Table 1 | Results of test sample reporting of 24 academic labs (1–24) and 3 vendors (A–C)

	Lab or vendor																									
	A	1	2	3	4	5	6	7	8	B	9	10	11	12	13	14	15	16	17	18	19	20	21	22	C	23	24
Abbreviation[a]	Group I							Group II							Group III						Group IV						
KHK	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	DB	+	+	+	+	+
ATPAF2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ST	N	TR	+	TR
SETD3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	DB	+	+	+	+	+
SPRY2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	TR
GLB1L3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	+	+	TR	+
FYTTD1	+	+	+	+	+	+	+	+	+	+	N	N	N	N	+	+	N	N	N	N	+	+	+	+	TR	+	TR
IHPK1	+	+	+	+	+	+	+	+	+	N	+	+	+	N	+	+	+	+	N	N	+	+	+	+	+	ST	TR
IFRD1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	N	+	+	N
GCNT3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	TR	+
EIF2S3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	A	+	+	+
F2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	AC	+	+	A	TR	ST	TR
FARP2	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+	+	+	N	+	+	+	+	+	+	+
ENOX1	+	+	+	+	+	+	+	N	+	N	+	N	N	+	+	+	N	+	N	N	+	N	+	+	+	+	N
KLHL13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+	N	+	ST	+
NIBP	+	+	+	+	+	+	+	+	N	+	N	+	+	+	+	N	+	N	+	+	+	+	+	+	+	+	+
MARS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	TR	+
NUP210	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
THBS4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ST	A	TR	+	+
KIAA0746	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	ST	+	TR	TR	+
HIRA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N
Reported[b]	20	20	20	20	20	20	20	20	20	20	20	20	20	20	24	22	21	22	30	25	22	22	21	21	20	18	18
Correct[b]	20	20	20	20	20	20	20	19	19	18	18	18	18	17	20	19	18	18	16	15	16	16	16	14	15	13	12
False positives[b]																			1	1			1	1			
Contaminants[b]															3	2	1	2	1	1	1	3	1	3	5	5	3
Redundant[b]															1				8	3	2	1	2				
Score (%)				100				90	90	81	81	81	81	72	83	82	77	74	42	45	58	58	61	47	56	47	40
SDS-PAGE[c]			+	+		+		+					+			+				+	+		+			
Mass spectrometer[d]	QT	IT	IT	QT	IT	H	TT	QT	QT	QT	IT	IT	H	QT	H	H	QT	H	IT	H	IT	IT	IT	TT	QT	H	IT
Peaklist sofware[e]	P	D	E	M	X	E	Ex	Di	Di	PP	E	B	DTA	PP	Xc	Ex	Di	Xc	B	E	Sp	B	Xc	Ex	Sp	Sp	Sp
Search engine[f]	O	M	S	Sp	M	M	M	M	M	O	M	S	M	O	S	M	M	O	S	S	Sp	S	O	M	Sp	Sp	S
Turnaround[g]	11	16	61	70	90	114	51	41	103	22	53	42	70	44	89	93	85	92	63	108	46	48	69	57	14	48	63

Groups I–IV identify labs scoring 100% (group I), those with naming (N) errors (group II) and those with naming errors as well as false positive, contaminant and redundant identifications (group III). Group IV includes labs with these errors as well as errors attributed to acrylamide alkylation (AC), database searching (DB), excessive stringency (ST), undersampling (A) or trypsinization (TR)-related errors. '+' indicates a correct identification.

[a]Notation used throughout the paper. [b]The number of proteins in each category are indicated. [c]+ here indicates which analyses used gel separation.

[d]Mass spectrometers used were: ion trap (IT); QToF (QT); hybrid (H) including LTQ-FT or LTQ-Orbitrap; and ToFToF (TT). [e]Peaklists were generated by using the following software: Bioworks Browser (Thermo Electron) (B); Data Analysis mzXML (D); Distiller (Matrix Science) (Di); DTA Supercharge (DTA); Extract_msn (Thermo Electron) (E); Explorer (Applied Biosystems) (Ex); Masslynx (Waters) (M); ProteinLynx Global Server (Waters) (P); Protein Pilot (Applied Biosystems) (PP); Spectrum Mill (Agilent) (Sp); X! Tandem (X); and Xcaliber (Thermo Electron) (Xc). [f]Database search engines used were: Mascot (Matrix Science) (M); Sequest (Thermo Electron) (S); Spectrum Mill (Sp); and other (O) that include IdentityE (PLGS, http://www.waters.com), ProteinPilot (Applied Biosystems) or X! Tandem. [g]Turnaround time is given in days.

of the 27 labs was to identify all 20 human proteins and all unique peptides (22) of mass 1,250 ± 5 Da and to report these to the lead investigator, A.W.B. We encouraged members of the labs to use whatever optimized procedures and instrumentation they routinely used, without constraints, which would allow us to assess any trends in those procedures or instruments that were the most effective. We had the labs use the same version of the National Center for Biotechnology Information (NCBI) nr human protein database (27 November 2006) so as to minimize variability in data matching and reporting.

For the first time, to our knowledge, in a proteomics test sample study, each of the participating laboratories is publicly identified here, though all data have been rendered anonymous to prevent tracking to any individual lab. This test sample experiment goes beyond previous efforts as after the findings from these the 27 labs were initially reported to us, we communicated back to them the potential sources of misidentification such that most errors could be corrected. Furthermore, we requested that members of each lab deposit all raw data, methodology, peak lists, peptide statistics and protein identifications into Tranche[17] for subsequent submission to the Proteomics Identifications Database (PRIDE)[18]. The availability of the raw data allowed us to centrally analyze all data. This analysis showed that even though members of most participating labs initially did not report all 20 proteins and the 22 1,250-Da peptides correctly, their raw data clearly indicated that most participants should have been able to identify all 20 proteins as well as most of the 22 1,250-Da peptides.

© 2009 Nature America, Inc. All rights reserved.

RESULTS

Test sample proteins

To create the test sample, we selected 20 proteins in the molecular weight range of 32–110 kDa from the open reading frame (ORF)[19] collection and the mammalian gene collection[20] (**Supplementary Methods** online). The criteria (**Supplementary Fig. 1a** online) for selection included a purity of about 95%, unique tryptic peptide sequences and the presence of at least one tryptic peptide of 1,250 ± 5 Da (**Supplementary Fig. 1b,c**). We expressed the candidate proteins in *Escherichia coli* and purified them following a production strategy by using ion exchange and reverse phase chromatography or by preparative electrophoresis purification from inclusion bodies (**Supplementary Methods**). One-dimensional SDS-PAGE revealed the purity of the 20 purified proteins (**Supplementary Fig. 1d**) at 95% or greater (**Supplementary Table 1** online) as evaluated by densitometry (**Supplementary Fig. 2** and **Supplementary Table 2** online). MS analysis of the 20 purified proteins revealed a vector-derived N-terminal extension of 7 amino acids present on each of the proteins (**Supplementary Fig. 3** online). MS analysis of the test sample confirmed quality (**Supplementary Fig. 4** and **Supplementary Tables 2,3** online) and stability (**Supplementary Fig. 5** and **Supplementary Table 4** online) before distribution to the 27 labs.

Protein identification

We instructed members of the 27 selected labs to use the NCBI nr human protein database of November 27, 2006 with exact matches for all 20 test sample proteins (**Supplementary Fig. 6** and **Supplementary Table 5** online) for protein identification. The individual results from the labs are reported in **Supplementary Table 6** online

Table 2 | Designed peptide mass complexity reporting of 24 academic labs and 3 vendors

	Lab or vendor																														
Abbreviation	A	1	2	3	4	5	6	7	8	B	9	10	11	12	13	14	15	16	17	18	19	20	21	22	C	23	24	22R	CR	23R	24R
KHK	+			+	+	+	+	+			+	+	+		+	+	+			+		+	+						+		+
ATPAF	+	+		+	+			+		+						+			+									+	+		
SETD3				+	+	+		+	+				+			+				+										+
SPRY2	+			+				+						+	+	+	+			+		+	+					+			+
GLB1L3	+			+	+	+					+		+			+				+			+								+
FYTTD1	+	+	+	+	+	+	+	+			+	+	+		+	+	+			+								+			+
IHPK1	+			+	+			+		+			+			+			+						+			+			
IFRD1				+				+		+			+		+	+			+						+				+		
GCNT3	+			+		+	+	+		+	+		+			+	+	+		+		+						+	+		
EIF2S3	+			+	+	+				+	+		+		+	+	+	+	+	+		+	+		+				+		+
F2	+	+		+	+	+		+	+	+	+		+			+	+	+											+		+
FARP2	+	+		+	+	+	+	+		+	+	+	+		+	+	+	+		+			+					+	+		
ENOX1	+	+		+		+	+	+		+	+	+	+		+	+	+	+	+	+		+			+				+		+
KLHL13	+	+		+		+	+	+		+	+	+	+	+		+	+	+		+	+	+							+		+
NIBP				+	+								+			+													+		
MARS	+			+	+	+	+	+			+	+	+	+	+	+	+		+	+		+	+						+		+
NUP210	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+		+	+		+			+	+		+
NUP210	+			+				+								+			+									+			
THBS4	+	+	+	+	+	+		+			+	+	+		+	+	+		+	+								+	+		
KIAA0746	+	+		+	+	+		+		+		+	+			+	+		+	+			+					+			+
HIRA	+	+		+	+	+	+	+			+	+	+		+	+	+	+	+	+					+			+	+		+
HIRA				+	+			+								+												+	+		
Total (initial)	15	9	3	16	12	15	9	14	3	11	12	10	15	4	10	22	14	8	11	15	1	8	8		4	0		7	12	0	13
Total (final)	18	10		22	16			19		11			18		11	22			11							2		11	16		
True positive						3		1					3										1								3
False positive																															
Contaminants				1		1							6		1					3	1	3									
Analysis scoring:																															
Initial score (%)	68	41	14	68	55	64	41	59	14	50	55	45	49	18	41	100	64	36	50	57	2	26	36		18	0	NR	32	55	0	59
Final score (%)	82	45	14	100	73	64	41	86	14	50	55	45	82	18	50	100	64	36	50	57	2	26	36		18	9	NR	50	73	0	59
Report scoring:																															
Centralized (count)	10	15	20	19	18	21	10	19	5	21	19	22	22	6	17	22	18	13	14	22	6	16	16		3	NRD	2	14	15	11	18
Score (%)	DRD	67	15	DRD	89	71	90	100	60	52	63	45	82	67	65	100	78	62	85	68	17	50	50		DRD	NRD	NR	79	DRD	0	72

Initial and final reporting of the number of peptides of mass 1,250 ± 5 Da with increases in totals related to tabulation of mass-shifted cysteine-containing peptides. + indicates a correct identification. Analysis scoring was calculated from the fraction of correct peptide identifications and the accuracy of reporting peptides of mass 1,250 Da, whereas the report scoring was based on the fraction of correct peptide identifications reported divided by the number identified by the centralized analysis. Results not reported, NR; no raw data, NRD; submitted and data reprocessing difference, DRD. DRDs are indicated by fewer peptides identified by the centralized analysis as compared to the number reported. Positive identifications included cysteine-containing peptides that have been alkylated, peptides including missed trypsin cleavage and oxidized methionine residues. Labs A, 4–9, 11 and 13 reported peptides assigned at <95% confidence. Lab 6 used iTRAQ.

© 2009 Nature America, Inc. All rights reserved.

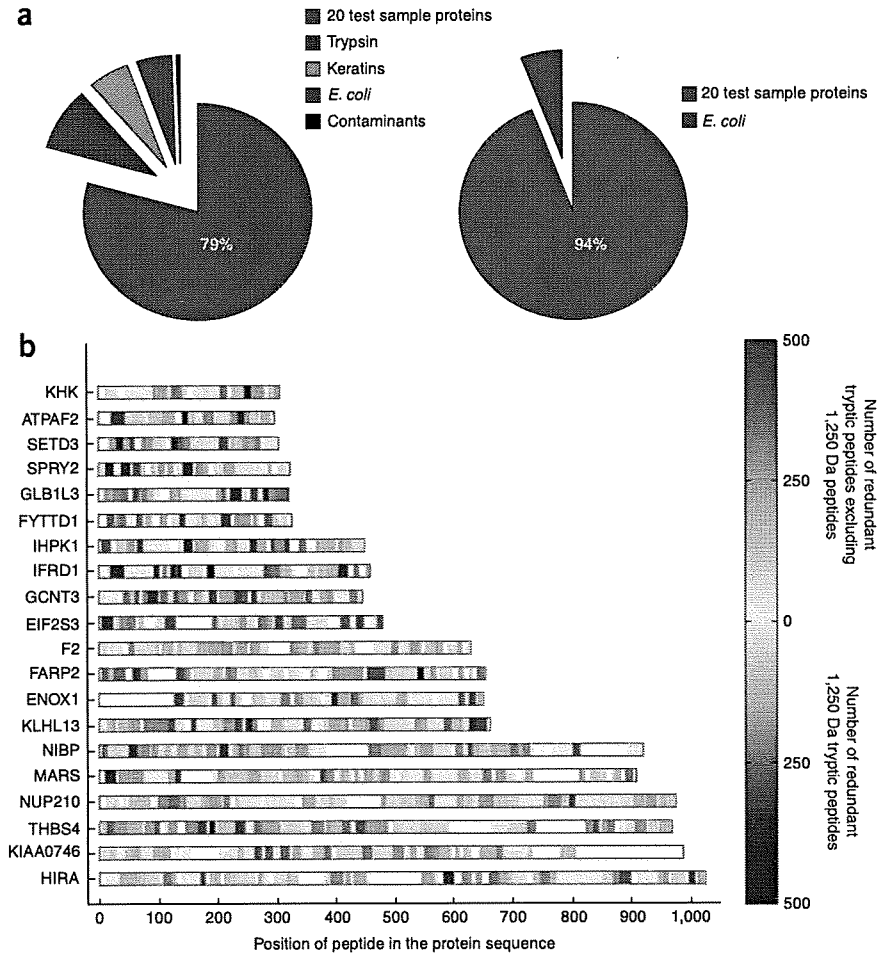

Figure 1 | Number of tandem mass spectra assigned to tryptic peptides. (**a**) Comparison of protein abundance (percent of total redundant peptides) from the centralized analysis of the raw data collected in the 27 labs (left) and after removal of individual lab contaminants including keratins as well as trypsin (right). (**b**) Peptide heatmap representation for each of the 20 proteins from the centralized analysis of the raw data from all 27 labs, revealing the frequency of observation of a given peptide as well as its position in the protein sequence. Raw data from lab 24 were excluded (Online Methods).

and are summarized in **Table 1**. Analysis of the reports revealed clear differences in the number of tandem MS spectra assigned based on the instrument used (**Supplementary Fig. 7** online) but incorrect reporting of false positive and contaminating proteins were not specifically linked to any MS platform or search engine.

Initially, members of only 7 labs (classified as group I) correctly identified all 20 proteins (**Table 1**). The labs classified as group II encountered naming errors. Labs classified as group III encountered naming errors, false positive and redundant identifications (**Supplementary Fig. 8** and **Supplementary Table 7** online). No redundant identifications were reported by members of any lab that used the Mascot (Matrix Science) search engine ($n = 11$) whereas labs using Sequest and SpectrumMill did report redundant identifications. Labs classified as group IV encountered several problems. We distributed another aliquot of the sample to labs that indicated trypsinization problems (labs C, 23 and 24; **Supplementary Table 8** online). Members of lab 22, who had a problem with undersampling, (**Supplementary Table 9** online) performed an additional analysis with their remaining sample. Other errors encountered by group IV included incomplete matching of tandem MS spectra resulting from acrylamide alkylation (**Supplementary Fig. 9** online), database search errors (**Supplementary Table 10** online) and the use of overly stringent identification criteria (**Supplementary Table 11** online), all of which resulted in missed identifications. We devised a scoring system to take incorrect reporting into account. After we discussed the problems with members of each laboratory (**Supplementary Table 12** online) and in some cases had them perform repeat analyses, all identified all 20 proteins, achieving a uniform score of 100% (data not shown).

Peptide sampling

We also assessed the completeness of peptide sampling and selection in the mass spectrometer by assessing the ability of the 27 labs to detect the 22 designed tryptic peptides of mass 1,250 ± 5 Da (**Supplementary Table 13** online), six of which contained cysteine residues whose mass increased as a consequence of reduction and alkylation as routinely used before protein trypsinization. Initially, members of only one lab (lab 14) reported detection of all 22 peptides (**Table 2**) and only an additional three groups (labs 17, B and CR; R indicates repeat analysis) reported detecting any peptides that contained cysteines. Several groups incorrectly reported peptides of 1,250 ± 5 Da derived from contaminating proteins. Several groups also reported peptides of 1,250 ± 5 Da as a result of a single missed trypsin cleavage (denoted as a true positive). We requested that these labs perform a reassessment as described above for protein reporting.

We used our scoring system to assess both the analysis and the reporting of the 1,250 ± 5 Da tryptic peptides. Initially, only members of lab 14 achieved a 100% score. After guidance, members of lab 3 achieved 100% success by correcting for cysteine-containing peptides and excluding peptides derived from contaminants. All other groups reported insufficient data. To distinguish between incomplete reporting and incomplete sampling, we compared the 1,250-Da peptides that were reported to those that were identified by the centralized analysis (see below). Results from labs 10, 11, 14 and 18 (but not lab 3) had data for all 22 1,250-Da peptides. However, members of labs 10, 11 and 18 could not report the peptides and our centralized analysis failed to identify the 22 peptides in the data from lab 3 (**Table 2**). Besides lab 14, only lab 7 achieved 100% reporting of all 1,250-Da peptides in their dataset (a total of 19 peptides, as assessed by our centralized analysis of the data) (**Table 2** and **Supplementary Table 13**).

Data deposition to Tranche and PRIDE

We asked members of the 27 labs to transfer their raw MS data, the methodologies used, peak lists, peptide statistics and protein

© 2009 Nature America, Inc. All rights reserved.

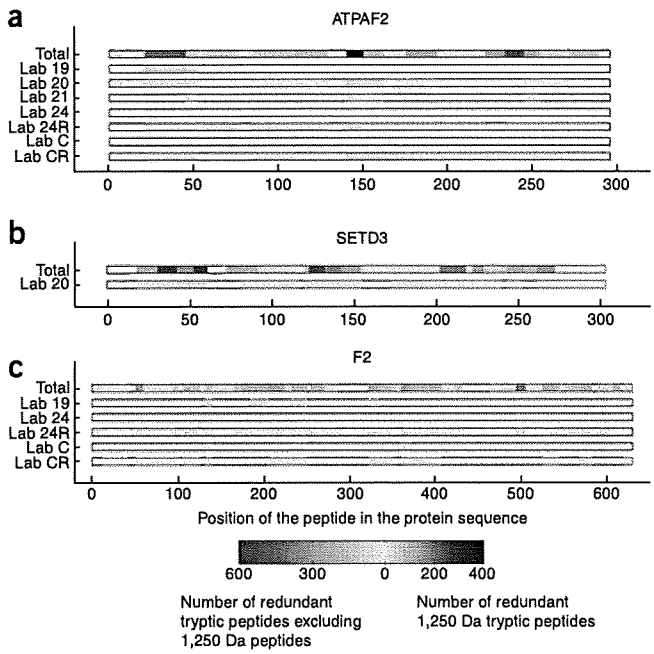

Figure 2 | Discrepancies between reported data and centralized analysis identify erroneous reporting. (**a**–**c**) Peptide heatmap comparisons of the centralized analysis compiled for all 27 labs (total), with the data from selected indicated individual labs for the proteins ATPAF2 (**a**), SETD3 (**b**) and F2 (**c**). Blue, the 1,250 Da peptides; red, all other tryptic peptides. Scale bar represents the number of redundant peptides. Missed cleavages account for the different degree of shading for peptides of 1,250 Da.

identifications to Tranche, a repository for raw data. Initial problems related to the transfer of data to Tranche were all overcome. Tranche hash and passphrase codes are available in **Supplementary Table 14** online. PRIDE personnel transferred a copy of all data from Tranche to PRIDE, a centralized public data repository for the standardized reporting of proteomics results. As evaluated by PRIDE personnel, the initially deposited data had several problems including incomplete files, proprietary software formats and screenshots of data displays in software rather than actual data files. The wide variety of data formats encountered faithfully represents the heterogeneity in the field concerning proteomics bioinformatics. It also appears that the implementation of community standards for data reporting and exchange is not yet at a level that accommodated the minimal requirements for these 20 test proteins.

Centralized analysis of the raw data

To independently assess the individual analyses of the 27 labs, we downloaded all raw data from Tranche. We reanalyzed the collective raw data centrally using a uniform protocol of database searching using X! Tandem[21] and post-processing with the Trans Proteomic Pipeline[22] to assign probabilities to all identifications and global false discovery rates as well as to determine the total number of tandem MS spectra assigned, number of distinct peptides and amino acid sequence coverage (**Supplementary Tables 13** and **15** online).

We found that members of the majority of the labs had in fact generated raw data of sufficient quality to identify all 20 proteins and most of the 22 1,250-Da peptides. We identified discrepancies between the submitted results (**Supplementary Table 12**) and the centrally reprocessed results (**Supplementary Table 15**) for labs 2, 4, 5, 8, 10, 11, 16, 19, 20, 21, 22R, 24 and CR, largely owing to the different data analysis strategies used in these labs. The centralized analysis included checks for experimental artifacts including pyro-Glu formation, deamidations and nontryptic cleavages.

For all 27 labs, the majority of tandem mass spectra (79%) were assigned to the 20 recombinant human proteins, but 21% of the spectra were assigned to contaminants that included *E. coli* proteins, trypsin, keratins and other proteins (**Fig. 1a** and **Supplementary Table 15**). The centralized analysis also revealed that all 22 predicted tryptic peptides of 1,250 Da were observed in only 4 labs, three of which used a Fourier transform ion cyclotron resonance (FTICR) instrument (**Tables 1** and **2**). These instruments reported the highest number of assigned tandem mass spectra, thereby increasing the likelihood of identifying all of the 1,250-Da peptides (**Supplementary Fig. 7**). Tandem mass spectra matching the 1,250-Da peptides were variable for each of the 20 proteins (**Fig. 1b**) and were variably detected in our centralized analysis (**Supplementary Fig. 10** online).

The centralized analysis also revealed (i) that the majority of tandem MS spectra assigned to keratins (human keratins KRT1, KRT2, KRT9 and KRT10 are commonly found in mature epidermal tissue and are also present in laboratory dust and fingerprints, rather than hair- or wool-derived keratins) were largely attributed to strategies that used one-dimensional PAGE (**Supplementary Fig. 11** and **Supplementary Table 15** online); (ii) that *E. coli* proteins were found by members of all but 2 labs (**Supplementary Fig. 11** and **Supplementary Table 15**) and most likely were present in the provided sample; (iii) that other protein contaminants (for example, albumin and casein) were found in datasets from a specific subset of labs (5 labs found albumin, 5 casein and 3 both proteins; albumin was incorrectly reported as human when in fact it was bovine, and both bovine serum albumin and casein are likely abundant proteins used in these labs for standardization); and (iv) that autolytic trypsin peptides resulted from added trypsin. Excluding the contaminants introduced in the labs, 94% of the tandem mass spectra were accounted for by the 20 recombinant proteins, and the remaining tandem MS spectra were assigned to the *E. coli* proteins (**Fig. 1a**). False negatives (one or more of the 20 recombinant proteins not detected) were likely a consequence of variability in trypsin digestion and the stochastic sampling of the mass spectrometry analysis.

Labs that used exclusively liquid phase separations in general had fewer spectra that could be assigned to epidermal keratins than labs that used a combination of protein separation by gel electrophoresis followed by in-gel digestion, peptide extraction and high-performance liquid chromatography peptide separation before tandem MS analysis (**Supplementary Fig. 11**). This trend is probably caused by the fact that each gel slice was exposed to the environment individually, effectively increasing the load of environmental contaminants. The number of spectra that could be assigned to keratins was also broadly correlated with the identification of low-concentration sample source contaminants (*E. coli* proteins) and reagent proteins (trypsin), suggesting that in most cases these proteins were present at substantially lower concentrations than the 20 test sample proteins (**Supplementary Table 15**).

Our centralized analysis confirmed that raw data initially reported by members of 4 labs were incomplete (**Supplementary Table 15**). Repeat analysis in these labs generated sufficient data to

© 2009 Nature America, Inc. All rights reserved.

identify the 20 proteins. No tandem mass spectra were initially observed for the ATAF2 protein in labs 24 and C (**Fig. 2**), but in a repeat analysis, they generated sufficient tandem mass spectra (marked as 24R and CR) to characterize the protein as well as the 1,250-Da peptide. However, members of labs 19, 20 and 21 generated sufficient tandem mass spectra for protein ATPAF2, members of lab 20 generated sufficient tandem mass spectra for protein SETD3, and members of labs 19 and C generated sufficient tandem mass spectra for protein F2 but still did not initially report the identification of these proteins. We determined that members of lab 20 had a database-matching problem for protein SETD3 and members of lab 19 had an acrylamide modification problem for protein F2. Lab 24 had a trypsinization problem for protein F2, which was fixed upon repeat analysis (24R). Although lab C initially reported a trypsinization problem for the F2 protein, the raw data proved otherwise. Lab C's repeat analysis (CR) revealed more tandem mass spectra assigned to protein F2 but insufficient data for the peptide of mass 1,250 Da. Detailed central analysis of each lab's data submitted to Tranche justified the removal of results from lab 24 (but not of this lab's repeat analysis, 24R) from the heat map shown in **Figure 1b**. Inspection of the results from lab 24 (**Supplementary Table 13**) revealed that ~95% of the tandem mass spectra were assigned to peptides with cyclized N-terminal glutamine amino acid (pyroGln), which is not typical for analysis of tryptic peptides. Additional in-depth analysis of the raw data did not identify tandem mass spectra; aberrant chemically induced modifications may have been introduced.

DISCUSSION

Our results demonstrate that, of 27 labs, members of only 7 labs initially characterized an equimolar sample of 20 human proteins. However, our centralized analysis of the raw data demonstrated that members of each of the labs, with a few exceptions, had in fact generated mass spectrometry data of very high quality, more than sufficient to identify all 20 proteins and most of the 22 1,250-Da peptides. This demonstrates the important need for education and training to properly apply such a complex technology.

Most notably, we found generic problems in databases to be the major hurdle for the correct characterization of proteins in the test sample. The search engines used in this study at present cannot distinguish among different identifiers for the same protein, deriving from the way the databases are constructed. Indeed, the search engines used either for the centralized data analysis or by the individual labs suggest an erroneous confidence to the assignments of peptides and proteins. This erroneous confidence necessitates the use of manual verification of both the peptide assignments and protein assignments for low-confidence identifications.

An extended standardized FASTA format (http://psidev.info/index.php?q=node/317) has been proposed by HUPO Proteomics Standards Initiative (PSI) that would resolve the problem of standardized annotation. Presently, manual curation of tandem MS data search results is needed for correct reporting. This includes the nonredundant assignments of tandem MS spectra to overcome the common errors in the apparent characterization of different proteins that are one and the same. We have observed that algorithms used by different search engines to calculate molecular weight are variable (data not shown). It is therefore reasonable to suggest that a common method for calculating molecular weight be chosen and used throughout the community. Additionally, the automatic matching of tandem mass spectra of high quality to a protein-coding genome with a single representative protein for each gene could overcome several of the current errors in protein naming and redundancies.

A test sample containing 20 proteins at 5 pmol equimolar abundance is not representative of a proteomics study with complex mixtures. However, a routine 100% success rate of protein and 1,250-Da peptide identification of such a test sample could be implemented as a standard, as well as the routine deposition of raw data into Tranche. This would enable a greater degree of trust in the conclusions deduced for proteomics studies in general. A limited number of the 20 test sample protein mixtures have been prepared and are available by contacting the lead author (A.W.B.). These samples, however, are stored in 7.5 M urea, which leads to variable carbamylation, and this may affect trypsinization as well as data analysis. Such test samples should be helpful as a benchmarking tool for researchers embarking on a proteomics study with complex mixtures. At the least, their abilities to collect sufficient data for unambiguous identification of 20 human proteins and 22 1,250-Da peptides can be assessed. A peptide-by-peptide comparison of results from any individual lab with those from a centralized analysis of the data should be informative to the inability of any lab's members to detect proteins or specific peptides. For any large-scale, multilab proteomics effort, we recommend the use of a centralized analysis, especially if data are generated on more than one platform, generated in more than one location or collected over time.

Our study allowed us to deduce several guidelines for performing any proteomics experiment. Sources of lab-derived contamination need to be identified and monitored closely, with the two major sources being environmental contamination carried over from prior experiments and keratins (largely from gel-based analysis). The use of target-decoy search strategies should be made mandatory, and false discovery rates should be reported. The monitoring of unique peptides and unique tandem mass spectra is needed to ensure that the minimum list of protein identifications is reported, to address the issue of redundant identifications (sequence variants of the same protein). A gene-centric database could ensure that only a single descriptive name would be assigned to each protein sequence, eliminating aliases. The creation of tools for transforming data (raw data, peak lists, peptide lists and protein lists) into standardized formats would aid the ease of submission to repositories such as Tranche. The distribution of all data deposited in Tranche to the community, via PRIDE, Human ProteinPedia, PeptideAtlas and GPM, would facilitate centralized data analysis which may help lead to new insights in proteomics experiments.

In summary, our analysis showed that even with a sample consisting of highly purified human proteins, members of many participating labs had difficulties in reporting data correctly. However, the majority of the participants deposited raw data, each with more than sufficient coverage of the 20 proteins. Thus a major contributing factor to erroneous reporting resides at the level of database and search engines used and once corrected for, provided an almost perfect score for most participants. Therefore, we expect that once databases and search engines have been improved and made compatible with MS-based proteomics, the accuracy of data reporting will increase and along with it, the fidelity of proteomics.

© 2009 Nature America, Inc. All rights reserved.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

Supported in part by Canadian Institutes of Health Research to the HUPO Head Quarters (S. Ouellette) for coordination of this HUPO test sample initiative. A.W.B. and C.E.A. were supported by Genome Quebec and McGill University. We thank D. Juncker, G. Temple, J. van Oostrum, G. Omenn, K. Colwill, J. Langridge and M. Hallett for their comments on the manuscript, and D.M. Desiderio for helpful comments on the manuscript. This test sample effort builds on pioneering efforts from several other groups and especially Association of Biomolecular Resource Facilities. This study is a HUPO test sample initiative and HUPO welcomes collaborative efforts to benefit proteomics. We acknowledge the following sources of grant support: E.W.D. is supported by the National Heart, Lung and Blood Institute, National Institutes of Health (NIH), under contract N01-HV-28179; the University of California, Los Angeles Burnham Institute for Medical Research NIH grant number RR020843; University of California, Los Angeles (National Heart, Lung and Blood Institute P01-008111); University of Michigan, NIH P41RR018627; Beijing Proteome Research Center, affiliated with The Beijing Institute of Radiation Medicine for National Key Programs for Basic Research grant 2006CB910801 and Hi-Tech Research grant 2006AA02A308. We acknowledge access and use of The University College Dublin Conway Mass Spectrometry Resource instrumentation, supported by Science Foundation, Ireland grant 04/RPI/B499. PRIDE, J.A.V. is a postdoctoral fellow of the "Especialización en Organismos Internacionales" program from the Spanish Ministry of Education and Science. L.M. is supported by the "ProDaC" grant LSHG-CT-2006-036814 of the EU. Samuel Lunenfeld Research Institute, Mount Sinai, Toronto is supported by Genome Canada through Ontario Genomics Institute. J.A.V. and L.M. thank H. Hermjakob and R. Apweiler for their support. A.W.B. thanks L. Roy and Z. Bencsath-Makkai for help in data submission and analysis.

AUTHOR CONTRIBUTIONS

A.W.B. coordinated all steps of the study. C.E.A., T.N. and J.J.M.B. coordinated data analysis and the final manuscript. E.W.D., R.B. and R.K. did the centralized analysis of the collective data retrieved from the raw data supplied from each lab to Tranche. S.A.C., P.P., L.M., E.K., C.D., S.S., X.Q., K.W., T.P.C., K.P. and T.A.B. provided comments. Invitrogen prepared, designed and distributed the test sample proteins.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/

Published online at http://www.nature.com/naturemethods/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. de Godoy, L.M. *et al.* Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. *Nature* **455**, 1251–1254 (2008).
2. Turck, C.W. *et al.* The Association of Biomolecular Resource Facilities Proteomics Research Group 2006 study: relative protein quantitation. *Mol. Cell. Proteomics* **6**, 1291–1298 (2007).
3. Boutilier, K. *et al.* Comparison of different search engines using validated MS/MS test datasets. *Anal. Chim. Acta* **534**, 11–20 (2005).
4. Elias, J.E., Haas, W., Faherty, B.K. & Gygi, S.P. Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. *Nat. Methods* **2**, 667–675 (2005).
5. Kapp, E.A. *et al.* An evaluation, comparison, and accurate benchmarking of several publicly available MS/MS search algorithms: sensitivity and specificity analysis. *Proteomics* **5**, 3475–3490 (2005).
6. Bell, A.W., Nilsson, T., Kearney, R.E. & Bergeron, J.J. The protein microscope: incorporating mass spectrometry into cell biology. *Nat. Methods* **4**, 783–784 (2007).
7. Gilchrist, A. *et al.* Quantitative proteomics analysis of the secretory pathway. *Cell* **127**, 1265–1281 (2006).
8. Klie, S. *et al.* Analyzing large-scale proteomics projects with latent semantic indexing. *J. Proteome Res.* **7**, 182–191 (2008).
9. Zubarev, R. & Mann, M. On the proper use of mass accuracy in proteomics. *Mol. Cell. Proteomics* **6**, 377–381 (2007).
10. Cortez, L. The implementation of accreditation in a chemical laboratory. *Trends Analyt. Chem.* **18**, 638–643 (1999).
11. Lander, E.S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921 (2001).
12. Yates, J.R. III., Gilchrist, A., Howell, K.E. & Bergeron, J.J. Proteomics of organelles and large cellular structures. *Nat. Rev. Mol. Cell Biol.* **6**, 702–714 (2005).
13. Shi, L., Perkins, R.G., Fang, H. & Tong, W. Reproducible and reliable microarray results through quality control: good laboratory proficiency and appropriate data analysis practices are essential. *Curr. Opin. Biotechnol.* **19**, 10–18 (2008).
14. Anonymous. Making the most of microarrays. *Nat. Biotechnol.* **24**, 1039 (2006).
15. Anonymous. Proteomics' new order. *Nature.* **437**, 169 (2005).
16. Domon, B. & Aebersold, R. Challenges and opportunities in proteomics data analysis. *Mol. Cell. Proteomics* **5**, 1921–1926 (2006).
17. Falkner, J.A., Hill, J.A. & Andrews, P.C. Proteomics FASTA archive and reference resource. *Proteomics* **8**, 1756–1757 (2008).
18. Martens, L. *et al.* PRIDE: the proteomics identifications database. *Proteomics* **5**, 3537–3545 (2005).
19. Liang, F. *et al.* ORFDB: an information resource linking scientific content to a high-quality Open Reading Frame (ORF) collection. *Nucleic Acids Res.* **32**, D595–D599 (2004).
20. Strausberg, R.L., Feingold, E.A., Klausner, R.D. & Collins, F.S. The mammalian gene collection. *Science* **286**, 455–457 (1999).
21. Craig, R. & Beavis, R.C. TANDEM: matching proteins with tandem mass spectra. *Bioinformatics* **20**, 1466–1467 (2004).
22. Keller, A., Eng, J., Zhang, N., Li, X.J. & Aebersold, R. A uniform proteomics MS/MS analysis platform utilizing open XML file formats. *Mol. Syst. Biol.* **1**, 2005.0017 (2005).

© 2009 Nature America, Inc. All rights reserved.

The full list of authors and affiliations is as follows:

Thomas A Beardslee[8], Thomas Chappell[9], Gavin Meredith[10], Peter Sheffield[11], Phillip Gray[12], Mahbod Hajivandi[10], Marshall Pope[10], Paul Predki[10], Majlinda Kullolli[13], Marina Hincapie[13], William S Hancock[13], Wei Jia[14], Lina Song[14], Lei Li[14], Junying Wei[14], Bing Yang[14], Jinglan Wang[14], Wantao Ying[14], Yangjun Zhang[14], Yun Cai[14], Xiaohong Qian[14], Fuchu He[14], Helmut E Meyer[15], Christian Stephan[15], Martin Eisenacher[15], Katrin Marcus[15], Elmar Langenfeld[15], Caroline May[15], Steven A Carr[16], Rushdy Ahmad[16], Wenhong Zhu[17], Jeffrey W Smith[17], Samir M Hanash[18], Jason J Struthers[18], Hong Wang[18], Qing Zhang[18], Yanming An[19], Radoslav Goldman[19], Elisabet Carlsohn[20], Sjoerd van der Post[20], Kenneth E Hung[21], David A Sarracino[22], Kenneth Parker[21], Bryan Krastins[22], Raju Kucherlapati[21], Sylvie Bourassa[23], Guy G Poirier[24], Eugene Kapp[25], Heather Patsiouras[25], Robert Moritz[25], Richard Simpson[25], Benoit Houle[26], Sylvie LaBoissiere[27], Pavel Metalnikov[28], Vivian Nguyen[29], Tony Pawson[29], Catherine C L Wong[30], Daniel Cociorva[30], John R Yates III[30], Michael J Ellison[31], Ana Lopez-Campistrous[31], Paul Semchuk[31], Yueju Wang[32], Peipei Ping[32], Giuliano Elia[33], Michael J Dunn[33], Kieran Wynne[33], Angela K Walker[34], John R Strahler[34], Philip C Andrews[34], Brian L Hood[35,36], William L Bigbee[35,37], Thomas P Conrads[35,36], Derek Smith[38], Christoph H Borchers[38], Gilles A Lajoie[39], Sean C Bendall[39], Kaye D Speicher[40], David W Speicher[40], Masanori Fujimoto[41], Kazuyuki Nakamura[41], Young-Ki Paik[42], Sang Yun Cho[42], Min-Seok Kwon[42], Hyoung-Joo Lee[42], Seul-Ki Jeong[42], An Sung Chung[42], Christine A Miller[43], Rudolf Grimm[43], Katy Williams[44], Craig Dorschel[45], Jayson A Falkner[34], Lennart Martens[46] & Juan Antonio Vizcaíno[46]

[8]Verdezyne, Inc., Carlsbad, California, USA. [9]BioGrammatics Incorporated, Carlsbad, California, USA. [10]Invitrogen Corporation, Carlsbad, California, USA. [11]Allergan, Irvine, California, USA. [12]Ambry Genetics, Aliso Viejo, California, USA. [13]Barnett Institute and Department of Chemistry and Chemical Biology,

Northeastern University, Boston, Massachusetts, USA. [14]State Key Laboratory of Proteomics, Beijing Proteome Research Center, Changping District, Beijing, China. [15]Bochum University, Ruhr-Universitaet Bochum, Bochum, Germany. [16]Proteomics, Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts, USA. [17]Burnham Institute for Medical Research, La Jolla, California, USA. [18]Fred Hutchinson Cancer Research Center, Seattle, Washington, USA. [19]Georgetown University, Department of Oncology, Washington, DC, USA. [20]Göteborg Proteomics Centre: The Proteomics Core Facility, Sahlgrenska Academy, University of Göteborg, Göteborg, Sweden. [21]Harvard Partners Center for Genetics and Genomics, Cambridge, Massachusetts, USA. [22]Thermo-Fisher BRIMS Center, Cambridge, Massachusetts, USA. [23]Proteomics Platform, Quebec Genomic Center, Laval University Medical Research Center, Quebec, Canada. [24]Health and Environment Unit, Laval University Medical Research Center, Quebec, Canada. [25]Joint Proteomics Laboratory, Ludwig Institute for Cancer Research and The Walter & Eliza Hall Institute for Medical Research, Parkville, Australia. [26]Genizon BioSciences Incorporated, Saint Laurent, Canada. [27]McGill University and Genome Quebec Innovation Centre, Montréal, Canada. [28]Ontario Cancer Biomarker Network, MaRS Centre, Toronto, Canada. [29]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada. [30]The Scripps Research Institute, Department of Chemical Physiology, La Jolla, California, USA. [31]Department of Biochemistry, University of Alberta, Edmonton, Canada. [32]Departments of Physiology, Medicine and Division of Cardiology, David Geffen School of Medicine, University of California, Los Angeles, California, USA. [33]Proteome Research Centre, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland. [34]Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan, USA. [35]Clinical Proteomics Facility, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania, USA. [36]Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. [37]Department of Pathology, University of Pittsburgh School of Medicine, Magee-Womens Research Institute, Pittsburgh, Pennsylvania, USA. [38]University of Victoria, Victoria, Canada. [39]Department of Biochemistry, University of Western Ontario, London, Ontario, Canada. [40]The Wistar Institute, Philadelphia, Pennsylvania, USA. [41]Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan. [42]Yonsei Proteome Research Center, Yonsei University, Sudaemoon-ku, Seoul, Korea. [43]Agilent Technologies Incorporated, Santa Clara, California, USA. [44]Applied Biosystems, Foster City, California, USA. [45]Waters Corporation, Milford, Massachusetts, USA. [46]EMBL Outstation, European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK.

© 2009 Nature America, Inc. All rights reserved.

ONLINE METHODS

Test sample generation and distribution. As more completely described in the **Supplementary Methods**, all test sample proteins were cloned[23] and expressed[24] in *E. coli*, purified from inclusion bodies under denaturing conditions and mixed in equimolar (5 pmol) amounts. A committee made up of funding agency representatives (NIH and Canadian Institutes of Health Research), journal editors and the HUPO Executive Committee proposed a list of 55 labs. Invitations to participate were extended to 41 labs and 24 accepted. Also, 6 mass spectrometer vendors were selected by the HUPO Industrial Advisory Board (IAB) and all agreed to participate but only 3 provided results. The 27 labs that participated are indicated here as co-authors. Dried samples containing 5 picomoles of each protein were shipped on dry ice, along with detailed examples of LC-MS proteomics analyses (http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf.Par.72904.File.tmp/HumanProteinStandardsforMassSpectrometry.pdf). Samples were shipped from Invitrogen and deliveries were overnight (by DHL in the USA and DHL International or FedEx International express overseas; 1 to 3 business day delivery). Delivery to Australia was delayed on two occasions owing to incomplete customs-related documentation that resulted in the samples attaining ambient temperatures and hence their replacement. Another two samples were received at the recipient institutes but did not arrive at the host lab. One vial was reported to be empty as negligible signal was observed by Coomassie blue staining of a two-dimensional gel. In all cases, more material was supplied. Participants were instructed to use a specified NCBI nr database (http://portal.proteomics.mcgill.ca:8080/hupo-standards/nr_human_20061127_v2.fasta), to report details of methodologies used and proteins identified and to deposit raw data and reports to Tranche (http://tranche.proteomecommons.org/) (**Supplementary Note** online).

Instructions to laboratories and vendors. Test samples were distributed to participating laboratories, who were instructed to (i) identify the 20 human proteins, (ii) report the details of the identifications (protein name, NCBI gi number, sequence coverage, number of peptides and number of tandem MS spectra) following the criteria of ref. 25 and (iii) report the details of methodology. The following description of the sample was supplied: "The sample is an equimolar mixture (5 pmol) of 20 human proteins that were expressed in *E. coli* under conditions to maximize inclusion body formation. The expression system results in an N-terminal extension of 7 amino acids (sequence MYKKAGT) followed by the encoded initiator methionine. The 20 proteins were purified by preparative SDS PAGE or 2D-LC (anion exchange and reversed phase) to > 95% purity. Trypsin digestion of the purified constructs results in the generation of a tripeptide (MYK) plus free K or a tetrapeptide (MYKK) resulting from 1 missed cleavage and an N-terminal extension of 3 (AGT) or 4 (KAGT, 1 missed cleavage) amino acids. Contaminants do not exceed 1% in the final mixture." Details regarding the proteomics MS analysis as well as the selection and purification of the test sample proteins by Invitrogen were also supplied (poster presentation (http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf.Par.72904.File.tmp/HumanProteinStandardsforMassSpectrometry.pdf) that was presented at the HUPO 5th Annual World Congress).

Protein identification reports were scored based on acceptable names as found in the specified database. For reassessment, each lab was instructed to make corrections based on naming; redundant, false positive and contaminant identifications; and acrylamide alkylation of cysteines. Labs that did not achieve 100% after reassessment were requested to repeat the analysis of another aliquot of the sample.

Reporting of peptides of mass 1,250 ± 5 Da was requested, with reassessment as above, and reports were scored twofold, for analysis and reporting completeness.

Database selection. To limit variation in data evaluation, a single database, the NCBInr human protein database of 27 November, 2006, was selected. The NCBInr database contained all 20 test proteins with their exact matches represented.

Previous efforts to benchmark proteomics through test samples have usually allowed participating labs to choose whatever database they felt might be the most appropriate to match their tandem mass spectra. As we have argued elsewhere[6,26], most databases are still in a constant flux changing from one release to another. These changes lead to increased variation in data evaluation. Here we compared the predicted amino acid sequence of the 20 test proteins selected as identified above with the NCBI nonredundant database, the Universal Protein Resource (UniProt) and the International Protein Index (IPI) databases (**Supplementary Table**). Comparisons were made by using blastp (http://www.ncbi.nlm.nih.gov/BLAST/). The reciprocal matching (database to ORF and ORF to database) process revealed differences in protein length as well as amino acid substitutions, most of which occurred in the IPI database and are likely to be related to the specific assembly process of the IPI[27]. Longer or shorter sequences in the database indicate extensions or truncations and/or differences in editing (removal of potential introns) the predicted DNA sequences. Amino acid substitutions are indicated by orange and green shading. An exact match is indicated by 100% identity in both directions. From this database assessment only the NCBInr database had all recombinant proteins with their exact matches represented.

Data reporting. The number of proteins reported and number correct are indicated as are the number of false positive (proteins identified by shared peptides) and contaminant (proteins not in the sample) identifications and those proteins identified more than once but reported as separate proteins (redundant). After the initial reporting by members of the 27 labs (numbers and letters are used to identify academic labs and vendors, respectively), one of us (A.W.B.) discussed with members of each lab problems associated with providing nondescriptive names (for example, hypothetical protein, ORF), and also the reporting of redundant identifications, and false positive and contaminating proteins. Problems associated with spurious alkylation of cysteine residues by acrylamide during preparative electrophoresis were also discussed. Participants were requested to reassess search results and to submit updated final reports. A scoring system was devised to take into account incomplete reporting as well as erroneous identifications. The score (**Table 1**) was calculated as follows: score = fraction identified (number correct / 20) × accuracy (number correct / number reported) × 100. For **Table 1**, details for the proteomics analyses on a lab-by-lab basis including protein separation, mass spectrometer, peaklist software and database

© 2009 Nature America, Inc. All rights reserved.

search engine as well as turn-around time (time from the lab receiving the sample until results were submitted by email (average 67 days)) are indicated. All labs used trypsin. Mass spectrometers used included: ion trap (IT); QToF (QT); hybrid (H) including LTQ-FT or LTQ-Orbitrap; and ToFToF (TT). Peaklists were generated by using the following software: Bioworks Browser (Thermo Electron) (B), Data Analysis mzXML (D), Distiller (Matrix Science) (Di), DTA Supercharge (DTA), Extract_msn (Thermo Electron) (E), Explorer (Applied Biosystems) (Ex), Masslynx (Waters) (M), ProteinLynx Global Server (Waters) (P), Protein Pilot (Applied Biosystems) (PP), Spectrum Mill (Agilent) (Sp), X! Tandem (X), and Xcaliber (Thermo Electron) (Xc), and all labs used default parameter with lab 5 including total ion current (TIC) threshold of 100 and a minimum of 10 peaks, and lab 7 including correlation threshold (CT) of 0.7, signal-to-noise ratio (SNR) of 20, reject width outliers and baseline correction. Database search engines included: Mascot (Matrix Science) (M), Sequest (Thermo Electron) (S), Spectrum Mill (Sp), and others (O) that include IdentityE (PLGS, http://www.waters.com), ProteinPilot (Applied Biosystems) or X! Tandem. All procedures used are reported in Tranche (**Supplementary Table 14**).

The methodology, the peak lists, the peptide statistics and protein identification data were transferred to Tranche, a repository for raw data. Detailed instructions (**Supplementary Note**) were provided to each participating lab with regards to the preparation and transferring of supporting data and information to Tranche (http://www.proteomecommons.org/dev/dfs/examples/hupo-2007/Tranche-HUPO.jsp). All problems in the transfer of data from host labs to Tranche (for example, compact disk and courier transmission, firewall problems, unresponsive servers) were overcome. The transfer of data culminated with the generation of a Tranche hash and passphrase codes that were returned by e-mail to the submitter and to one of us (A.W.B.). The final set of codes is listed in **Supplementary Table 14**).

Transfer of peaklists, search results, peptide statistics and protein identification data from Tranche to PRIDE by the PRIDE personnel led to the successful transfer of 29 datasets (accession numbers: 8130–8158). The data can be accessed by these accession numbers or by project name (HUPO test samples) from the 'Browse experiments' portal at PRIDE. The information in PRIDE comprises protein identifications and spectra from all the groups involved, and all the associated metadata.

Centralized analysis of the collective data. To provide an independent assessment of all individual analyses, we reanalyzed all data collectively by using a uniform protocol of searching with X! Tandem[21] and post-processing with the Trans Proteomic Pipeline[22] to assign probabilities to all identifications and global false discovery rates.

Raw data and supporting documentation as deposited by each lab to Tranche were downloaded by using Tranche hash and passphrase codes (**Supplementary Table 14** online). For labs 1–5, 7, 9–14, 15_1, 16–21, 23R, 24, 24R and A, raw mass spectrometer output files were deposited in the native instrument vendor format. These files were transformed into the open XML format mzXML[28]. Labs 6, 8, 15_2, 22R and B did not provide mass spectrometer output files, and in these cases, the text-format peak list files were used in the centralized analysis. For labs C and CR, mzData files were submitted and used for the analysis. Lab A data were acquired in MS^e (ref. 29) mode that include low energy (MS scans) and high energy (fragmentation scans) scans without peptide ion selection. Standard processing techniques cannot be applied to the output MS^e spectra because co-eluting peptide ions are fragmented simultaneously. For the centalized analysis, lab A provided PKL files with time-deconvolved peaklists. These PKL files were converted to mzXML and processed in the same manner as the others. For lab 7, the conversion from vendor format to mzXML did not sum consecutive scans, which would have resulted in approximately twice as many identified spectra. For this reason, the MGF files provided by the lab that already contained summed scans were used for the analysis.

All of the datasets were subjected to a uniform processing and validation to provide a homogeneous analysis environment in an attempt to minimize data processing differences among the groups. The tandem mass spectra were searched against a reference database constructed from a) the human IPI 3.50 protein list (http://www.ebi.ac.uk/IPI/), b) the non-redundant *E. coli* database distributed by NCI ABCC dated 2008-02-06 (ftp://ftp.ncifcrf.gov/pub/nonredun/), c) the cRAP set of common contaminant proteins from the Global Proteome Machine database (GPMDB) dated 2008-10-01 (http://www.thegpm.org/cRAP/index.html), d) the 20 recombinant proteins present in the test samples with the vector-derived N-terminal extension of 7 amino acids and e) finally an appended set of decoy proteins derived by scrambling all tryptic peptides in the target sequences described above. A copy of this constructed database is available at http://www.peptideatlas.org/tmp/HsIPI3.50_Ec_cRAP_20_TargetDecoy.fasta. The spectra were searched using the X! Tandem search engine[21] with the K-score plugin[30].

The search parameter files used for each experiment are available in the centalized reanalysis Tranche project file (**Supplementary Table 14** online). In general, the search parameters were: 2 allowed missed cleavages, precursor *m/z* tolerance from −2.1 to +4.1, fragment *m/z* tolerance 0.4. Searches were performed with variable methionine oxidation, pyro-glutamic acid formation (from N-terminal glutamic acid and glutamine) and variable iodoacetamide and acrylamide modifications on cysteine or iTRAQ modifications, if appropriate. If the native data contained charge state information, it was used; when charge state information was not available, either +1 or both +2, +3 were searched. Consideration for potential ion pairs that might degrade MS analysis (that is, glutamic acid and aspartic acid residues in carboxylate form and ion-paired with Na^+ or K^+) revealed a negligible contribution, and these ion pairs were not included.

Validation of the search results was performed using the Trans Proteomic Pipeline (TPP) software suite[22]. The TPP tool PeptideProphet[31] modeled the correct and incorrect spectrum assignments, calculating a probability of being correct to each match based on the models. The ProteinProphet tool[32] was then used to adjust the identification probabilities based on corroborating evidence of other identifications that include tandem MS of similar matching characteristics but of lower quality within each dataset and, notably, perform a protein-inference step that coalesces the identifications that map to multiple proteins into single consensus identifications. This processing and validation produced a high-quality set of identifications for each lab. A final centralized processing of all PeptideProphet results through a single ProteinProphet run yields a global picture of all proteins detected by the 27 labs in the mass spectrometry analyses.

© 2009 Nature America, Inc. All rights reserved.

doi:10.1038/nmeth.1333

23. Khan, S. *et al.* Identification of the dominant translation start site in the *attB1* sequence of the pET-DEST42 Gateway vector. *Protein Expr. Purif.* **49**, 102–107 (2006).
24. Fahnert, B., Lilie, H. & Neubauer, P. Inclusion bodies: formation and utilisation. *Adv. Biochem. Eng. Biotechnol.* **89**, 93–142 (2004).
25. Carr, S. *et al.* The need for guidelines in publication of peptide and protein identification data: Working Group on Publication Guidelines for Peptide and Protein Identification Data. *Mol. Cell. Proteomics* **3**, 531–533 (2004).
26. Au, C.E. *et al.* Organellar proteomics to create the cell map. *Curr. Opin. Cell Biol.* **19**, 376–385 (2007).
27. Kersey, P.J. *et al.* The International Protein Index: an integrated database for proteomics experiments. *Proteomics* **4**, 1985–1988 (2004).
28. Pedrioli, P.G. *et al.* A common open representation of mass spectrometry data and its application to proteomics research. *Nat. Biotechnol.* **22**, 1459–1466 (2004).
29. Silva, J.C. *et al.* Quantitative proteomic analysis by accurate mass retention time pairs. *Anal. Chem.* **77**, 2187–2200 (2005).
30. MacLean, B., Eng, J.K., Beavis, R.C. & McIntosh, M. General framework for developing and evaluating database scoring algorithms using the TANDEM search engine. *Bioinformatics* **22**, 2830–2832 (2006).
31. Keller, A., Nesvizhskii, A.I., Kolker, E. & Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **74**, 5383–5392 (2002).
32. Nesvizhskii, A.I., Keller, A., Kolker, E. & Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **75**, 4646–4658 (2003).

© 2009 Nature America, Inc. All rights reserved.

doi:10.1038/nmeth.1333

Corrigendum: A HUPO test sample study reveals common problems in mass spectrometry–based proteomics

Alexander W Bell, Eric W Deutsch, Catherine E Au, Robert E Kearney, Ron Beavis, Salvatore Sechi, Tommy Nilsson, John J M Bergeron & HUPO Test Sample Working Group
Nat. Methods 6, 423–430 (2009); published online 17 May 2009; corrected after print 29 June 2009.

In the version of this article initially published, the author name Steven A. Carr was spelled incorrectly, and the name of an organization described in the text, the HUPO Proteomics Standards Initiative (PSI), was given incorrectly. These errors have been corrected in the PDF and HTML versions of this article.

Erratum: Transposon-mediated genome manipulation in vertebrates

Zoltán Ivics, Meng Amy Li, Lajos Mátés, Jef D Boeke, Andras Nagy, Allan Bradley & Zsuzsanna Izsvák
Nat. Methods 6, 415–422 (2009); published online 28 May 2009; corrected after print 11 June 2009.

In the version of this article initially published, a part of Figure 1b was incorrectly labeled. The error has been corrected in the HTML and PDF versions of the article.

© 2009 Nature America, Inc. All rights reserved.

Proteomics finding heat shock protein 27 as a biomarker for resistance of pancreatic cancer cells to gemcitabine

SAYAKA MORI-IWAMOTO[1,2], YASUHIRO KURAMITSU[2], SHOMEI RYOZAWA[1], KUNIKO MIKURIA[2], MASANORI FUJIMOTO[2], SHIN-ICHIRO MAEHARA[3], YOSHIHIRO MAEHARA[3], KIWAMU OKITA[1], KAZUYUKI NAKAMURA[2] and ISAO SAKAIDA[1]

Departments of [1]Gastroenterology and Hepatology, and [2]Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi; [3]Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi Higashiku, Fukuokashi, Fukuoka, Japan

Received June 27, 2007; Accepted August 24, 2007

Abstract. Pancreatic cancer remains a devastating disease and >96% of patients with pancreatic cancer do not survive for more than 5 years. Gemcitabine (2'-deoxy-2'-difluoro-deoxycytidine: Gemzar) appears to be the only clinically effective drug for pancreatic cancer, but it has little impact on outcome. Proteomic analysis of gemcitabine-sensitive cells (KLM1) and resistant pancreatic cells (KLM1-R) was performed to identify target proteins of the gemcitabine. We found seven proteins, HSP27, peroxiredoxin 2, endoplasmic reticulum protein ERp29 precursor, 6-phosphogluconolactonase, triosphospate isomerase, α enolase, and nucleophosmine that could play a role in determining the sensitivity of pancreatic cancer to gemcitabine. We knocked down HSP27 in KLM1-R and the sensitivity to gemcitabine was restored. In addition, increased HSP27 expression in tumor specimens was related to higher resistibility to gemcitabine in patients of pancreatic cancer. HSP27 may play an important role in the resistibility to gemcitabine, and it could also be a possible biomarker for predicting the response of pancreatic cancer patients to treatment with gemcitabine.

Introduction

Pancreatic cancer is characterized by difficulties in diagnosis, its aggressiveness, and the lack of effective systemic therapy, Only 4% of patients with adenocarcinoma of the pancreas survive for more than 5 years after diagnosis (1,2). Surgical resection is the sole curative treatment that is currently available, but only 10-15% of patients are free from metastasis at the time of diagnosis. Gemcitabine (2'-deoxy-2'-difluoro-deoxycytidine: Gemzar) is a deoxycytidine analogue with structural and metabolic similarities to cytarabine. Currently, this nucleoside analogue appears to be the only clinically effective drug for pancreatic cancer (3). However, the median survival time of patients treated with gemcitabine is only 6.3 months (range: 1.6-19.2 months) (4). Intrinsic or acquired resistance of pancreatic cancer to apoptosis is an important factor in the failure of this treatment (5). Better understanding of the cellular and molecular mechanisms of gemcitabine resistance is required to allow this drug to be used more effectively.

There have been reports that selenoprotein P contributes to gemcitabine resistance (6), that apoptosis-regulating genes control tumor sensitivity to gemcitabine (5,7), and that deoxycytidine kinase (dCK) deficiency is responsible for gemcitabine resistance (8). However, there has been no comprehensive study on protein expression in tumors that have developed gemcitabine resistance. The combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) is powerful for high-throughput analysis of proteomic profiling of cancer.

In this study, we investigated the differential expression of proteins in a gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer cell line, and identified a protein participating in gemcitabine sensitivity. The protein was knocked down and we examined gemcitabine sensitivity. In addition we studied whether the protein can become a biomarker of gemcitabine sensitivity clinically with tumor specimens obtained by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA).

Materials and methods

Tumor cell lines and culture conditions. Two human pancreatic cancer cell lines, gemcitabine-sensitive KLM1 cells and gemcitabine-resistant KLM1-R cells, were kindly provided by the Department of Surgery and Science at Kyushyu University Graduate School of Medical Science. KLM1-R was established by exposing KLM1 cells to gemcitabine, as described previously (7). The tumor cells were cultured in RPMI-1640

Correspondence to: Dr Yasuhiro Kuramitsu, Department of Biochemistry and Functional Proteomics, Yamaguchi University School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi 755-8505, Japan
E-mail: climates@yamaguchi-u.ac.jp

Key words: pancreatic cancer, gemcitabine, proteomics, heat shock protein 27

medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FCS. All cells were kept in a water-saturated atmosphere containing 5% CO_2 at 37˚C and without endotoxin.

Cell proliferation assay. Cells were seeded onto 96-well plates at a density of 1,000 cells/well, incubated for 24 h, and exposed to different concentrations of gemcitabine for 72 h. After incubation with gemcitabine, 10 μl of a 5 mg/ml solution of MTT (3-[4,5-dimethythazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to each well and the plates were incubated for another 4 h. Then the formazan product was dissolved by adding 100 μl of DMSO and keeping it in the dark for 1 h to completely dissolve the crystals. Finally, the absorbance was measured at a wavelength of 570 nm with an ELISA plate reader (Model 550 Microplate Reader; Bio-Rad, Hercules, CA). Absorbance showed a linear relationship with the number of cells and each experiment was repeated three times.

Sample preparation. Suspensions of cultured cells were centrifuged at 1,500 rpm for 5 min. The pellet was washed three times with 10 mM PBS(-), pH 7.4, and then lysed in lysis buffer (1% NP-40, 1 mM sodium vanadate, 1 mM PMSF, 50 mM Tris, 10 mM NaF, 10 mM EDTA, 165 mM NaCl, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) at 4˚C for 1 h. The lysate was centrifuged at 15,000 x g for 30 min to obtain the supernatant, which was stored at -80˚C. Samples of the cell lines were prepared and stored three times each.

Two-dimensional gel electrophoresis (2-DE). Three hundred micrograms of protein was used for each 2-DE assay. First-dimension IEF was performed on 7-cm immobilized pH gradient strips with a linear pH gradient from 3 to 10 (GE Healthcare Bio-Science Corp., Piscataway, NJ) at 20˚C and 50 mA. Then the strips were rehydrated with 125 μl of sample solution (8 M urea, 2% CHAPS, and 0.5% IPG buffer) for 14 h. IEF was performed in three steps, which were 500 V for 1 h, 1,000 V for 1 h, and 8,000 V for 2 h. Voltage increases were carried out according to a gradient. The second-dimension was run on precast polyacrylamide gels (2-D homogeneous 12.5; GE Healthcare) in two steps (600 V, 20 mA for 30 min and 600 V, 50 mA for 70 min). After electrophoresis, the gels were stained with CBB R-250 (Nacalai Tesque, Kyoto, Japan) for 24 h. Subsequently, the gels were destained with 10% acetic acid in water containing 30% methanol for 30 min and then destained with 7% acetic acid and used for in-gel digestion.

Image analysis. The positions of the protein spots on the gels obtained using samples of KLM1 and KLM1-R cells were recorded with an Agfa ARCUS 1200 image scanner (Agfa-Gevaert N.V., Mortsel, Belgium) and were analyzed with Progenesis software (Progenesis PG240; Perkin-Elmer Inc., Wellesley, MA). Spots that showed at different intensities were excised from the gels and stored in 100 μl of ultrapure water at -80˚C as samples for MS analysis.

In-gel digestion. After cutting out the target protein spots from the gels, CBB dye was removed by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM DTT for 15 min, and twice in 50% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel pieces were dehydrated in 100% acetonitrile twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml of sequencing grade modified trypsin (Promega, Madison, WI) in 30% acetonitrile, 50 mM ammonium bicarbonate, and 5 mM DTT. In-gel digestion was performed overnight at 30˚C. The samples were rinsed in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT for 2 h and lyophilized overnight at -30˚C.

LC-MS/MS analysis. Lyophilized samples were dissolved in 20 ml of 0.1% formic acid and centrifuged at 15,000 x g for 5 min. Sequencing of the identified protein spots was performed by LC-MS/MS with a Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Santa Clara, CA).

Immunoblot analysis. Samples (30 μg) were separated by SDS-PAGE at 15 mA, and then transferred electrophoretically from the gels to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) and blocked overnight at 4˚C with TBS containing 5% skim milk. The primary antibody was an anti-heat shock protein (HSP) 27 monoclonal antibody (1:600, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with this antibody for 1 h at room temperature, washed three times with TBS containing 0.05% Tween-20 and once with TBS, then incubated for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibody (1:2000, ICN Pharmaceuticals, Aurora, OH). The reaction products were visualized with a chemiluminescence reagent (ECL Western blotting detection reagents; GE Healthcare).

Transfection with siRNA. KLM1-R cells were seeded in 6-well plates at a density of approximately 50% in 2.0 ml of complete medium. At 24 h after seeding, either specific HSP27 siRNA (Santa Cruz Biotechnology) or control siRNA (Santa Cruz Biotechnology) was added at a final concentration of 520 nM and incubation was performed for 30 h. Then the medium was exchanged for 2.0 ml of fresh growth medium containing 10% FCS and cells were incubated for 24 h. For the MTT assay, cells were trypsinized and transferred to 96-well plates. For protein extraction, cells were kept in the 6-well plates and used for Western blot analysis at 48 h after transfection. All experiments were repeated three times.

EUS-FNA. All procedures were carried out by one endosonographer using a linear endoscopic ultrasound (EUS) scanner (GF UCT240; Olympus Optical Co., Tokyo, Japan) with an EU-C2000 ultrasound platform (Olympus Optical Co.). EUS was performed to localize the pancreatic tumor and to obtain samples for histological diagnosis. Fine needle aspiration (FNA) was performed with a 22-gauge needle (EchoTip; Wilson-Cook Medical Inc., Winston-Salem, NC) under direct EUS guidance.

Immunohistochemistry. Formalin-fixed and paraffin-embedded samples were cut into 4-μm thick sections, which were treated with anti-HSP27 monoclonal antibody (1:200, Santa Cruz Biotechnology). Then avidin-biotin (Vector, Burlingame, CA) and 3,3-diaminobenzidine (Dojindo, Kumamoto, Japan) were used for detection.

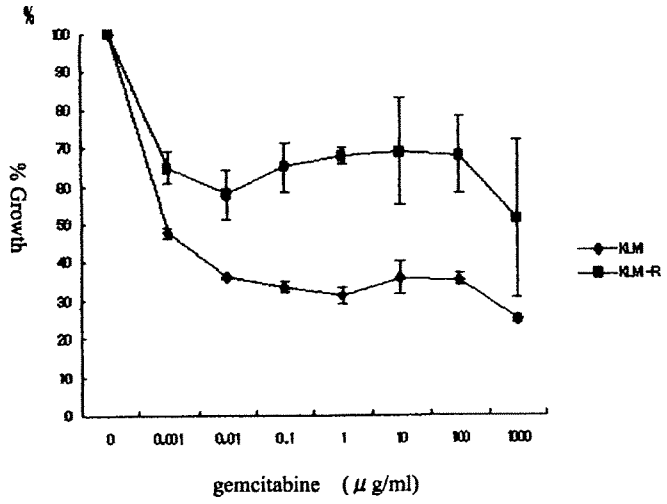

Figure 1. Cytotoxicity of gemcitabine against KLM1 and KLM1-R cells. KLM1 and KLM1-R cells were continuously exposed to various concentrations of gemcitabine for 72 h. KLM1-R cells were much less sensitive to gemcitabine.

Evaluation of HSP27 staining. HSP27 protein was stained brown in the cytoplasm of cancer cells. We counted the percentage of section with immunostaining signals of tumor in three different fields at a magnification of x400. We calculated the ratio of immunopositive area to cancerous area in three arbitrary fields of vision.

Results

Cytotoxicity of gemcitabine for KLM1 and KLM1-R cells. To evaluate the cytotoxicity of gemcitabine for KLM1 and KLM1-R cells, continuous exposure to various concentrations of the gemcitabine was performed for 72 h (Fig. 1). KLM1-R cells exhibited 2.2-fold (1 μg/ml gemcitabine) and 1.9-fold (10 μg/ml gemcitabine) greater viability compared with KLM1 cells ($p<0.05$), so the KLM1-R cell line was much less sensitive to gemcitabine.

Detection and identification of proteins. Protein expression was assessed using three samples each of KLM1 and KLM1-R cells cultured under the same conditions. More than 1,000 protein spots were visualized on the 2-DE gels. Differences in the intensity of these spots between KLM1 and KLM1-R were compared visually and analyzed with Progenesis PG240. As a result, 5 spots showed increased intensity on gels from KLM1-R cells (spots No. 1-5) and three spots showed decreased intensity (spots No. 6-8) (Fig. 2). The above-mentioned eight spots were excised from each gel, and identified by LC-MS/MS analysis. Information about the eight proteins thus identified is summarized in Table I. HSP27 was identified at three spots whose ratio was significantly different between the two cell lines. Expression comparisons of three spots of HSP27 by CBB dye are shown in Fig. 3.

Verification of protein expression by Western blot analysis. The expression of HSP27 was confirmed by immunoblot analysis, and it was shown to be up-regulated in KLM1-R cells (Fig. 3).

Effect of HSP27 on the response of KLM1-R cells to gemcitabine. To determine whether a decrease of HSP27 expression affected the sensitivity of pancreatic cancer cells to gemcitabine, we knocked down HSP27 using siRNA. When gemcitabine-resistant KLM1-R cells were treated with specific siRNA targeting HSP27, a significant reduction of HSP27 protein expression was detected compared with negative control siRNA-treated KLM1-R cells (Fig. 3). When normal KLM1 cells, normal KLM1-R cells, control KLM1-R cells (treated with control siRNA), and HSP27-silenced KLM1-R cells were exposed to various concentrations of gemcitabine for 72 h, the HSP27-silenced KLM1-R cells showed increased gemcitabine sensitivity (Fig. 4).

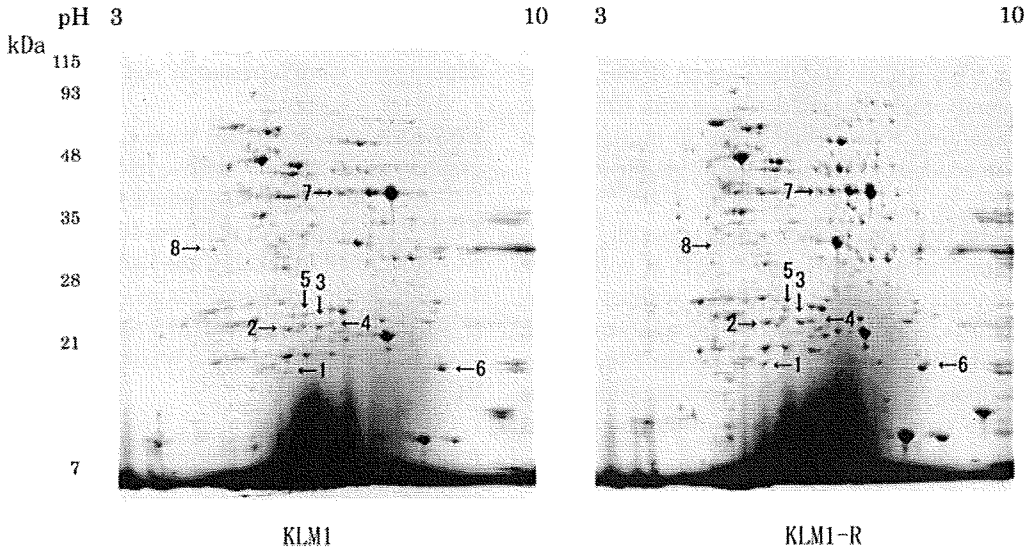

Figure 2. Two-dimensional gel images of KLM1 and KLM1-R cells. More than 1,000 protein spots were visualized on the 2-DE gels. Five spots showed increased intensity on gels from KLM1-R cells (spots No. 1-5) and three spots showed decreased intensity (spots No. 6-8).

Table I. Identification of proteins which are expressed differentially between KLM1 and KLM1-R.

Protein number[a]	Molecular mass (Da)	pI	Protein identification	Spot intensity[b] (% average ± SD)	p-value[c]
1	21892	5.66	Peroxiredoxin 2	2.69±0.69	0.013
2	22782	5.98	Heat shock protein 27	2.50±1.51	0.16
3	22482	5.98	Heat shock protein 27	2.13±1.00	0.12
4	28993	6.77	Endoplasmic reticulum protein ERp29 precursor	1.84±0.19	0.0014
	22782	5.98	Heat shock protein 27		
5	27547	5.70	6-phosphogluconolactonase	1.83±0.32	0.011
6	26538	6.51	Triosphosphate isomerase	0.40±0.068	0.0002
7	47038	6.99	α enolase	0.60±0.28	0.068
8	32575	4.64	Nucleophosmine	0.74±0.049	0.0008

[a]Spot numbers correspond to those in Fig. 2. [b]Percentage of spot intensitiy of KLM1-R to KLM1. [c]The differences in expression between KLM1and KLM1-R were analyzed by Student's t-test.

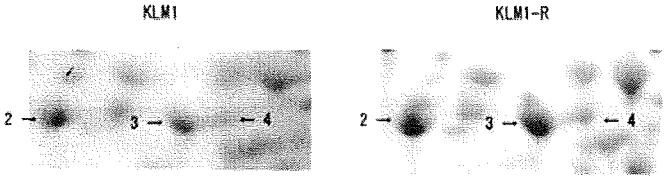

Figure 3. Comparison of spots of HSP27 between KLM1 and KLM1-R. The 2-DE pattern of KLM1 is on the left and that of KLM1-R is on the right. The spot numbers correspond to those in Fig. 2. HSP27 was shown to be up-regulated in KLM1-R cells compared with KLM1 cells.

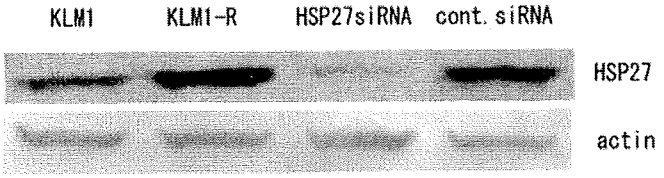

Figure 4. Immunoblotting of HSP27. HSP27 was shown to be up-regulated in KLM1-R cells compared with KLM1 cells. When gemcitabine-resistant KLM1-R cells were treated with specific siRNA targeting HSP27, a significant reduction of HSP27 protein expression was detected compared with negative control siRNA-treated KLM1-R cells and normal KLM1-R cells.

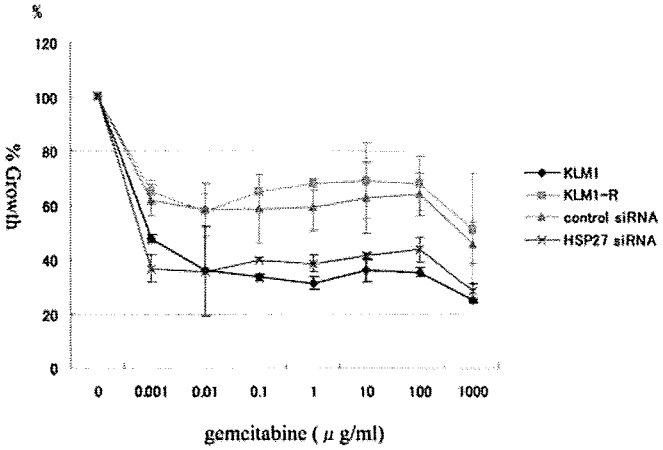

Figure 5. Sensitivity of HSP27-silenced KLM1-R cells to gemcitabine. The HSP27-silenced KLM1-R cells showed increased drug sensitivity as well as KLM1.

Immunohistochemistry of HSP27 in pancreatic cancer tissues and correlation with gemcitabine effects and survival. To evaluate the expression of HSP27 in clinical specimens, we performed immunohistochemical analysis of pancreatic cancer tissues that were obtained by EUS-FNA. Connective tissue was prominent in the specimens obtained by EUS-FNA. We observed the tumors under a microscope (x400) in three arbitrary fields of vision. The rate of HSP27-stained cancer area to total cancer area was calculated. The mean value in three views was calculated. The therapeutic gain of gemcitabine was judged according to guidelines of Response Evaluation Criteria in Splid Tumor (RECIST) (9). We examined the eleven patients who were diagnosed with EUS-FNA as having pancreatic cancer and treated with gemcitabine. Seven patients had progressive disease (PD) (Fig. 6a), and 4 patients had stable disease (SD) (Fig. 6b) in 11 patients. The PD group's positive ratio for HSP27 was higher than that of the SD group (p=0.0066) (Fig.6c). When the overall survival analysis of these patients was performed according to the HSP27 immunoreactivity, a shorter survival of pancreatic cancer patients correlated with high HSP27 expression (positive rate >30%) rather than with low HSP27 expression (positive rate <30%) (p=0.0025) (Fig. 6d).

Discussion

In the present study, proteomic analysis revealed that expression of HSP27 was increased in a gemcitabine-resistant pancreatic cancer cell line, while HSP27-silenced cells showed increased sensitivity to gemcitabine. These findings suggest that increase of expression of HSP27 by advanced pancreatic cancer might contribute to gemcitabine resistance, and silenced

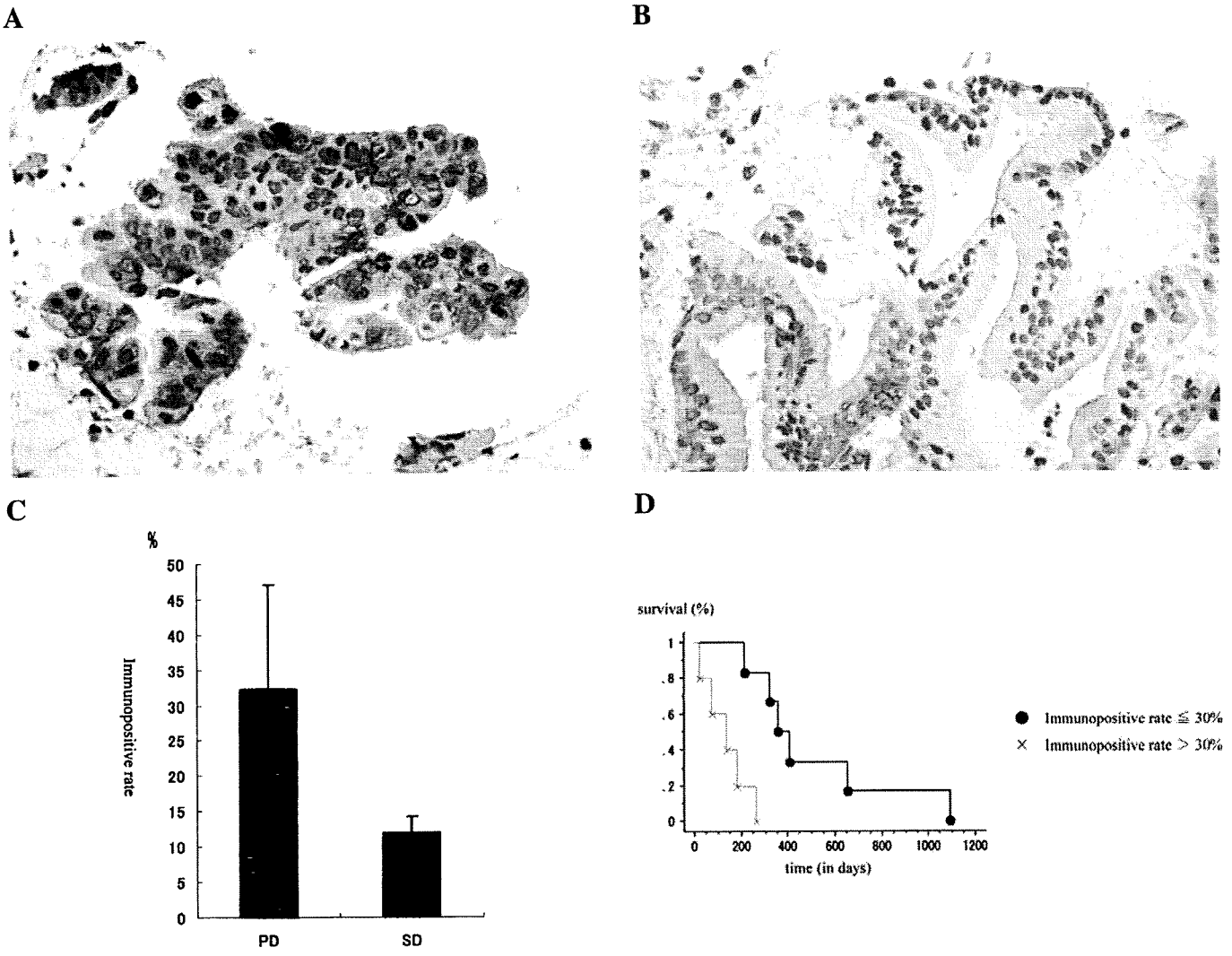

Figure 6. Immunohistochemistry of HSP27 in pancreatic cancer tissues and its correlation with survival rate of patients. (a) Tissue from a patient with progressive disease (PD). Fifty-one percent of the cancerous area was immunopositive (x400). (b) Tissue from a patient with stable disease (SD). Eleven percent of the cancerous area was immunopositive (x400). (c) The positive rate of HSP27 in PD and SD. The PD group's ratio of positive for HSP27 was higher than that of the SD group. (d) Immunohistochemistry positive rate of HSP27 and patient survival. Patients whose positive rate was >30% had a shorter survival than those with a rate <30%.

expression of HSP27 of gemcitabine-resistant pancreatic cancer might increase the gemcitabine sensitivity.

The mechanisms of gemcitabine resistance are still controversial, although many studies have been performed. The apoptosis-regulating proteins of the bcl-2 family and P-glycoprotein have been reported to have a role in resistance to chemotherapy (7,10-14), as well as various other proteins. However, none of these proteins showed any difference between gemcitabin-sensitive and -resistant tumor cells in our proteomic analysis. One possible reason for this may be that we used a cell line with acquired gemcitabine resistance rather than intrinsic resistance, while the other reason would be the limitations of 2DE. However, we found that gemcitabine-resistant cells showed increased expression of 4 other proteins in addition to HSP27 and decreased expression of 3 proteins, suggesting that various proteins may participate in gemcitabine resistance as well as HSP27. By knocking down HSP27 using siRNA, the gemcitabine sensitivity of pancreatic cancer cells was increased, confirming that HSP27 has a role in gemcitabine resistance.

HSP27 belongs to the family of small heat shock proteins, which are molecular chaperones that modulate the ability of cells to respond to several types of injury and are expressed in virtually all organisms from prokaryotes to mammals (15). Evidence has been obtained that HSP27 regulates apoptosis by interacting with key components of the apoptotic signaling pathway (16). HSP27 inhibits etoposide-induced apoptosis by preventing cytochrome c and dATP-triggered activation of caspase-9, which occurs downstream of cytochrome c release (17,18). Increased expression of antiapoptotic factor enhances the resistance of tumor cells to chemotherapy. Thus, the overexpression of HSP27 inhibits doxorubicin-induced apoptosis of human breast cancer cells (19), as well as apoptosis of prostate cancer cells induced by etoposide, diethyl-maleate, cycloheximide, or radiation (20), and etoposide-induced apoptosis of neuroblastoma cells (21). In the present study, HSP27 was shown to be overexpressed by KLM1-R cells, as is the case with the above-mentioned cancers. It is suggested that pancreatic cancer develops resistance through the antiapoptotic action of HSP27, and that

this is an important component of resistance to gemcitabine. In fact, HSP27-silenced KLM1-R cells showed an increase of sensitivity to gemcitabine, which reached the same level as that of parental KLM1 cells.

EUS-FNA has come into widespread use, mainly in Western countries, as an efficient and safe method for the cytologic or histologic diagnosis of pancreatic cancer (22-26). Although EUS-FNA is only employed to make a histological diagnosis of pancreatic cancer at present, it may also contribute to tailor-made medicine in future by evaluating gemcitabine sensitivity. We can possibly expect an improved response to gemcitabine by combining it with a method of reducing HSP27 expression in pancreatic cancer.

Proteomic analysis was useful for finding intracellular proteins with differential expression between pancreatic adenocarcinoma cell lines showing sensitivity and resistance to gemcitabine. HSP27 may be involved in the mechanism of resistance to gemcitabine, and it could also be a possible biomarker for predicting the response of pancreatic cancer to treatment.

Acknowledgements

This project was supported by the Pancreas Research Foundation of Japan.

References

1. Williamson RC: Pancreatic cancer: the greatest oncological challenge. Br Med J (Clin Res Ed) 296: 445-446, 1988.
2. Gordis L and Gold EB: Epidemiology of pancreatic cancer. World J Surg 8: 808-821, 1984.
3. Burris HA, Moore MJ III, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD and Von Hoff DD: Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 15: 2403-2413, 1997.
4. Carmichael J, Fink U, Russell RC, Spittle MF, Harris AL, Spiessi G and Blatter J: Phase II study of gemcitabine in patients with advanced pancreatic cancer. Br J Cancer 73: 101-105, 1996.
5. Shi X, Liu S, Kleeff J, Friess H and Buchler MW: Acquired resistance of pancreatic cancer cells towards 5-Fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. Oncology 62: 354-362, 2002.
6. Maehara S, Tanaka S, Shimada M, Shirabe K, Saito Y, Takahashi K and Maehara Y: Selenoprotein P, as a predictor for evaluating gemcitabine resistance in human pancreatic cancer cells. Int J Cancer 112: 184-189, 2004.
7. Xu Z, Friess H, Solioz M, Aebi S, Korc M, Kleeff J and Buchler MW: Bcl-x(L) antisense oligonucleotides induce apoptosis and increase sensitivity of pancreatic cancer cells to gemcitabine. Int J Cancer 94: 268-274, 2001.
8. Ruiz van Haperen VW, Veerman G, Eriksson S, Boven E, Stegmann AP, Hermsen M, Vermorken JB, Pinedo HM and Peters GJ: Development and molecular characterization of a 2',2'-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res 54: 4138-4143, 1994.
9. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC and Gwyther SG: New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 92: 205-216, 2000.
10. Bold RJ, Chandra J and McConkey DJ: Gemcitabine-induced programmed cell death (apoptosis) of human pancreatic carcinoma is determined by Bcl-2 content. Ann Surg Oncol 6: 279-285, 1999.
11. Friess H, Lu Z, Graber HU, Zimmermann A, Adler G, Korc M, Schmid RM and Buchler MW: bax, but not bcl-2, influences the prognosis of human pancreatic cancer. Gut 43: 414-421, 1998.
12. Chang GC, Hsu SL, Tsai JR, Wu WJ, Chen CY and Sheu GT: Extracellular signal-regulated kinase activation and Bcl-2 downregulation mediate apoptosis after gemcitabine treatment partly via a p53-independent pathway. Eur J Pharmacol 502: 169-183, 2004.
13. Erkan M, Kleeff J, Esposito I, Giese T, Ketterer K, Buchler MW, Giese NA and Friess H: Loss of BNIP3 expression is a late event in pancreatic cancer contributing to chemoresistance and worsened prognosis. Oncogene 24: 4421-4432, 2005.
14. Bergman AM, Pinedo HM, Talianidis I, Veerman G, Loves WJ, van der Wilt CL and Peters GJ: Increased sensitivity to gemcitabine of P-glycoprotein and multidrug resistance-associated protein-overexpressing human cancer cell lines. Br J Cancer 88: 1963-1970, 2003.
15. Arrigo AP: Hsp27: novel regulator of intracellular redox state. IUBMB Life 52: 303-307, 2001.
16. Concannon CG, Gorman AM and Samali A: On the role of Hsp27 in regulating apoptosis. Apoptosis 8: 61-70, 2003.
17. Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP and Solary E: HSP27 inhibits cytochrome c-dependent activation of procaspase-9. FASEB J 13: 2061-2070, 1999.
18. Samali A, Robertson JD, Peterson E, Manero F, van Zeijl L, Paul C, Cotgreave IA, Arrigo AP and Orrenius S: Hsp27 protects mitochondria of thermotolerant cells against apoptotic stimuli. Cell Stress Chaperones 6: 49-58, 2001.
19. Hansen RK, Parra I, Lemieux P, Oesterreich S, Hilsenbeck SG and Fuqua SA: Hsp27 overexpression inhibits doxorubicin-induced apoptosis in human breast cancer cells. Breast Cancer Res Treat 56: 187-196, 1999.
20. Gibbons NB, Watson RW, Coffey RN, Brady HP and Fitzpatrick JM: Heat-shock proteins inhibit induction of prostate cancer cell apoptosis. Prostate 45: 58-65, 2000.
21. Urbani A, Poland J, Bernardini S, Bellincampi L, Biroccio A, Schnolzer M, Sinha P and Federici G: A proteomic investigation into etoposide chemo-resistance of neuroblastoma cell lines. Proteomics 5: 796-804, 2005.
22. Chang KJ, Nguyen P, Erickson RA, Durbin TE and Katz KD: The clinical utility of endoscopic ultrasound-guided fine-needle aspiration in the diagnosis and staging of pancreatic carcinoma. Gastrointest Endosc 45: 387-393, 1997.
23. Gress FG, Hawes RH, Savides TJ, Ikenberry SO and Lehman GA: Endoscopic ultrasound-guided fine-needle aspiration biopsy using linear array and radial scanning endosonography. Gastrointest Endosc 45: 243-250, 1997.
24. Bhutani MS, Hawes RH, Baron PL, Sanders-Cliette A, van Velse A, Osborne JF and Hoffman BJ: Endoscopic ultrasound guided fine needle aspiration of malignant pancreatic lesions. Endoscopy 29: 854-858, 1997.
25. Fritscher-Ravens A, Sriram PV, Bobrowski C, Pforte A, Topalidis T, Krause C, Jaeckle S, Thonke F and Soehendra N: Mediastinal lymphadenopathy in patients with or without previous malignancy: EUS-FNA-based differential cytodiagnosis in 153 patients. Am J Gastroenterol 95: 2278-2284, 2000.
26. Ryozawa S, Kitoh H, Gondo T, Urayama N, Yamashita H, Ozawa H, Yanai H and Okita K: Usefulness of endoscopic ultrasound-guided fine-needle aspiration biopsy for the diagnosis of pancreatic cancer. J Gastroenterol 40: 907-911, 2005.