P, Merino M, Orfao A. Gene expression profiles of meningiomas are associated with tumor cytogenetics and patient outcome. *Brain Pathol* 2009; **19**: 409–20
7 Espinosa AB, Tabernero MD, Maillo A, Sayagues JM, Ciudad J, Merino M, Alguero MC, Lubombo AM, Sousa P, Santos-Briz A, Orfao A. The cytogenetic relationship between primary and recurrent meningiomas points to the need for new treatment strategies in cases at high risk of relapse. *Clin Cancer Res* 2006; **12**: 772–80
8 Domingues PH, Teodosio C, Ortiz J, Sousa P, Otero A, Maillo A, Barcena P, Garcia-Macias MC, Lopes MC, de Oliveira C, Orfao A, Tabernero MD. Immunophenotypic identification and characterization of tumor cells and infiltrating cell populations in meningiomas. *Am J Pathol* 2012; **181**: 1749–61
9 Paz-Bouza JI, Orfao A, Abad M, Ciudad J, Garcia MC, Lopez A, Bullon A. Transrectal fine needle aspiration biopsy of the prostate combining cytomorphologic, DNA ploidy status and cell cycle distribution studies. *Pathol Res Pract* 1994; **190**: 682–9
10 Cruz I, Ciudad J, Cruz JJ, Ramos M, Gomez-Alonso A, Adansa JC, Rodriguez C, Orfao A. Evaluation of multiparameter flow cytometry for the detection of breast cancer tumor cells in blood samples. *Am J Clin Pathol* 2005; **123**: 66–74
11 Matarraz S, Fernandez C, Albors M, Teodosio C, Lopez A, Jara-Acevedo M, Cervero C, Caballero G, Gutierrez O, Orfao A. Cell-cycle distribution of different cell compartments in normal versus reactive bone marrow: a frame of reference for the study of dysplastic hematopoiesis. *Cytometry B Clin Cytom* 2011; **80**: 354–61
12 Tabernero MD, Espinosa AB, Maillo A, Rebelo O, Vera JF, Sayagues JM, Merino M, Diaz P, Sousa P, Orfao A. Patient gender is associated with distinct patterns of chromosomal abnormalities and sex chromosome linked gene-expression profiles in meningiomas. *Oncologist* 2007; **12**: 1225–36
13 Rushmere NK, Knowlden JM, Gee JM, Harper ME, Robertson JF, Morgan BP, Nicholson RI. Analysis of the level of mRNA expression of the membrane regulators of complement, CD59, CD55 and CD46, in breast cancer. *Int J Cancer* 2004; **108**: 930–6
14 Ravindranath NM, Shuler C. Cell-surface density of complement restriction factors (CD46, CD55, and CD59): oral squamous cell carcinoma versus other solid tumors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2007; **103**: 231–9
15 Spendlove I, Ramage JM, Bradley R, Harris C, Durrant LG. Complement decay accelerating factor (DAF)/CD55 in cancer. *Cancer Immunol Immunother* 2006; **55**: 987–95
16 Shamah SM, Alberta JA, Giannobile WV, Guha A, Kwon YK, Carroll RS, Black PM, Stiles CD. Detection of activated platelet-derived growth factor receptors in human meningioma. *Cancer Res* 1997; **57**: 4141–7
17 Yang SY, Xu GM. Expression of PDGF and its receptor as well as their relationship to proliferating activity and apoptosis of meningiomas in human meningiomas. *J Clin Neurosci* 2001; **8** (Suppl. 1): 49–53
18 Mosnier JF, Perret AG, Brunon J, Boucheron S. Expression of the bcl-2 oncoprotein in meningiomas. *Am J Clin Pathol* 1996; **106**: 652–9
19 Roessler K, Dietrich W, Kitz K. Expression of BCL-2 oncoprotein on tumor cells and tumor-infiltrating lymphocytes (TIL) in meningiomas. *Neurosurg Rev* 1999; **22**: 205–9
20 Tyagi D, Sharma BS, Gupta SK, Kaul D, Vasishta RK, Khosla VK. Expression of Bcl2 proto-oncogene in primary tumors of the central nervous system. *Neurol India* 2002; **50**: 290–4
21 Funa K, Sasahara M. The roles of PDGF in development and during neurogenesis in the normal and diseased nervous system. *J Neuroimmune Pharmacol* 2013; doi: 10.1007/s11481-013-9479-z
22 Doucette T, Yang Y, Zhang W, Fuller GN, Suki D, Fults DW, Rao G. Bcl-2 promotes malignant progression in a PDGF-B-dependent murine model of oligodendroglioma. *Int J Cancer* 2011; **129**: 2093–103
23 Mina-Osorio P. The moonlighting enzyme CD13: old and new functions to target. *Trends Mol Med* 2008; **14**: 361–71
24 Mawrin C, Wolke C, Haase D, Kruger S, Firsching R, Keilhoff G, Paulus W, Gutmann DH, Lal A, Lendeckel U. Reduced activity of CD13/aminopeptidase N (APN) in aggressive meningiomas is associated with increased levels of SPARC. *Brain Pathol* 2010; **20**: 200–10
25 Rempel SA, Ge S, Gutierrez JA. SPARC: a potential diagnostic marker of invasive meningiomas. *Clin Cancer Res* 1999; **5**: 237–41
26 Hemler ME. Tetraspanin functions and associated microdomains. *Nat Rev Mol Cell Biol* 2005; **6**: 801–11
27 Ovalle S, Gutierrez-Lopez MD, Olmo N, Turnay J, Lizarbe MA, Majano P, Molina-Jimenez F, Lopez-Cabrera M, Yanez-Mo M, Sanchez-Madrid F, Cabanas C. The tetraspanin CD9 inhibits the proliferation and tumorigenicity of human colon carcinoma cells. *Int J Cancer* 2007; **121**: 2140–52

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28 Funakoshi T, Tachibana I, Hoshida Y, Kimura H, Takeda Y, Kijima T, Nishino K, Goto H, Yoneda T, Kumagai T, Osaki T, Hayashi S, Aozasa K, Kawase I. Expression of tetraspanins in human lung cancer cells: frequent downregulation of CD9 and its contribution to cell motility in small cell lung cancer. *Oncogene* 2003; **22**: 674–87
29 Mazzocca A, Liotta F, Carloni V. Tetraspanin CD81-regulated cell motility plays a critical role in intrahepatic metastasis of hepatocellular carcinoma. *Gastroenterology* 2008; **135**: 244–56
30 Hirano C, Nagata M, Noman AA, Kitamura N, Ohnishi M, Ohyama T, Kobayashi T, Suzuki K, Yoshizawa M, Izumi N, Fujita H, Takagi R. Tetraspanin gene expression levels as potential biomarkers for malignancy of gingival squamous cell carcinoma. *Int J Cancer* 2009; **124**: 2911–16
31 Wiley HS. Trafficking of the ErbB receptors and its influence on signaling. *Exp Cell Res* 2003; **284**: 78–88
32 Sorkin A. Internalization of the epidermal growth factor receptor: role in signalling. *Biochem Soc Trans* 2001; **29**: 480–4
33 Laurendeau I, Ferrer M, Garrido D, D'Haene N, Ciavarelli P, Basso A, Vidaud M, Bieche I, Salmon I, Szijan I. Gene expression profiling of ErbB receptors and ligands in human meningiomas. *Cancer Invest* 2009; **27**: 691–8
34 Wickremesekera A, Hovens CM, Kaye AH. Expression of ErbB-1 and ErbB-2 in meningioma. *J Clin Neurosci* 2010; **17**: 1155–8
35 Mahzouni P, Movahedipour M. An immunohistochemical study of HER2 expression in meningioma and its correlation with tumor grade. *Pathol Res Pract* 2012; **208**: 221–4
36 Stamenkovic I, Yu Q. Merlin, a 'magic' linker between extracellular cues and intracellular signaling pathways that regulate cell motility, proliferation, and survival. *Curr Protein Pept Sci* 2010; **11**: 471–84
37 Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H, Herrlich P. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev* 2001; **15**: 968–80
38 Tabernero MD, Maillo A, Nieto AB, Diez-Tascon C, Lara M, Sousa P, Otero A, Castrillo A, Patino-Alonso Mdel C, Espinosa A, Mackintosh C, de Alava E, Orfao A. Delineation of commonly deleted chromosomal regions in meningiomas by high-density single nucleotide polymorphism genotyping arrays. *Genes Chromosomes Cancer* 2012; **51**: 606–17
39 Tabernero M, Jara-Acevedo M, Nieto AB, Caballero AR, Otero A, Sousa P, Goncalves J, Domingues PH, Orfao A. Association between mutation of the NF2 gene and monosomy 22 in menopausal women with sporadic meningiomas. *BMC Med Genet* 2013; **14**: 114
40 Clark VE, Erson-Omay EZ, Serin A, Yin J, Cotney J, Ozduman K, Avsar T, Li J, Murray PB, Henegariu O, Yilmaz S, Gunel JM, Carrion-Grant G, Yilmaz B, Grady C, Tanrikulu B, Bakircioglu M, Kaymakcalan H, Caglayan AO, Sencar L, Ceyhun E, Atik AF, Bayri Y, Bai H, Kolb LE, Hebert RM, Omay SB, Mishra-Gorur K, Choi M, Overton JD, Holland EC, Mane S, State MW, Bilguvar K, Baehring JM, Gutin PH, Piepmeier JM, Vortmeyer A, Brennan CW, Pamir MN, Kilic T, Lifton RP, Noonan JP, Yasuno K, Gunel M. Genomic analysis of non-NF2 meningiomas reveals mutations in TRAF7, KLF4, AKT1, and SMO. *Science* 2013; **339**: 1077–80
41 Lewy-Trenda I, Omulecka A, Janczukowicz J, Papierz W. CD44 expression in human meningiomas: an immunohistochemical analysis. *Pol J Pathol* 2004; **55**: 33–7
42 Ruiz J, Martinez A, Hernandez S, Zimman H, Ferrer M, Fernandez C, Saez M, Lopez-Asenjo JA, Sanz-Ortega J. Clinicopathological variables, immunophenotype, chromosome 1p36 loss and tumour recurrence of 247 meningiomas grade I and II. *Histol Histopathol* 2010; **25**: 341–9
43 Lagaudriere-Gesbert C, Le Naour F, Lebel-Binay S, Billard M, Lemichez E, Boquet P, Boucheix C, Conjeaud H, Rubinstein E. Functional analysis of four tetraspans, CD9, CD53, CD81, and CD82, suggests a common role in costimulation, cell adhesion, and migration: only CD9 upregulates HB-EGF activity. *Cell Immunol* 1997; **182**: 105–12
44 Zumkeller W, Westphal M. The IGF/IGFBP system in CNS malignancy. *Mol Pathol* 2001; **54**: 227–9
45 Lichtor T, Kurpakus MA, Gurney ME. Expression of insulin-like growth factors and their receptors in human meningiomas. *J Neurooncol* 1993; **17**: 183–90
46 Wick MR. Immunohistochemical approaches to the diagnosis of undifferentiated malignant tumors. *Ann Diagn Pathol* 2008; **12**: 72–84
47 Ishizawa K, Komori T, Shimada S, Hirose T. Olig2 and CD99 are useful negative markers for the diagnosis of brain tumors. *Clin Neuropathol* 2008; **27**: 118–28
48 Renshaw AA. O13 (CD99) in spindle cell tumors: reactivity with hemangiopericytoma, solitary fibrous tumor, synovial sarcoma, and meningioma but rarely with sarcomatoid mesothelioma. *Appl Immunohistochem* 1995; **3**: 250–6
49 Vaz R, Borges N, Cruz C, Azevedo I. Cerebral edema associated with meningiomas: the role of peritumoral brain tissue. *J Neurooncol* 1998; **36**: 285–91
50 Arismendi-Morillo G, Castellano A. Tumoral micro-blood vessels and vascular microenvironment in human astrocytic tumors. A transmission electron microscopy study. *J Neurooncol* 2005; **73**: 211–17
51 Brown RC, Davis TP. Calcium modulation of adherens and tight junction function: a potential mechanism for blood-brain barrier disruption after stroke. *Stroke* 2002; **33**: 1706–11
52 Brastianos PK, Horowitz PM, Santagata S, Jones RT, McKenna A, Getz G, Ligon KL, Palescandolo E, Van Hummelen P, Ducar MD, Raza A, Sunkavalli A, Macconaill LE, Stemmer-Rachamimov AO, Louis DN,

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Hahn WC, Dunn IF, Beroukhim R. Genomic sequencing of meningiomas identifies oncogenic SMO and AKT1 mutations. *Nat Genet* 2013; **45**: 285–9

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Impact of tumour WHO grade and cytogenetics on relapse-free survival of meningioma patients. Relapse-free survival curves of meningioma patients classified according to tumour grade ($n = 78$; panel **A**) and cytogenetics ($n = 77$; panel **B**) are shown.

Table S1. Relevant clinical, histopathological and genetic characteristics of the 78 meningioma samples studied by multiparameter flow cytometry immunophenotyping ($n = 51$) and gene expression profiling by oligonucleotide arrays ($n = 40$).

Table S2. Identification (ID) number of the 78 samples included in the present study and that have also been included in other previous reports with their corresponding code number.

Table S3. Antibody reagents used for the immunophenotypic analysis of tumour cells in meningioma.

Table S4. Immunophenotypic characterization of meningeal neoplastic cells evaluated by multiparameter flow cytometry: percentage of positive cases, positive cells per case and amount of expression – mean fluorescence intensity (MFI) for each analysed marker ($n = 51$).

Received 24 September 2013
Accepted after revision 5 February 2014
Published online Article Accepted on 24 February 2014

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