



ORIGINAL RESEARCH

Candidate Biomarker Discovery for Angiogenesis by Automatic Integration of Orbitrap MS1 Spectral- and X!Tandem MS2 Sequencing Information

Mark K. Titulaer *

Academic Medical Center, University of Amsterdam, 1100 DD Amsterdam, The Netherlands

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Abstract Candidate protein biomarker discovery by full automatic integration of Orbitrap full MS1 spectral peptide profiling and X!Tandem MS2 peptide sequencing is investigated by analyzing mass spectra from brain tumor samples using Peptrix. Potential protein candidate biomarkers found for angiogenesis are compared with those previously reported in the literature and obtained from previous Fourier transform ion cyclotron resonance (FT-ICR) peptide profiling. Lower mass accuracy of peptide masses measured by Orbitrap compared to those measured by FT-ICR is compensated by the larger number of detected masses separated by liquid chromatography (LC), which can be directly linked to protein identifications. The number of peptide sequences divided by the number of unique sequences is $9248/6911 \approx 1.3$. Peptide sequences appear 1.3 times redundant per up-regulated protein on average in the peptide profile matrix, and do not seem always up-regulated due to tailing in LC retention time (40%), modifications (40%) and mass determination errors (20%). Significantly up-regulated proteins found by integration of X!Tandem are described in the literature as tumor markers and some are linked to angiogenesis. New potential biomarkers are found, but need to be validated independently. Eventually more proteins could be found by actively involving MS2 sequence information in the creation of the MS1 peptide profile matrix.

Introduction

Orbitrap mass spectrometry (MS) plays an increasingly important role in proteomics research. Orbitrap combines great mass

accuracy (< 10 ppm) with a high processing speed for peptide mixtures separated by nano-liquid chromatography (nano-LC). A comparable instrument, the Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer, has a slightly better mass accuracy of ≤ 1 ppm [1]. However, the performance of FT-ICR depends on the magnet strength chosen, *e.g.*, 9.4 T, and thus it takes up a lot more space because of the sizeable magnet. Orbitrap has a shorter time scale of measuring than FT-ICR and can therefore be more conveniently linked to a nano-LC column to measure peptide fractions of a trypsin-digested sample.

Comparison of mass spectra from different samples is called peptide profiling. Peptide profiling creates a matrix of

* Corresponding author.

E-mail: mktitulaer@telfort.nl (Titulaer MK).

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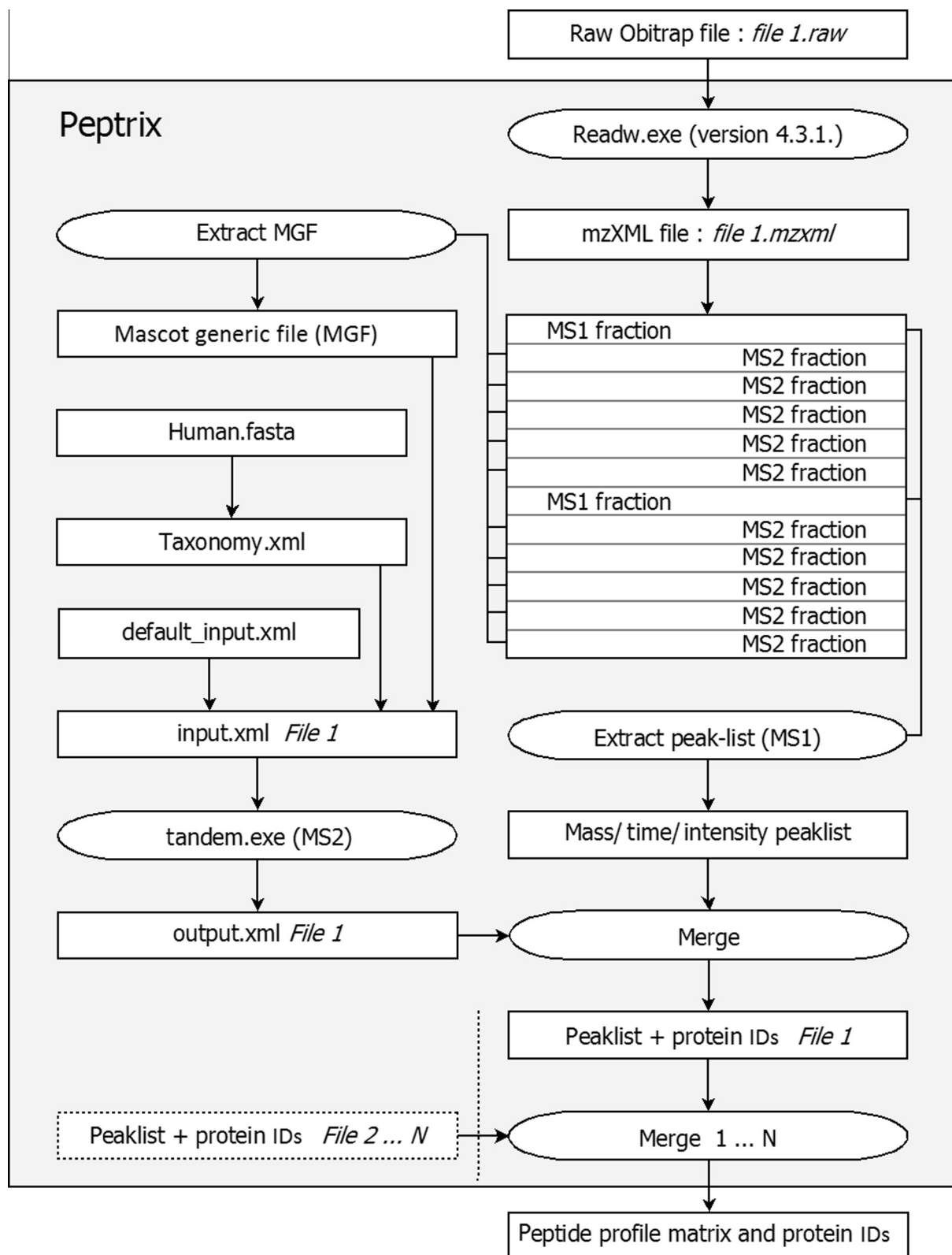


Figure 1 Architecture of Peptrix

Peptrix processes raw Orbitrap files to a peptide profile matrix of all the intensities measured for every peptide mass with a certain retention time in the different samples with protein identifications. Peptrix implements Readw.exe (version 4.3.1), *.fasta file, and tandem.exe. Peptrix generates a Mascot generic file (MGF) for each Orbitrap file. The *.fasta file is the text database to correlate MS2 fragmentation masses to a protein.

Table 1 Differentially expressed peptides of proteins between GV and NV groups

Mass MH+ (Da)	Time (s)	Calculated MH+ (Da)	ppm	Mod	Sequence	Miss	P	Up	Ave. intensity ($\times 100$)			
									GV/NV	GV	NV	
<i>sp O00468 AGRIN_HUMAN agrin</i>												
1051.5951	11,281	1051.5935	1.5		SFLAFPTLR	0	0.0022	+	High	124	0	
1154.6514	6352	1154.6527	1.1		LELGIGPGAATR	0	0.1207	+	3.1	364	117	
1188.6501	8036	1205.7000	19.4	1	QLLTPEHVLR	0	0.0448	+	2.2	552	255	
1246.6788	5956	1246.6790	0.2		GPSGLLLYNGQK	0	0.9692	–	0.8	175	215	
1295.6452	15,752	1295.6477	1.9		SIESTLDDLFR	0	0.0283	+	13.8	219	16	
1323.6219	5687	1323.6175	3.3		SAGDVTTLAFDGR	0	0.0072	+	11.7	4942	422	
1628.8763	9105	1628.8754	0.6		ALEPQGLLLYNGNAR	0	0.0110	+	10.5	145	14	
<i>sp O75891 ALIL1_HUMAN aldehyde dehydrogenase family 1 member L1</i>												
1363.6835	10,783	1363.6852	1.2		DLGEAALNEYLR	0	0.0060	+	High	146	0	
1787.9414	5112	1787.9174	13.4		LPQPEEGATYEGIQKK	1	0.0187	+	39.1	312	8	
<i>tr E9PR44 E9PR44_HUMAN Uncharacterized protein (CRYAB_HUMAN.alpha-crystallin B chain)</i>												
921.5027	5749	921.5040	1.4		FSVNLDVK	0	0.0858	+	8.7	431	50	
921.5040	7157	921.5040	0.0		FSVNLDVK	0	0.0277	+	3.6	2940	820	
1213.6566	5147	1213.6575	0.7		HFSPEELKVK	1	0.0058	+	18.2	2431	134	
1454.8235	5121	1454.8213	1.5	5	TIPITREEKPAVT	2	0.0403	+	28.4	257	9	
1822.0476	5108	1822.0432	2.4		TIPITREEKPAVTAAPK	2	0.0022	+	High	907	0	
<i>tr E9PJJ7 E9PJJ7_HUMAN Uncharacterized protein (CRYAB_HUMAN.alpha-crystallin B chain)</i>												
1374.7080	5710	1374.7065	1.1		RPFPPFHSPSR	1	0.0022	+	High	461	0	
1446.7278	7155	1388.7255	5.5	2,3	MDIAIHPWIR	0	0.1183	+	14.8	867	59	
1639.7568	5629	1639.7571	0.2		HEERQDEHGFISR	1	0.0079	+	20.5	358	17	
1716.9039	5148	1716.9027	0.7		VLGDVIEVHGKHEER	1	0.0376	+	2.4	1745	733	
<i>tr E9PNH7 E9PNH7_HUMAN Uncharacterized protein (CRYAB_HUMAN.alpha-crystallin B chain)</i>												
1165.6595	5443	1165.6575	1.7		VLGDVIEVHGK	0	0.0100	+	14.6	1054	72	
<i>tr E7ETU3 E7ETU3_HUMAN Uncharacterized protein (cell division control protein 42 homolog)</i>												
1224.6482	6133	1241.6736	0.9	1	QKPIPTETAEK	1	0.7910	+	1.1	168	153	
1472.8491	13,709	1472.8471	1.4		TPFLLVGTQIDLR	0	0.0298	+	17.2	109	6	
<i>sp P10909 CLUS_HUMAN clusterin</i>												
1117.6073	7057	1117.6099	2.3		TLLSNLEEAK	0	0.0125	+	25.5	876	34	
1245.6996	5854	1245.7049	4.3		TLLSNLEEAKK	1	0.0146	+	80.7	1019	13	
1296.7549	7499	1296.7521	2.2	6	PITVTPVEVSR	0	0.0060	+	High	460	0	
1360.7441	5927	1360.7430	0.8		TLEIKTNEERK	2	0.5036	+	58.5	288	5	
1373.8000	5205	1373.7998	0.1		KTLLSNLEEAKK	2	0.0022	+	High	1404	0	
1575.8515	5619	1575.8489	1.6		YVNKEIQNAVNGVK	1	0.0022	+	High	417	0	
1576.8315	5260	1575.8489	0.9	4	YVNKEIQNAVNGVK	1	0.0426	+	14.4	621	43	
1842.8973	5748	1842.9079	5.8	7	SHTSDSDVPSGVTEVVVK	0	0.1564	–	0.4	52	125	
1873.9827	12,680	1873.9905	4.2		LFSDPITVTPVEVSR	0	0.0187	+	141.2	1721	12	
2009.8660	6065	2009.8716	2.8		DQTVSDNELQEMSNQGSK	0	0.0889	+	14	1469	105	
2010.8479	6275	2009.8716	3.8	4	DQTVSDNELQEMSNQGSK	0	0.0666	+	18.2	246	14	
2025.8692	6109	2009.8716	0.9	2	DQTVSDNELQEMSNQGSK	0	0.0077	+	14.5	400	28	
<i>tr B4DN87 B4DN87_HUMAN Uncharacterized protein (collagen-binding protein 2 P50454)</i>												
1224.6574	5092	1224.6582	0.7		GVVEVTHDLQK	0	0.1613	+	1.5	353	239	
1337.7452	6553	1337.7423	2.2		HLAAGLGLTEAIDK	0	0.1722	+	8.4	102	12	
1659.8017	11,433	1659.8013	0.2		LYGPSVSFADDFVR	0	0.3059	+	16.2	409	25	
1887.0880	9896	1887.0810	3.7		DTQSGSLLFIGRLVRPK	2	0.0779	+	High	65	0	
<i>tr B4DF14 B4DF14_HUMAN Uncharacterized protein (excitatory amino acid transporter 1)</i>												
1723.7509	6962	1723.7513	0.2		DVEMGNSVIEENEMK	0	0.0139	+	11.8	168	14	
1739.7299	6837	1723.7513	12.1	2	DVEMGNSVIEENEMK	0	0.0229	+	16.3	449	28	
2040.0745	11,079	2040.0720	1.2		TTTNVLGDSLGAIVGHELSR	0	0.0060	+	High	211	0	
<i>sp Q16658 FSCN1_HUMAN fasciclin</i>												
1076.5133	5760	1076.5119	1.3		YSVQTADHR	0	0.2247	+	2.3	272	120	
1076.5315	5880	1076.5119	18.2		YSVQTADHR	0	0.5149	–	0.2	1159	4652	
1190.6392	5446	1190.6415	1.9		YLAPSGPSGTLK	0	0.0448	+	2.7	404	148	
1200.6740	5289	1200.6735	0.4		YLKGDHAGVLK	1	0.0489	+	5.6	241	43	
1240.7888	6908	1240.7888	0.0		LINRPIIVFR	1	0.0008	+	High	225	0	
1819.9725	7243	1819.9701	1.3		LVARPEPATGYTLEFR	1	0.1012	+	48.2	294	6	

Table 1 continued

Mass MH+ (Da)	Time (s)	Calculated MH+ (Da)	ppm	Mod	Sequence	Miss	P	Up	Ave. intensity (×100)		
									GV/NV	GV	NV
<i>sp O15540 FABP7_HUMAN fatty acid-binding protein, brain</i>											
963.5163	5668	963.5258	9.9	8	T FGDVVAVR	0	0.3994	+	2.2	279	129
1489.6637	6212	1489.6628	0.6		LTNSQNFDEYMK	0	0.0008	+	High	676	0
1489.6672	5901	1489.6628	3.0		LTNSQNFDEYMK	0	0.1826	-	0.4	58	157
1505.6539	6215	1489.6628	5.6	2	LTNSQNFDEYMK	0	0.0022	+	High	324	0
1554.8059	12,106	1538.8069	0.3	2	MV M TLTFGDVVAVR	0	0.8942	+	1.9	26	14
1637.9231	6219	1637.9220	0.7		SVVSLDGDGKLVHVIQK	1	0.0147	+	137.3	474	3
1896.0122	7155	1913.0338	2.6	1	Q VGVNVTKPTVIISQEGDK	1	0.0149	+	High	53	0
2363.3206	9030	2380.3558	3.7	1	Q VGVNVTKPTVIISQEGDKVVIR	2	0.0149	+	High	113	0
2380.3550	7027	2380.3558	0.3		QVGVNVTKPTVIISQEGDKVVIR	2	0.0837	+	215.1	379	2
<i>sp P49327 FAS_HUMAN fatty acid synthase</i>											
1038.6568	7667	1038.6557	1.1		GTPLISPLIK	0	0.0403	+	30.6	119	4
1115.6675	8470	1115.6670	0.4		SEGVVAVLLTK	0	0.0350	+	High	30	0
1263.7421	6778	1263.7419	0.2		LQVVDQPLPVR	0	0.0299	+	2.6	123	47
1298.6487	5094	1298.6699	16.3		VGDPQELNGITR	0	0.2694	+	6.9	582	84
1308.6212	14,642	1250.6119	0.6	3,2	M EEVVIAG M SGK	0	0.0041	+	9.7	49	5
1386.7491	9654	1386.7474	1.2		GVDLVLNSLAEEK	0	0.0030	+	9	82	9
1426.7769	8686	1426.7722	3.3		SLLVNPEGPTLMR	0	0.0366	+	22	113	5
1469.6989	7733	1469.7019	2.0		FPQLDSTSFANSR	0	0.0522	+	4.9	97	20
1622.9367	10,585	1622.9363	0.2		VVVQVLSAEPEAVLK	0	0.0366	+	11.1	69	6
<i>tr E9PE77 E9PE77_HUMAN Uncharacterized protein (FINC_HUMAN, fibronectin)</i>											
808.4306	5311	808.4312	0.7		AQITGYR	0	0.0530	+	6.3	641	101
997.5225	5423	997.5200	2.5	9	TKTETITG E	1	0.9356	+	1.4	219	152
1109.5254	6194	1109.5221	3.0	10	S SGSGPFTDVR	0	0.9097	+	1.3	274	208
1110.5407	5185	1110.5426	1.7		STTPDITGYR	0	0.7913	+	1.8	4345	2454
1113.6136	5923	1113.6150	1.3	11	S TAIPAPTDLK	0	0.0639	+	3.8	452	118
1275.6244	5075	1275.6116	10.0		TYHVGEQWQK	0	0.0113	-	0.3	685	2203
1283.7390	5171	1283.7317	5.7	12	L VAIKGNQESPK	1	0.0350	+	High	62	0
1293.6728	8904	1292.6732	12.1	4	D LQFVEVTDVK	0	0.1010	+	3.9	109	28
1323.6652	7053	1323.7127	35.9		LGVRPSQGGEAPR	1	0.0385	+	3.3	288	88
1323.6722	5683	1323.7127	30.6		LGVRPSQGGEAPR	1	0.0746	+	4.3	6728	1560
1323.6730	5297	1323.7127	30.0		LGVRPSQGGEAPR	1	0.1036	-	0.8	8441	10,627
1323.6737	14,140	1323.7127	29.5		LGVRPSQGGEAPR	1	0.1975	-	0.6	23	37
1341.6769	5739	1341.6757	0.9		DGQERDAPIVNK	1	0.9699	+	1.1	1779	1600
1341.6773	7209	1341.6757	1.2		DGQERDAPIVNK	1	0.2247	+	2.5	1240	504
1355.6969	7262	1355.6954	1.1		IYLYTLNDNAR	0	0.0521	+	12.9	2909	226
1356.6406	6614	1356.6668	19.3		HHPEHFSGRPR	1	0.2083	+	46.1	187	4
1356.6728	6473	1356.6668	4.4		HHPEHFSGRPR	1	0.0125	+	10.5	409	39
1357.6465	5734	1357.6859	29.0		IAWESPQGQVSR	0	0.0051	+	9.9	1966	199
1379.7041	7364	1379.7052	0.8		GLAFTDVDVDSIK	0	0.5907	-	0.5	55	114
1431.7515	5652	1431.7491	1.7		WSRPQAPITGYR	1	0.1028	+	21.1	3335	158
1461.7908	6160	1461.7907	0.1		VPGTSTSATLTGLTR	0	0.2729	+	4.8	3209	670
1543.7547	7635	1543.7638	5.9		SYTITGLQPGTDYK	0	0.1354	-	0.4	97	247
1543.7706	6869	1543.7638	4.4		SYTITGLQPGTDYK	0	0.0290	+	14.9	2740	184
1591.8081	5246	1591.8074	0.4		GDSPASSKPISINYR	1	0.0530	+	10.9	3078	283
1593.8131	8693	1593.8118	0.8		VTDATETITISWR	0	0.0837	+	27.1	246	9
1593.8246	9706	1593.8118	8.0		VTDATETITISWR	0	0.3219	+	1.5	52	36
1629.8716	7026	1629.8707	0.6		VDVIPVNLPGHEGQR	0	0.0111	+	39.8	875	22
1732.9435	9993	1732.9479	2.5		NLQPASEYTVSLVAIK	0	0.0858	+	21.3	744	35
1768.9796	5514	1768.9816	1.1		IGFKLGVSRPSQGGEAPR	2	0.0049	+	692.4	1426	2
1819.0136	5600	1819.0112	1.3		ITGYIIKYEKPGSPPR	2	0.0366	+	88.4	1853	21
1863.8806	5183	1863.8831	1.3		HTSVQTTSSGSGPFTDVR	0	0.1494	+	5.2	845	163
1913.9780	9350	1912.9974	1.8	4	SSPVVIDASTAIDAP S NLR	0	0.0803	+	7.9	140	18
1955.0102	8432	1955.0080	1.1		EESPLLIGQQSTVSDVPR	0	0.4509	+	7	151	22
2168.0326	9262	2168.0506	8.3		ITYGETGGNSPVQEFTVPGSK	0	0.6087	-	1	44	45
2478.2029	6598	2462.2079	1.8	2	TEIDKPSQ M QVTDVQDNSISVK	1	0.2627	+	9.2	354	38
2478.2034	6016	2462.2079	1.6	2	TEIDKPSQ M QVTDVQDNSISVK	1	0.0585	+	2	359	183
2573.3187	7827	2572.3365	0.7	4	TKTETITGFQVDAVPAN G QTPIQR	1	0.2755	+	37.1	1167	31

Table 1 continued

Mass MH+ (Da)	Time (s)	Calculated MH+ (Da)	ppm	Mod	Sequence	Miss	P	Up	Ave. intensity (× 100)		
									GV/NV	GV	NV
<i>sp Q13423 NNTM_HUMAN NAD(P) transhydrogenase, mitochondrial</i>											
953.5322	5046	953.5315	0.7		FGIHPVAGR	0	0.0366	+	10.4	127	12
1072.6238	6122	1072.6248	0.9		EVLASDLVVK	0	0.0149	+	High	123	0
1270.6883	7837	1270.6889	0.5		SLGAEPLEVDLK	0	0.0049	+	15.8	86	5
1429.7437	5824	1429.7434	0.2		GITHIGYTDLPSR	0	0.0277	+	4.8	191	40
1524.9084	9253	1524.9107	1.5		ILIVGGGVAGLASAGAAK	0	0.0237	+	7.6	151	20
1561.7473	8847	1578.7758	1.3	1	Q GFNVVVESGAGEASK	0	0.0060	+	High	53	0
<i>sp O43175 SERA_HUMAN D-3-phosphoglycerate dehydrogenase</i>											
1099.6110	6981	1099.6105	0.5		GGIVDEGALLR	0	0.0022	+	93.5	7167	77
1298.7301	6156	1298.7314	1.0		ILQDGGGLQVVEK	0	0.0149	+	High	153	0
1345.7718	6477	1345.7685	2.5		GTIQVITQGTSLK	0	0.0187	+	58.4	290	5
<i>sp Q71U36 TBA1A_HUMAN tubulin alpha-1A chain</i>											
1015.5805	5968	1015.5782	2.3		DVNAAIATIK	0	0.0538	+	4.5	11,321	2513
1380.6974	5847	1380.6980	0.4		LDHKFDLMYAK	1	0.0054	+	30.1	1786	59
1400.8227	5309	1400.8219	0.6		DVNAAIATIKTKR	2	0.2667	+	4.1	877	216
1457.8696	16,184	1457.8686	0.7		LIGQIVSSITASLR	0	0.0149	+	High	57	0
1507.6710	9724	1507.6699	0.7	13	D SFNTFFSETGAGK	0	0.1012	+	7.7	23	3
1536.7840	5378	1536.7991	9.8		LDHKFDLMYAKR	2	0.2550	–	0.7	112	163
1552.7914	5455	1536.7991	4.6	2	LDHKFDLMYAKR	2	0.0152	+	3.6	233	64
1622.6928	10,812	1622.6969	2.5	13	D SFNTFFSETGAGK	0	0.0149	+	High	63	0
1718.8808	6237	1718.8820	0.7		NLDIERPTYTNLNR	1	0.0277	+	13.7	807	59
2008.8721	10,534	2007.8930	2.4	4	TIGGGDDSFNTFFSETGAGK	0	0.1681	+	High	55	0
2185.8381	8495	2185.8414	1.5	14	DYEEVGVDSVEGEGEEEGEE	0	0.4562	+	4.8	34	7
2399.1689	10,388	2415.2051	4.3	1,4	Q LFHPEQLITGKEDAANNYAR	1	0.1722	+	6.6	113	17
2416.2020	7906	2415.2051	5.3	4	Q LFHPEQLITGKEDAANNYAR	1	0.0803	+	11.3	176	16
<i>tr F5H5D3 F5H5D3_HUMAN Uncharacterized protein (tubulin alpha-1C chain)</i>											
814.4665	5435	814.4669	0.5		APVISAEEK	0	0.2742	+	1.6	62	38
1149.6202	5231	1149.6150	4.5		ATYAPVISAEEK	0	0.1494	–	0.8	402	530
1221.6031	5836	1221.6110	6.5		NLDIERPTYT	1	0.0088	+	3.9	532	136
1241.5144	5244	1241.5103	3.3		GMEEGEFSEAR	0	0.1066	+	7.7	152	20
1285.6621	8031	1285.6634	1.0		DLEPTVIDEVR	0	0.0147	+	81.1	134	2
1309.6394	5455	1309.6131	20.1		TGKEDAANNYAR	1	0.2413	+	1.3	764	584
1448.7374	8034	1448.7380	0.4		NLDIERPTYTNL	1	0.8712	+	17.1	123	7
1679.7192	9832	1679.7183	0.5		GD DSFNTFFSETGAGK	0	0.0934	+	3.4	49	15
1689.7023	6470	1689.7060	2.2		YVGE GMEEGEFSEAR	0	0.7093	+	3.8	287	76
1705.6973	5522	1689.7060	4.8	2	YVGE M EEGEFSEAR	0	0.2343	+	8.9	37	4
1718.8422	7066	1718.8820	23.2		NLDIERPTYTNLNR	1	0.0919	+	5.9	187	32
1736.7429	9841	1736.7398	1.8		GG DSFNTFFSETGAGK	0	0.0350	+	High	31	0
1825.9652	9180	1824.9854	2.3	4	VG I NYQPPTVVPGGDLAK	0	0.0121	+	12.7	425	33
2008.8725	12,260	2007.8930	2.2	4	TIGGGDDSFNTFFSETGAGK	0	0.0779	+	High	31	0
2398.1713	9981	2415.2051	3.0	1	Q LFHPEQLITGKEDAANNYAR	1	0.0041	+	47.1	625	13

Note: MH+ indicates the protonated peptide mass. Amino acids with modifications are highlighted. Mod indicates modification: (1) –NH₃, (2) +O, (3) +COCH₂, (4) –NH₂ + OH, (5) non-tryptic, TA, (6) DP, (7) AS, (8) LT, (9) FQ, (10) TS, (11) QS, (12) SL, (13) GDD, (14) EY. Miss indicates number of missed cleavages. In Up column, + indicates intensity GV > NV while – indicates intensity GV < NV.

all the intensities measured for every peptide mass with a certain retention time in different samples [2]. There are a number of open-source software applications for comparing Orbitrap mass spectra from large numbers of samples in different groups [3–5]. Open-source software applications running on Windows operating system (OS), which combine the full spectral MS1 peptide masses and protein identifications by fragmentation of peptide masses (MS2 or MS/MS), are scarce. A few such examples are MsInspect, MSight, MSQuant and MaxQuant [3–6]. The majority of open-source packages use commercial search engines such as Mascot and Sequest to correlate MS2 fragmentation spectra with proteins in *.fasta databases, such as MSQuant and the early version of MaxQuant [4,6,7]. Other open-source software packages that compare

MS1 spectra from various samples with each other can only be installed on the Linux OS [5]. There are applications which use statistics to first determine differentially expressed MS1 peptide masses between the samples [8]. The masses that are differentially expressed with peak intensities in the groups are linked to a protein using MS2 fragmentation spectra.

Some applications determine relative quantities of a protein between samples on the basis of the number of times that a protein's peptide sequence is detected in an MS2 scan in a nano-LC Orbitrap measurement. For example, in MaxQuant [7], for label-free quantification, the maximum number of peptides between any two samples is compared, resulting in a matrix of protein ratios. There are a number of drawbacks associated with this spectral count technique. The sequence

Table 2 Differentially expressed proteins with number of peptides between GV and NV groups

Classification	Protein name	No. of peptides	References	Classification	Protein name	No. of peptides	References
Major blood proteins	Fibrinogen	96	[11,12,14,16]	Cytoskeleton	Actin-related protein 2	4	[14]
	Hemoglobin subunit alpha	22	[1,14]		Actin-related protein 2/3 complex subunit 3	2	[14,15]
	Ig kappa chain C	3			Actin-related protein 3	2	[14,15]
	Serum albumin	22	[11]		Alpha-internexin	2	[11]
Extracellular matrix/cell membrane	Agrin	7	[10]	Calponin-3	5	[13,16]	
	Alpha-1-antitrypsin	18	[11,14]	Catenin beta-1	3		
	Alpha-2-macroglobulin	22	[14]	Cell division control protein 42 homolog	2	[14]	
	Annexin A2	24	[12,14]	Cofilin-1	6	[14]	
	Basement membrane-specific heparan sulfate proteoglycan core protein	25	[10,15]	Collapsin response mediator protein 4 long variant	14		
	Basigin	6	[14]	Cytoplasmic dynein 1 heavy chain 1	15		
	Brevican core protein	2		Cytoplasmic dynein 1 light intermediate chain 2	2		
	CD44 antigen	5	[12]	Cytoskeleton-associated protein 4	10		
	CD99 antigen, isoform	4		Differentiation-related gene 1 protein	2		
	Cell surface glycoprotein MUC18	3		Dihydropyrimidinase-related protein 2	11	[14]	
	Chondroitin sulfate proteoglycan 4	4		Ezrin	5	[11,13,14]	
	Collagen	43	[12]	Fascin	6	[13]	
	Complement component C9	8		Gamma-adducin	2	[14]	
	Erythrocyte band 7 integral membrane protein	4	[14]	Glial fibrillary acidic protein	135	[1,11,14]	
	Fibronectin	49	[1,11,12,14,15]	Keratin, type II cytoskeletal 78	14		
	Galectin-3	4	[12]	Lamin-B1	11	[13]	
	Glypican-1	3	[12]	Microtubule-associated protein	2		
	Integrin alpha-V light chain	4	[10,12]	Microtubule-associated protein 1B	38	[14]	
	Inter-alpha-trypsin inhibitor heavy chain H1	8		Myosin regulatory light chain 12B	3	[14,15]	
	Inter-alpha-trypsin inhibitor heavy chain H2	6		Nestin	27	[10]	
	Laminin	30	[10,12,14]	Neurofilament light polypeptide	5		
	Major histocompatibility complex, class I, C	2		Plectin	80	[14]	
	Neuronal membrane glycoprotein M6-a	2		Profilin-2	2	[11]	
	Nidogen-1	10	[10,14]	Septin-7	4	[14]	
	Nidogen-2	5	[10,12,14]	Septin-8	2		
	Periostin	16	[10,12,15]	Spectrin alpha chain, non-erythrocytic 1	25		
	Protein MAL2	3		Spectrin beta chain, brain 1	21	[14]	
	Reticulon-4	6	[14]	Synemin	3		
	Tenascin	42	[10,12,15]	Talin-1	21	[14]	
	Thrombospondin-1	4	[14,15]	Transgelin-2	7	[14,15]	
	Thy-1 membrane glycoprotein	2		Tubulin	106	[11,14,15,22]	
	Transforming growth factor-beta-induced protein ig-h3	9	[10,12,15]	Vimentin	155	[1,11]	

Table 2 continued

Classification	Protein name	No. of peptides	References	Classification	Protein name	No. of peptides	References
	Vitronectin	12	[14]	Protein folding/ chaperone/transport/ channel function	4F2 cell-surface antigen heavy chain	3	
Lipid and fatty acid metabolic process and regulation	3-Hydroxyacyl-CoA dehydrogenase type-2	6	[14]		60 kDa heat shock protein, mitochondrial	14	[14]
	Acid ceramidase subunit beta	2			78 kDa glucose-regulated protein	29	[14,15]
	Enoyl-CoA hydratase 2	2	[14]		Annexin A6	23	
	Fatty acid synthase	9	[14]		Apolipoprotein E	6	
	Perilipin-3	7			Aquaporin-4	5	[11]
	Peroxisomal multifunctional enzyme type 2	2	[14]		Band 3 anion transport protein	9	[10]
Mitochondrial transport	ADP/ATP translocase 2	10			Clusterin	15	[14]
	ADP/ATP translocase 3	2	[15]		Coatomer subunit alpha	3	[14]
	Phosphate carrier protein, mitochondrial	4	[14]		Collagen-binding protein 2	4	[1,15,16]
	Voltage-dependent anion- selective channel protein 1	11	[12,14,15]		Cytochrome b-245 heavy chain	2	
	Voltage-dependent anion- selective channel protein 2	4	[14]		Cytochrome c oxidase subunit 2	4	
Metabolic enzymes	2',3'-Cyclic-nucleotide 3'- phosphodiesterase	2			Electrogenic sodium bicarbonate cotransporter 1	2	
	26S Protease regulatory subunit 4	2	[14]		Endoplasmic reticulum resident protein 29	2	
	26S Proteasome non-ATPase 2 regulatory subunit 11	2			Excitatory amino acid transporter 1	3	
	26S Proteasome non-ATPase 3 regulatory subunit 3	3			Fatty acid-binding protein, brain	9	[1,11,13]
	3-Ketoacyl-CoA thiolase	5			Heat shock cognate 71 kDa protein	17	
	6-Phosphogluconolactonase	2	[14]		Heat shock protein HSP 90	57	[12,14,15]
	Acetyl-CoA acetyltransferase, mitochondrial	3			Hsc70-interacting protein	3	[14]
	Adipocyte plasma membrane- associated protein	3	[14]		Lactotransferrin	8	
	Alanyl-tRNA synthetase	4	[14]		Prohibitin	7	[11,14]
	Aldehyde dehydrogenase family 1 member	2	[12]		Ragulator complex protein LAMTOR1	2	
	Alpha-enolase	7	[12,14]		Serotransferrin	8	[14]
	Amine oxidase (flavin- containing) B	9	[14]		Sideroflexin 3	3	
	ATP synthase	38	[11,12,14]		Solute carrier family 2 (facilitated glucose transporter), member 1	3	
	ATP-dependent RNA helicase DDX3Y	3			Sorting nexin 1, isoform	2	
	Coagulation factor XIII A chain	7	[14]		Sorting nexin-3	2	[14]
	Cytosol aminopeptidase	3			T-complex protein 1	16	[10,14]
	Cytosolic non-specific dipeptidase	4	[14]		V-type proton ATPase subunit B, brain	2	
	D-3-phosphoglycerate dehydrogenase	3	[13]	Protein processing in endoplasmic reticulum	40S ribosomal protein S23	6	[10,14,15]

Table 2 continued

Classification	Protein name	No. of peptides	References	Classification	Protein name	No. of peptides	References
	<u>Endonuclease domain-containing 1 protein</u>	4	[14]		<u>60S ribosomal protein</u>	5	[14,15]
	Extracellular signal-regulated kinase-2 splice variant	4			<u>ADP-ribosylation factor 3</u>	2	
	Farnesyl pyrophosphate synthase	3			<u>Alpha-crystallin B chain</u>	9	[11,13]
	<u>Fructose-bisphosphate aldolase A</u>	20	[14,15]		<u>Calnexin</u>	11	[14]
	<u>Glutamate dehydrogenase 1, mitochondrial</u>	5	[10,11,14]		<u>Calreticulin</u>	5	[14,15]
	<u>Glyceroldehyde-3-phosphate dehydrogenase</u>	15	[12,14,15]		Coatmer protein complex, subunit gamma	5	
	<u>Glycogen phosphorylase, brain</u>	2	[14]		<u>Cullin-associated NEDD8-dissociated protein 1</u>	4	[14]
	<u>Haptoglobin</u>	3	[14]		<u>Dolichyl-diphosphooligosaccharide-protein glycosyltransferase</u>	6	[14,15]
					<u>Elongation factor 1-gamma</u>	4	[14,15]
	<u>Iso citrate dehydrogenase (NADP)</u>	3	[14]		<u>Eukaryotic initiation factor 4A-I</u>	4	[11,15]
	<u>L-lactate dehydrogenase</u>	7	[14,15]		Eukaryotic initiation factor 4A-III	2	
	<u>Malate dehydrogenase, mitochondrial</u>	3	[14]		<u>Eukaryotic translation initiation factor 4H</u>	3	[14]
	<u>Methyltransferase-like protein 7A</u>	2			Heat shock 70 kDa protein 9	10	
	<u>NAD(P) transhydrogenase, mitochondrial</u>	6	[14]		<u>Isoform 3 of Heterogeneous nuclear ribonucleoprotein Q</u>	2	[15]
	Peptidylprolyl isomerase A (Cyclophilin A)	3			<u>Protein disulfide-isomerase</u>	58	[11,14,15]
	<u>Peroxiredoxin-2</u>	6	[14]		<u>Ubiquitin-conjugating enzyme E2N</u>	2	[14]
	Phosphofructokinase, platelet	2			<u>Vesicle-trafficking protein SEC22b</u>	3	[14]
	<u>Phosphoglycerate kinase</u>	12	[14]		<u>Clathrin heavy chain 1</u>	14	[14,15]
	Polyadenylate-binding protein 1	5		Vesicular trafficking	<u>Rab GDP dissociation inhibitor alpha</u>	6	[14,15]
	<u>Puromycin-sensitive aminopeptidase-like protein</u>	2	[14]		<u>Ras-related protein Rab-10</u>	3	[14]
	<u>Pyruvate kinase isozymes M1/M2</u>	35	[14,15]		<u>Ras-related protein Rab-2A</u>	3	[14]
	<u>Pyruvate kinase</u>	6	[12]		Secernin 1, isoform	3	
	<u>Sarcoplasmic/endoplasmic reticulum calcium ATPase 2</u>	7	[14]		<u>Transitional endoplasmic reticulum ATPase</u>	15	[14,15]
	<u>Splicing factor 3A subunit 3</u>	2	[14]		<u>Transmembrane emp24 domain-containing protein 10</u>	7	[14,15]
	<u>Thioredoxin-dependent peroxide reductase, mitochondrial</u>	2	[14]		<u>Vesicle-fusing ATPase</u>	4	
Signal transduction	<u>14-3-3 Protein beta/alpha</u>	6	[14]		FACT complex subunit SSRP1	3	
	<u>14-3-3 Protein epsilon</u>	26	[14]	Other	<u>Isoform 4 of Myelin basic protein</u>	2	
	<u>14-3-3 Protein zeta/delta</u>	4	[14,15]		<u>Receptor expression-enhancing protein 5</u>	2	
	<u>Adenylyl cyclase-associated protein</u>	4	[10,12]				

Table 2 continued

Classification	Protein name	No. of peptides	References	Classification	Protein name	No. of peptides	References
	Guanine nucleotide binding protein (G protein), beta polypeptide 2	4			Serine/arginine-rich splicing factor 1	5	
	STAT1-alpha/beta	3	[15]		Single-stranded DNA binding protein 1, isoform CRA_c	4	
	<u>Vesicle-associated membrane protein-associated protein A</u>	2	[14]				

Note: Proteins in bold indicate proteins that have previously been named in the literature as a tumor biomarker, or linked with angiogenesis. The underlined proteins are also up-regulated in GT compared to NT.

and therefore protein identification is measured relatively less often for peptide masses with a low intensity in the MS1 spectrum [3]. The Xcalibur instrument software uses exclusion criteria in a nano-LC MS measurement to exclude selected MS1 parent masses for MS2 fragmentation in order to obtain as many protein identifications as possible (www.thermoscientific.com).

Performance of Peptrix was compared with that of MsInspect [2] and the results were remarkably different, since the software applications differ greatly in their techniques for processing the mass spectra. Peptrix runs on an average computer system with the Windows OS. Peptrix is written in Java and uses around 1 GB of memory with a maximum memory heap size, -Xmx1024 M, setting of the Java executable. Peptrix does not use any statistics to make the peptide profile matrix [1]. Peptrix uses the freely available MS2 sequencing application X!Tandem (<http://www.thegpm.org/tandem/>) [9] for linking the protein identifications through MS2 peptide sequences to the MS1 peptide masses. The interesting points and results from this new link will be discussed.

Gliomas are among the most vascularized tumors. Therefore, identification of new angiogenesis-related proteins is important for the development of anti-angiogenic therapies [10]. A glioma type brain tumor dataset containing glioma and endometrium control samples is analyzed using Peptrix in this study. To discriminate between physiological and pathological angiogenesis, protein expression profiles of proliferating vessels in glioma are compared with those of endometrium tissue where physiological angiogenesis takes place. The potential protein biomarkers for glioma angiogenesis obtained are compared with proteins that have previously been reported in the literature [11–16]. The Orbitrap analysis results are also compared with FT-ICR MS analysis results from a comparable sample set [1].

Results

Peptrix performance

The processing of the 40 mass spectra in a peptide profile matrix shown in Figure 1 takes a total of 70.5 or 1.75 h per file on average. A peptide profile matrix for peptide masses with a sequence and protein label, and the average peak intensities of the masses in the spectra for the groups GV, GT, NV and NT are created. The peptide profile matrix consists of 24,249 mass-retention time bins, of which 9248 (38%) have a sequence

and protein label. A large part of the low intensity peptide masses in the MS1 spectra is not selected for MS2 sequencing or sequencing is not successful. Among the 24,249 peptides, 52% are up-regulated with intensity ratio GV/NV > 1 (+), and 48% were down-regulated with intensity ratio GV/NV < 1 (-).

The instrumental coefficient of variance (CV) of the Orbitrap mass spectrometer is 10% for measurements of high intensity peaks of technical replicates [2]. When working with peak lists, peak finding and matching low intensity peaks increase the CV. The average CV of measured peak intensities of technical replicates is 25% for Peptrix [2]. The biological variability of peak intensities is large. The CV of peak intensities when measured for all samples of a group, 7350 times in the 24,249 mass retention time bins of the peptide profile matrix, is about 100% of the mean intensity.

A small selection of proteins that have differentially expressed peptide peak intensities between GV and NV is shown in Table 1. The average spectrum peak intensities for the peptide masses for myosin-9 in the four groups examined are shown in Table S1. The average mass accuracy of all identified peptide masses was 4 ppm compared to the calculated value. The Orbitrap peptide masses are approximately less accurate by a factor of 4–7 than those measured with FT-ICR MS.

The number of unique sequences is 6911 in the Orbitrap peptide profile matrix, which means that sequences appear redundant 1.3 times on average in the matrix. The number of peptide sequences divided by the number of unique sequences is 9248/6911 ≈ 1.3. Majority of the sequences appear once (about 85%) or twice (about 10%), while some sequences appear three times (about 4%) or more (about 1%) (Tables 1 and S1).

There are three reasons why peptide sequences appear in the peptide profile matrix more than once. Firstly, in approximately 40% of cases, the repetition of the sequences is caused by tailing possibly combined with mismatching of the peptide mass with other masses in the elution profile for the nano-LC. For example, the sequence FSVNLDVK for protein CRYAB_HUMAN (alpha-crystallin B chain) is shown twice with a difference in retention time binning of 1408 s (7157 – 5749 s), which is > 300 s (5 min) (Table 1). Another example is the peptide mass with the sequence IAQLEEQLDNETK for myosin-9 in Table S1. The difference in retention time between two bins is 1357 s (7199 – 5842 s), which is > 300 s (5 min) too. Secondly, in approximately 40% of cases, the sequence is present more often in the peptide profile matrix

through mass modifications of the peptide. The sequence QEEEMMAKEELVK for myosin-9 is present in three mass retention time bins in Table S1, *i.e.*, with mass 1705.7642, 1721.7544 and 1722.7951 Da. The mass of the peptide without modification is 1722.7951 Da. The first modification of peptide with mass 1705.7642 Da is an N-terminal loss of NH_3 with -17.0265 Da and cyclization of glutamine (Q) [17], while the second modification of peptide with mass 1721.7544 Da is an extra oxidation of methionine (M), which leads to an increase of the mass by 15.999 Da (net change $15.999 - 17.0265 \approx -1$ Da). Finally, in approximately 20% of cases, the sequence is measured more often because of faults in the determinations or mismatching of the masses of the peptides. The mass difference is slightly greater than 10 ppm and the peptide appears in a different mass retention time bin in the matrix, while it should be present in one bin. The peptide with sequence YSVQTADHR for fascin (Table 1) is such an example. The difference in mass is 17 ppm (> 10 ppm binning), while the retention time binning is not more than 5 min different, *i.e.*, the bins differ by $5880 - 5760 = 120$ s.

The number of unique proteins linked to the peptide profile matrix is 1873. Each protein is identified with approximately 4 (6911/1873) unique peptide sequences. The MS2 protein labels from the peak lists from the individual samples are currently passively matched with the MS1 peptide profile matrix in the last step of the process shown in Figure 1. The number of unique peptide sequences in the peak lists from the individual samples is 10,259 and the number of protein labels is 2569. The peptide profile matrix has approximately 67% (6911/10,259) of the peptide sequence and 73% (1873/2569) of the protein information from the peak lists from the individual samples.

Modifications detected by X!Tandem

Compared to the commercial search engine Mascot, the search engine X!Tandem detects a large number of non-tryptic peptide fragments due to a different search algorithm employed, approximately 11% of the total (Tables 1 and S1). Two such examples are the sequence DYEEVGVDSVEGEGEEEGEE from tubulin alpha-1A chain split at EY (Table 1) and the sequence KTELEDTLDSTAAQQELR from myosin-9 split at LK in Table S1.

Approximately 20% of the sequences have a modification (Tables 1 and S1). In approximately 1/3 of the cases, this involves an N-terminal loss of $-\text{NH}_3$ and cyclization of Q for -17.0265 Da, while in approximately 1/3 of the cases, oxidation of M (+O) adds $+15.999$ Da. In addition, in approximately 1/4 of the cases there occurs deamidation ($-\text{NH}_2 + \text{OH}$) of asparagine (N) or Q to increase $+0.984$ Da, and in some cases (the remaining approximately 1/10), acetylation ($+\text{COCH}_3$) of M or alanine (A) confers an augmentation of $+42.0106$ Da.

An N-terminal loss of $-\text{NH}_3$ and Q cyclization increases the hydrophobicity of the peptide. Consequently, the retention time of the peptide masses clearly increases by approximately 2200 s (36 min) through this N-terminal loss [17]. For peptide with sequence QAQQERDELADEIANSSGK from myosin-9, the increased retention time is $7610 - 5496 = 2114$ s and $7610 - 5363 = 2247$ s (Table S1). There are two different values due to binning (errors), since the retention times 5496 and 5363 should have been in one mass-retention time bin.

From all observed modifications, only M oxidation is given as an input variable in the graphical user interface (GUI) of Peptrix. Peptrix stores the modifications in the file *default_input.xml* used by tandem.exe (Figure 1). The other modifications are detected by X!Tandem as standard, when the template *default_input.xml* is used, which can be downloaded together with tandem.exe in the distribution of X!Tandem. The *default_input.xml* can be changed according to personal needs. The modifications including N-terminal loss of ammonia and Q cyclization, oxidation of M, acetylation of M or A, as well as which amino acid this concerns, are reported in the file *output.xml* (Figure 1). The deamidation of N or Q is not detected as such, but is reported as an increase of mass of approximately 1 Da, compared to the theoretical mass.

Selection of candidate biomarkers for glioma angiogenesis

Candidate biomarkers for glioma angiogenesis (Table S2) are selected from the peptide profile matrix based on the following criteria: (1) at least two unique peptide sequences are up-regulated in the GV versus NV group with intensity ratio $\text{GV}/\text{NV} > 1$ (+). This results in 597 protein labels, which are about 32% of the total number of 1873 protein labels; (2) no more than 1 of 6 sequences exclusively down regulated for each protein (−), this is 17% of the peptide sequences for each protein. This results in 328 protein labels, which are about 18% of the total number of protein labels; (3) at least one or preferably more peptides with a Wilcoxon–Mann–Whitney *P* value < 0.1 . This results in 235 protein labels (Table S2), which are about 13% of the total number of protein labels.

Apparent down-regulated peptide masses with intensity $\text{GV} < \text{NV}$ (−) due to tailing in retention time are not considered. In most cases, the peptides of proteins that are up-regulated in GV are also up-regulated with peak intensities $\text{GV} > \text{NV}$ (+). For some sequences of peptide masses in Table 1, a different pattern can be observed with peptide peak intensity $\text{GV} < \text{NV}$ (−). This usually concerns sequences that appear more than once in the peptide profile matrix through tailing of the peptide mass in the elution profile from LC, modifications of the peptide or errors in determining the mass. Such examples include the sequences YSVQTADHR from fascin, LTNSQNFDEYMK from fatty acid-binding protein, brain, LGVRPSQGGEAPR and SYTITGLQPGETDYK from fibronectin and LDHKFDLMYAKR from tubulin alpha-1A chain.

Proteins can be selected from the list of 235 up-regulated protein labels (Table S2) and proteins that were previously named in the literature as a tumor biomarker or linked with angiogenesis [1,10–16] are displayed in bold in Table 2. The up-regulated proteins in GV were classified according to a scheme set-up [15] and information provided at www.uni-prot.org. As presented in Table 2, a large number of proteins are cytoskeleton proteins, involved in cell migration and cell shape or cross linking of actin [1] in bundles, the filopodia [18], such as fascin, cell division control protein 42 homolog and spectrin beta chain, brain 1. Fibronectin (Table 2) is an example of the KEGG hsa04512 ECM–receptor interaction pathway, connecting with cell surface protein integrins, regulating cell-to-ECM and cell-to-cell adhesion. Other members of this pathway including agrin, integrin, laminin, tenascin [10,12,14,15] are also listed in Table 2.

New potential biomarkers for glioma angiogenesis can be selected from the list of up-regulated proteins in Table 2, but need to be validated independently. For example, clusterin was previously reported to be related to multiple sclerosis [19], while excitatory amino acid transporter 1 is perhaps associated with the glutamate dehydrogenase 1, mitochondrial (Accession No. P00367, Table S2) [10,11], since both glutamate transporters and glutamate dehydrogenase play roles in the developing brain [20]. In addition, 3-hydroxyacyl CoA dehydrogenase is an example of a candidate involved in the lipid and fatty acid metabolic process and regulation. It is interesting to note that angiogenesis and metastasis are reduced by the inhibition of fatty acid synthesis with the anti obesity drug Orlistat [21].

In the previous study, the comparison of GT and NT also shows a sharp skewed distribution toward low Wilcoxon–Mann–Whitney *P* values [2]. A large number of peptides are differentially expressed between GT and NT. These proteins are underlined in Table 2. 69 of the selected 235 protein labels (29%) appear also up-regulated in GT, compared to NT. Differences observed between GV and GT are not presented in Table 2, while no significant differences appear between NV and NT at all [2].

The candidate biomarker Cdc42 effector protein 3 [1] is not found in the peptide profile matrix. Peptide sequences from Cdc42 effector protein 4 (B3KUS7) are however defined in the peak lists of GV. The peak intensity of a peptide mass with sequence TPFLLVGTQIDLR from a related protein Cdc42 homolog (E7ETU3) is up-regulated in GV compared to NV with a factor of 17.2 (Table 1). Myosin-9 [15] is also not present in the list of selected candidates. The considerably lower Wilcoxon–Mann–Whitney *P* values and greater intensity ratios GV/NV measured by FT-ICR [1] are not observed in the present analysis. The Wilcoxon–Mann–Whitney *P* values between GV and NV of peptide masses (marked with * in Table S1) remain constantly high at about 0.6 from position 712 up to position 1755 of the primary structure of myosin-9. In addition, annexin A5 [1,12,14] is up-regulated in GV compared to NV, but absent from the list of selected candidates in Table 2, due to lack of peptide mass with Wilcoxon–Mann–Whitney *P* value < 0.1. Different from an earlier finding [1], desmin (Swiss-Prot Accession code P17661) does not appear to be up-regulated in GV compared to NV, but is instead down-regulated (–) as reported in another study [15]. The down regulation of desmin could be attributed to the use of a different control sample set, proliferating endometrium (representing physiological angiogenesis) instead of the normal control hemispheric brain used in the FT-ICR study. Even so, desmin is related to angiogenic micro vessels and is localized together with vimentin, a marker for pericytes [15,22].

Estimation of the FDR

The false discovery rate (FDR) of a protein is estimated based on how many single peptides of the protein are up- or down regulated.

All proteins mentioned in Table S2 are taken up-regulated and the majority of the proteins have been reported to be associated with tumor growth in the literature (Table 2). The 235 protein labels represent 2133 mass intensity bins in the peptide profile matrix (Table S2), from which 312 appear down-regulated (–) in GV versus NV group. The chance of having

a single peptide measured as down-regulated by mistake is estimated as 0.15 (312/2133) for an assumed up-regulated protein.

Since approximately half of the peptides are either up-regulated or down-regulated in the peptide profile matrix, the FDR is therefore set as 0.15. The FDR of protein with two positive (+) peptides is 0.02. The FDR of fascin (Table 1) found with 5 positive (+) and one negative (–) peptide for example is $0.0004 [(0.15)^5 \times (0.85) \times 6!/(5! \times 1!)]$.

Discussion

Peptrix implements the MS2 sequencing application X!Tandem and detects label-free differentially expressed candidate biomarkers for angiogenesis in a small dataset, by comparing the average peak intensities in combination with the non-parametric Wilcoxon–Mann–Whitney test. In this way, Peptrix is capable of detecting meaningful biomarkers, despite the large biological variability of peak intensities and zero values (peptide peak intensity is below detection limit or peak detection fails). As a result, biomarkers that have been reported previously in the literature are identified.

Peptide profiling from Orbitrap™ MS files results in less pronounced intensity ratios between GV and NV than with previous FT-ICR measurements. There is therefore no sign (Table S1) of the supposed up-regulation of peptide masses on the C-terminus of myosin-9 [1]. The level of up-regulation can now be determined with peptide masses at more positions in the protein, because more peptide masses are measured from myosin-9 through LC separation than those measured by FT-ICR.

The lower mass accuracy of Orbitrap™ MS compared to FT-ICR MS is compensated by greater number of masses and protein identifications, which can be directly linked to the peptide profile matrix. Through LC separation, the Orbitrap™ peptide profile matrix contains approximately 10 times more bins (24,249/2275) than the FT-ICR peptide profile matrix obtained from a comparable dataset. The signal is averaged over more MS1 measurements than with FT-ICR MS as well.

A distorted image of up- or down-regulated peptide masses from a protein is however sometimes created through tailing, modifications, incorrectly-determined masses and mismatching. Peptide masses from up-regulated protein intensity $GV > NV$ (+) in some mass retention time bins can appear down-regulated with intensity $GV < NV$ (–). The peptide matrix contains approximately 70% of the MS2 labels of the individual peak lists together. Loss of MS2 sequencing information occurs when matching the individual peak lists in the last step of the creation of the peptide profile matrix (Figure 1). It is therefore important not to match the MS2 sequencing information passively, but to actively involve it in the creation of the matrix, so that all MS2 sequencing information is retained. This active matching should be implemented while retaining maximal speed of Peptrix and the minimal memory usage of the work station.

Materials and methods

Dataset

A glioma type brain tumor dataset containing glioma and endometrium control samples is analyzed in this study. The dataset consists of 10 micro-dissected tissue samples from

glioma blood vessels (GV), 10 from tissue around these blood vessels (GT), 10 from normal endometrium blood vessels (NV), and 10 from endometrium tissue around these blood vessels (NT). The origins and preparation of the micro-dissected tissue samples are described in [10]. In the present analysis, NV and NT of proliferating endometrium (representing physiological angiogenesis) are used as control samples [10], whereas normal control hemispheric brain samples were used in the FT-ICR study [1,16].

Peptrix label-free peptide profiling software

The Orbitrap™ MS measurements are described previously in [2,10]. Forty *.RAW files exported by the Xcalibur instrument software with an average size of 523 MB are imported in Peptrix for analysis. The Peptrix architecture is described in [23]. The imported *.RAW files are saved on an FTP server. Records with the file names of these *.RAW are created in the Table *Sample* in a MySQL database (Supplementary File 1). The files are assigned to GV, GT, NV or NT group, respectively, in the Java Swing graphic user interface (GUI) of Peptrix. The links between file name and group code are saved as records in the table *Results* of the MySQL database (Supplementary File 1). The mass and retention time window for binning the peptide masses in the peptide profile matrix, which is set as 10 ppm and 5 min, respectively, are entered in the GUI. The expected modifications of the peptide masses can also be entered. Only the (fixed) modification carbamidomethyl cysteine (C), mass + C₂H₃NO 57.022 Da, and (variable) oxidation of M, mass + O 15.999 Da, are currently implemented. The precursor mass tolerance of the parent peptide and MS2 fragment mass tolerance, which are set as 10 ppm and 0.6 Da, respectively, are entered in the GUI.

The processing of the MS files is displayed in Figure 1, which is done by pressing the button once without any further user interaction. Peptrix uses the following applications and files invisibly: (1) R.exe (<http://www.r-project.org/>) to trace differentially expressed peptides in the groups with the Wilcoxon–Mann–Whitney module; (2) Readw.exe (version 4.3.1, <http://sourceforge.net/projects/sashimi/files/>) for converting the *.RAW files into *.mzXML files; (3) the *.fasta file and HUMAN.fasta in this study, which is the text database to correlate MS2 fragmentation masses to a protein and (4) tandem.exe to search for the most likely protein. Tandem.exe reads both Mascot generic files, *.MGF, with the peptide parent mass and the MS2 fragmentation masses, and the 38.1 MB HUMAN.fasta file. The HUMAN.fasta file can be downloaded as HUMAN.fasta.gz archive (<ftp://ftp.uniprot.org/pub/databases/uniprot/>) in the directory *current_release/knowledgebase/peptomes/*. Peptrix generates a MGF file for each Orbitrap™ file.

At first, a pop-up window is displayed by Peptrix to search for the files and programs in the computer file system: (1) R.exe; (2) Readw.exe (version 4.3.1); (3) *.fasta file and (4) tandem.exe. The paths to the files in the file system and file names are saved as records in the Table *Itemvalue* of the MySQL database (Supplementary File 1). Peptrix once again prompts for the file or the program when the files or programs are not found in a subsequent analysis, for example, because they have been deleted from the computer file system.

Availability and requirements

Peptrix is freely available. It requires Microsoft Windows 2000 OS or higher, R (R-2.15.1-win.exe or higher), Quick 'n Easy FTP Server Lite version 3.2 or higher, MySQL 5.5.27 (mysql-5.5.27-win32.msi) or higher database, Java Runtime Environment (JRE) 7 Update 7 or higher (jre-7u7-windows-i586.exe), Eclipse Classic (Eclipse Juno 4.2.0) – Windows, edtfpj-2.3.0 or higher (edtfpj.jar), Connector/J (mysql-connector-java-5.1.22-bin.jar or higher), X!Tandem (tandem-win-11-12-01-1.zip) (tandem.exe), HUMAN.fasta (HUMAN.fasta.gz) and Readw.exe (version 4.3.1) (ReAdW-4.3.1.zip) for running. The source code of Peptrix is available as a zip file (<http://sourceforge.net/projects/peptrix/files/>), as well as the database script (Supplementary File 1), with detailed installation and running instructions and URLs of the software providers. The raw Orbitrap™ files conversion to mzXML formatted files was tested with Readw.exe version 4.3.1. Because the Readw.exe program depends on Windows-only vendor libraries from Thermo, the code for Orbitrap™ data handling will only work under Windows with Thermo Fischer Scientifics' Xcalibur software installed. If the Readw.exe program does not work properly, zlib1.dll should be downloaded (<http://sourceforge.net/projects/peptrix/files/>). The library zlib1.dll can be placed in the c:/windows/system32/directory and “regsvr32 c:/windows/system32/zlib1.dll” or “regsvr32 zlib1.dll” can be executed in the Windows command prompt (MSDOS box). If a 64-bit version of Windows is used, zlib1.dll should be copied in C:/Windows/SysWOW64/.

Competing interests

The author has declared that he has no competing interests.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.gpb.2013.02.002>.

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