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Review

Proteome analysis of Epstein–Barr virus-transformed B-lymphoblasts and the proteome database

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Abstract

The proteome is the entire protein complement of the genome expressed in a particular cell, tissue, or organism at a given time under a specific set of environmental conditions. Proteomics is a combinatorial methodology to comprehensively analyze the proteome. The general protocol of the expression proteomics consists of advanced methods of high-resolution protein separation, high-quality image analysis and high-throughput protein identification. Although Epstein–Barr virus-transformed B-lymphoblastoid cell lines (LCLs) have long been believed to be immortalized, recent studies have provided ample evidence that a large proportion of LCLs have limited life spans due to shortening of telomeres, and that part of them are truly immortalized by developing strong telomerase activity to maintain telomeres. Differential proteome analysis of pre- and post-immortal LCLs would provide a powerful tool to analyze proteins participating in the process of immortalization. We focus in this review on cumulative data of proteomic information on pre- and post-immortal LCLs.

Keywords: Reviews; Proteome databases; Epstein–Barr virus; B-lymphoblasts

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1. Introduction

The term proteome is defined as the entire protein complement expressed by a genome, or by a cell or tissue type. Thus proteomics, the method to investigate proteome, gives supplementary information of, and more diverse information than, genomics. Expression proteomics [1,2] exactly follows the initial object of the original proteomics. Comparative proteomics [3,4] or differential expression proteomics [5] corresponds to the differential display of mRNA that is useful to investigate suspected proteins causing a specific cell behavior. Structural analysis by nuclear magnetic resonance or X-ray diffraction, and computer simulation of functional domains of protein molecules, will be included in the most advanced functional proteomics [6]. Among these various types of proteomics, comparative proteomics is considered to be the most appropriate for research on mechanisms of biological and pathological processes such as immortalization of a specific cell type, because we can analyze proteins participating in immortalization by comparing protein profiles between pre- and post-immortal cells.

In the general procedure of comparative proteomics, proteins in a complex mixture are separated by high-resolution two-dimensional gel electrophoresis (2-DE). Protein spots on a set of 2-DE patterns are arranged to be matched, and the quantitative or qualitative, or both, differences of protein spots among the 2-DE patterns are shown by computer-aided image processing. Proteins with a marked difference are subsequently subjected to in-gel digestion and to mass spectrometry by using mass fingerprinting to identify them [7]. The comprehensive identification of proteins by peptide mass fingerprinting in proteomics began with Williams and his students in 1995 [8]. The term proteome was invented by Wilkins when he was a graduate student of Williams. Before proteomics, molecular mass search of peptide fragments to identify proteins was reported by Henzel and his coworkers in 1993 [9]. Henzel's group originally generated a database of peptide fragments from a latest version of protein sequence databases to achieve the efficient identification. They also developed their original software to find the most homologous proteins from the peptide fragment database. Their method of protein identification used simultaneous processing of a large number of 2-DE spots.

The use of the immobilized pH gradient (IPG) in first dimensional isoelectric focusing (IEF) has also contributed to a sudden extensive spread of proteomics. Görg et al. [10] originally reported the application of IPG to IEF for 2-DE. The combination of IPG–IEF and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) improved the resolution and reproducibility of 2-DE. The commercial release of excellent software to process 2-DE images also has pushed us forward to perform comparative proteomics in our laboratory.

Immortalization of cells to attain an infinite proliferating ability is an important characteristic of malignant cells. Thus, the proliferative transformation and succeeding immortalization of mammalian cells after the infection are attractive subjects in the cellular physiology and pathology of carcinogenesis by tumor viruses. Indeed, many clinical research scientists have focused on these fields.

In clinical investigation, many tumorigenic viruses, such as mammary tumor viruses, papilloma viruses and human T-cell leukemia viruses, have been isolated, and the viral genes causing the proliferative transformation and immortalization have been investigated. Among them, Epstein–Barr virus (EBV) is a typical B-lymphotropic herpesvirus widespread in human populations. EBV infection usually occurs in early childhood and causes a wide spectrum of diseases. EBV was isolated initially from a cultured cell line of Burkitt lymphoma, and has been thought to etiologically link to the development of Burkitt lymphoma [11]. EBV is also implicated in a variety of other diseases, such as X-linked lymphoproliferative syndromes, AIDS- and transplant-related B-cell lymphomas, T-cell non-Hodgkin's lymphoma, Hodgkin's disease, and natural killer-cell granular lymphoproliferative disorder. Recently, lymphoepithelial carcinoma of digestive organs and smooth muscle tumors were also recognized as EBV-associated diseases.

The complete sequence of the genome of a typical EBV strain has been submitted to GenBank as Accession number NC_001345. The major genes that encode elements causing transformation such as

latent membrane protein 1 (LMP1) [12–14] and EBV nuclear antigen 2 (EBNA2) [15], have been assigned to the coding regions of the viral genome.

EBV infection is associated with both benign and malignant tumors, and the direct effect of viral gene products is suggested to be insufficient to induce the complete phenotypes of immortalization in the transformed cells [16]. Many groups have reported their evidence suggesting participation of host gene expression in the EBV-associated B-cell immortalization [17,18]. Recent data of our research group and others have also indicated that EBV transformation and the succeeding immortalization are closely related but distinct events [19–24]. Sugimoto et al. [20,21] reviewed data on cellular senescence and immortalization of human lymphoblastoid cell lines transformed by EBV. Their data provided strong evidence that the majority of EBV-transformed LCLs (85% of total LCLs) finish their life spans by shortening the telomers, while only a small proportion of LCLs (15%) were immortalized accompanied by a marked increase in telomerase activity to maintain telomere length. Some pre-immortal LCLs have unusually long life spans, but they all die till 160 population doubling levels (PDLs). Another noteworthy difference between pre- and post-immortalized LCLs is with the karyotypes: most pre-immortal LCLs have normal karyotypes whereas all eight immortalized LCLs thus far examined have clonally abnormal karyotypes [22–24]. Post-immortal LCL cells may show proteome profiles distinct from those of pre-immortal LCL cells for proteins other than telomerase. Thus, we formed the working hypothesis that comparative analyses of proteome profiles of EBV-transformed LCLs before and after immortalization gives us novel information about the cellular mechanisms of virus-induced immortalization.

2. Genomics of EBV and proteomics of EBV-transformed LCLs

Approximately 40–50% of cases of Hodgkin's disease in Western populations are associated with EBV. Transformation induced by EBV may have a major role in the pathogenesis of Hodgkin's disease by activating anti-apoptotic factors in pre-malignant germinal B-lymphocytes [25]. In the majority of malignant cells of Hodgkin's disease tumors, the increased expression of the gene of tumor necrosis factor receptor-associated factor 1 was observed in EBV-positive tumors [26].

Among cases of Burkitt's lymphoma, EBV association varies geographically. For example, EBV DNA was detected in 47% of cases from Argentina and 80% of cases from India by in situ hybridization by using the RNA probe of EBV-encoded nuclear RNAs-1 (*EBER-1*). The complete coding sequence of genomic DNA of a sub-strain of EBV has been submitted to the GenBank Database as Accession number NC_001345 (http://www.ncbi.nlm.nih.gov:80/entrez/viewer.fcgi?val=NC_001345).

EBV DNA encodes several functional proteins, including EBV-encoded nuclear antigen (*EBNA*)*1–6* and latent membrane protein (*LMP*)*1, 2*. Recent studies have shown that *EBNA1, 2, 5, 3A* and *3C* work cooperatively in transformation. In Burkitt's lymphoma-type EBV infection, *EBNA1*, *EBER*, *BARF0* and *LMP2* are expressed, but *EBNA2* nor *LMP1* are not observed [27,28]. Komano et al. [29] concluded that *EBER*s produce the phenotypes of transformed cells. In all cases of BL cells, a chromosomal translocation that includes the *c-myc* locus results in constitutive activation of the *c-myc* gene [30]. In mammalian cells, overexpression of *c-myc* contributes not only to tumorigenesis [31] but also induces apoptosis [32–34]. EBV transformation upregulates the expression of *bcl-2* to protect cells from *c-myc*-induced apoptosis [11].

In recent studies on the genomics of EBV, target genes of *EBNA2* were isolated and characterized by a subtractive cDNA cloning [35]. The proteins that cause immortalization after EBV transformation remain to be clarified. To discover them, the study of proteomic profiling of LCL cells before and after immortalization is a very effective method.

Poirier et al. [36] constructed a proteome database of Burkitt lymphoma cell line DG75, and reported the changes of protein profiles after treatment with 5′-azycytidine The cell line was judged to be immortalized before starting their experiment because it had long been maintained by subculture. The lym-

phoma cell line was apparently EBV-negative, suggesting that the EBV-gene expression was not necessarily required to maintain the immortalized state; some unknown cellular processes may have contributed the immortalization. Various cytokines are induced by EBV infection [37,38], and may have an important role in immortalization. These facts suggest that modification of the host gene expression contributes to immortalization of EBV-transformed LCLs.

The proteome database prepared by Poirier et al. [36] did not contain sufficient information about the protein factors in the process of immortalization. Our proteome database of EBV-transformed human LCLs in the TMIG-2DPAGE web site (http://proteome.tmig.or.jp/2D/) was prepared to gather information about proteins contributing to the mechanisms of immortalization of these cell lines.

3. Proteome analysis of EBV-transformed LCLs

3.1. Background

The recent progress in proteomics is indebted to both the high-resolution protein mapping by 2-DE and the high-throughput protein identification by mass spectrometry. Separation techniques other than 2-DE, such as high-pressure liquid chromatography [39,40], capillary zone electrophoresis [41], surface interaction micro-fluidics [42], are also used in proteomics. However, no other separation method has achieved the highest resolution by 2-DE.

EBV-transformed LCLs have been widely used for various researches, such as production of human monoclonal antibody and studies on differentiation and carcinogenic transformation of B-cells. LCLs are mostly mortal in nature at the early passages after transformation, and only part of them are immortalized after long term sub-cultivation [19–24]. Thus, immortalization of LCLs is closely related to, but distinct from, the transformation by EBV. Thus we started to analyze alterations of protein profiles accompanied by immortalization EBV-transformed LCLs.

Sugimoto and co-workers [19–24] made a series of experiments to establish immortalized LCLs collected from the peripheral blood of 58 normal individuals and 50 Werner syndrome patients. Although no immortalized cell line was established from LCLs from Werner syndrome patients, eight immortalized LCLs were established. Among them, we chose the immortalized LCLs N0003, N0005 and N6803 and their pre-immortal counterpart LCLs for a proteome analysis [24]. All the immortalized LCLs developed strong telomerase activity, maintained telomere length and showed clonally abnormal karyotypes, and are still actively proliferating over 300 PDLs today. Table 1 shows the characteristics of the three LCLs. They secreted IgM (N0005), IgG (N6803) or IgA (N0003) into the medium at the early passages between 17 and 20. The N6803 and N0003 cell lines continued to secrete each class of immunoglobulin at decreased levels, but the N0005 cell line secreted almost no immunoglobulin after the immortalization. Karyotype analysis of the immortalized cell lines showed that they were derived from a single cell because they shared a set of abnormal chromosomes within each cell population (Table 2).

We then studied proteome profiles of the three pairs of pre- and post-immortal LCLs [43]. In the conventional method of 2-DE established by O'Far-

Table 1
Immunoglobulin secretion by the B-lymphoblastoid cell lines

Cell line	PDLs	IgM	IgG				IgA	
			1	2	3	4	1	2
N0003	17	−	−	−	−	−	++	+
	151	−	−	−	−	−	++	−
N0005	17	+++	−	−	−	−	−	−
	151	−	−	−	−	−	−	−
N6803	14	−	+++	−	−	−	−	−
	210	−	+	−	−	−	−	−

The data are cited from Ref. [23].

Table 2
Karyotypes of immortalized B-lymphoblastoid cell lines

Cell line	PDLs[a]	Type	Chromosomal analysis
N0003	120	1	45, XX,der(13;14)(q10;q10)
		2	and tas
		3	and r(7)(p22q36)
		4	and del(20(q32), add(19)(p13.3)
N0005	120	1	46, XY, der(3)t(1;3)(q23);p25),der(8)t(1;8)(q23;q24.3)
		2	and +12
		3	and +mar
N6803	14	1	46, XY (normal)
		2	and add(16)(q24)
		3	and +12
N6803	72	1	47, XY, +2, add(3)(q27;q13)
		2	and del(1)(q11)
N6803	179	1	47, XY, +2, add(3)(q27;q13)
		2	and +8

[a] PDLs at the time of analysis. The data are cited from Ref. [23].

rell [44], IEF was done on a gel column, in which the pH gradient was generated with carrier ampholytes in a field of electric potential. The pH gradient was not stable enough to assure the reproducibility of electro-focusing, because the carrier ampholytes were still mobile in the gel column. Furthermore, a special skill was required to handle the fragile gel column. In the advanced method of 2-DE, IEF is done on a gel strip, which may maintain an IPG on a plastic film backing. The immobilized pH gradient has allowed us to reproduce excellent separation of proteins by the advanced method of 2-DE. Details of the method of 2-DE in our proteome analysis are given in the TMIG-2DPAGE home page at the URL: http://proteome.tmig.or.jp/2D/.

3.2. 2-D gel electrophoresis of EBV-transformed cells

3.2.1. Sample preparation

The reproducibility of proteome profiling by 2-DE depends largely on the quality control of the sample preparation. In the case of tumor marker surveillance by proteome analysis, for example, contamination of different cell types in the tumor tissue specimen to be analyzed interferes with making correct conclusions of tumor cell-specific protein expressions. Micro-dissection might be effective to isolate tumor cells from surrounding normal tissues. Quality control of in vitro transformed cells is much easier than of clinical tissue samples. Therefore, we chose EBV-transformed LCLs to analyze proteins participating in immortalization.

The preparation of EBV-transformed LCLs and passage of LCL cells of N6803, N0003 and N0005 were reported previously [22,23]. The procedure of the succeeding protein extraction is also very important for the reproducibility. The most efficient buffer for protein solubilization should be used, especially in the quantitative analysis of differences between cell preparations in a set. Although the stepwise extraction may have advantage over the single step extraction in efficacy especially for membrane protein analysis, it has disadvantage in the quality control of protein to make a reliable comparison of proteome profiles of many samples quantitatively. Thus, a single step extraction was used.

Cultured cells were harvested by centrifugation at 2000 rpm, and were suspended in a phosphate-buffered saline (PBS) to remove serum protein. The cells were washed three times and were suspended again in a small volume of PBS. The cell suspension was transferred to a microfuge tube, which had been weighted before use. The cell volume was determined roughly by weighting the cell pellet in the tube, and cells were suspended in three volumes of cell-lysis buffer (8 *M* urea, 3% (v/v) 2-mercaptoethanol, 0.2% (w/v) SDS, 0.8% (v/v) Triton X-

100). The cells were disrupted by ultrasonication, and the supernatant was removed by centrifugation.

3.2.2. First-dimensional IPG–IEF

In our proteomic profiling of LCL cells, the IEF was done on an IPG. Immobiline DryStrip (pH 4–7, 18 cm long) purchased from Amersham Bioscience was re-hydrated in the buffer (8 *M* urea, 3% (v/v) 2-mercaptoethanol, 0.2% (w/v) SDS, 0.8% (v/v) Triton X-100) overnight at 20 °C. A small piece of filter paper to absorb the protein sample solution was put on the re-hydrated gel strip near the end of the cathode side. Electrofocusing was done under the program of a stepwise voltage increase: 500 V for 2 h, 700 V for 1 h, 1000 V for 1 h, 1500 V for 1 h, 2000 V for 1 h, 2500 V for 1 h, 3000 V for 1 h, and 3500 V for 8 h.

3.2.3. Treatment of IPG–IEF gel strip

After finishing the IPG–IEF, the gel strip was incubated in the buffer for SDS treatment and reduction of protein disulfide bridges (50 m*M* Tris–HCl, pH 6.8, 6 *M* urea, 0.5% 1,4-dithiothreitol) for 30 min. The strip was incubated in an alkylating reagent (50 m*M* Tris–HCl, pH 6.8, 6 *M* urea, 4.5% iodoacetamide) for 10 min to block the free SH groups. The reductive alkylation is not necessary if the gel is applied to the comparative image analysis alone. The alkylation is indispensable for proteins when they are analyzed by a succeeding peptide mass fingerprinting.

3.2.4. Second-dimensional SDS–PAGE

After the SDS treatment and reductive alkylation, the IPG–IEG gel strip was placed on top of a 7.5% T, 5% C polyacrylamide gel slab (19×18 cm, 1 mm thick), and was fixed by pressing with a shark's tooth comb. The second-dimensional SDS–PAGE was done under a constant current at 40 mA/gel slab. After reaching the dye front near the bottom of the gel slab, protein spots were visualized by staining with silver, Coomassie Brilliant Blue or Cypro Ruby, depending on the purpose of the gel to be run.

3.3. Differential image analysis

The resolution of protein spots on a 2-D gel image is too fine to find by eye differences in a large number of spots on many sheets of gel. Computer-aided image analysis using an effective software, such as PDQuest, is helpful to detect spots by eliminating noise and background, quantify spots in relative abundance, match spots among a set of gel plates, and find distinct differences above a given threshold level.

In our first series of experiment [43], we compared protein spots detected by silver-staining of the 2-DE images for three pairs of pre- and post-immortal LCLs. The spots were acquired by scanning and were analyzed by computer-aided imaging. Fig. 1 summarized some of the results of the differential image. We found no spot that behaved commonly in all three lines before or after immortalization, but the standard spot protein (ssp) 7001 showed a marked decrease after immortalization in N0003 and N0005. This spot, however, was extremely down regulated in both pre- and post-immortal N6803 LCLs. These facts suggested that the down regulation of ssp7001 protein participated in the process of immortalization, and that a part of immortalization process is already started in pre-immortal N6803 LCL. The ssp7001 protein was identified as stathmin by Edman degradation peptide sequencing. Other results of the image analysis including minor alterations, which are not referred in this review, are given on our web site.

We analyzed the same three pairs of LCLs for a sheet of Cypro Ruby-stained gels: 32 spots, including eight spot proteins that changed relatively more with immortalization, were excised out, and were then digested in the gel for peptide mass fingerprinting as mentioned in Section 3.4.

3.4. Protein identification

The recent development in the method of high-throughput protein identification by peptide mass fingerprinting is another important event in the progress of proteomics. In the early days of biomedical applications of 2-DE, which was initiated by O'Farrell [44], proteins were identified by an Edman degradation peptide sequencing. Western blotting was also used to identify proteins speculated of being relevant from their physico-chemical properties and by using specific antibodies. Edman degradation peptide sequencing has been a sure method for

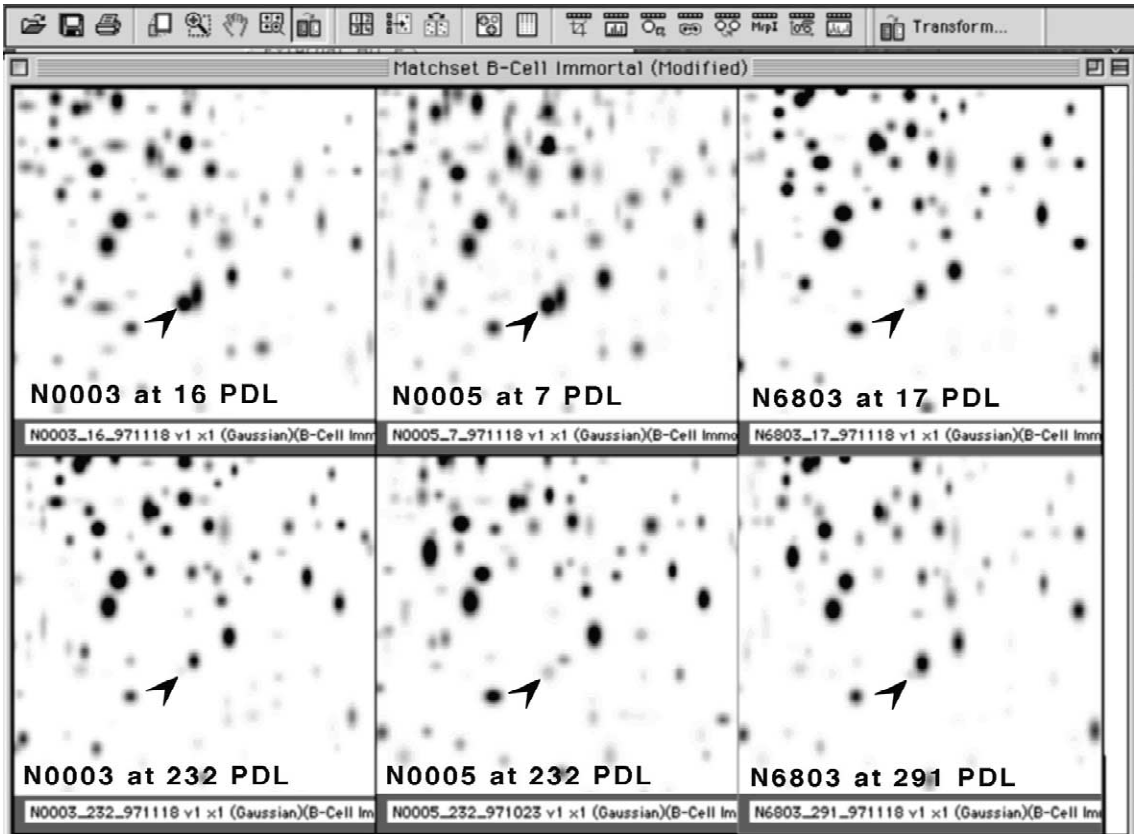

Fig. 1. 2-D gel protein maps in a local area of 2-D gel plate in three lines of pre- and post-immortalized EBV-transformed LCLs. The ssp7001 protein (arrow head) showed extremely low levels of expression in all immortalized cells (lower frames). The pre-immortal LCL N6803 might have partly shifted to an immortal cell.

protein identification. However, N-terminal deblocking or isolation of an internal peptide is required, because the N-terminal amino acid is blocked in most proteins separated by 2-DE. Peptide mass fingerprinting has been recently used for protein identification in advanced proteomics, not only because it is superior to Edman degradation sequencing in its sensitivity, but also it does not require isolation of a single peptide after proteolytic digestion, which is the major reason why peptide mass fingerprinting is preferred in advanced proteomics. When peptide mass fingerprinting is not accurate enough to identify a protein, the protein can be identified if information about the peptide sequence is obtained by post-source decay (PSD)–mass spectrometry (MS)–MS or collision-induced dissociation (CID)–MS–MS analysis.

For the 32 spots in a sheet of Cypro Ruby-stained gel, peptide masses obtained by tryptic digestion were analyzed by MALDI–TOF–MS. As a result of a database search by consulting with the peptide mass fingerprint data, 20 proteins were assigned to known proteins (Fig. 2). The up-regulation of proliferating cell nuclear antigen (PCNA) and nucleoside diphosphate kinase (NDPK)-A might be the result of growth stimulation in immortalized cells. The up regulation of ubiquitin c-terminal hydrolase and the down regulation of ubiquitin ligase N are interesting, and suggests a change in proteasome function after immortalization. Down regulation of stathmin sug-

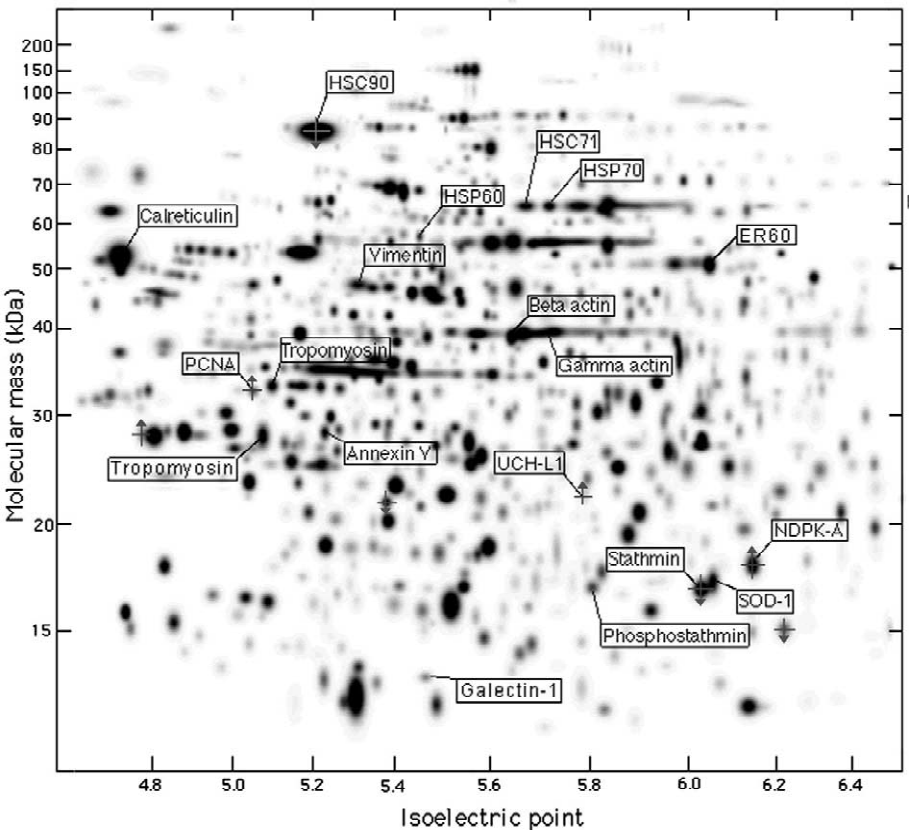

Fig. 2. Identified proteins on the 2-D gel protein map of EBV-transformed human B-lymphoblastoid cells. The ssp7001 protein was assigned to stathmin on the map. The upward and downward arrows indicate increase and decrease of the protein with immortalization, respectively.

gests a possible change in stability of microtubules in immortalization.

3.5. Proteome database of EBV-transformed LCLs

Because EBV-transformed LCLs are useful in various biomedicine, freely accessible proteome databases of researchers are expected. Poirier et al. [36] reported the 2-DE protein database of Burkitt lymphoma. Joubert-Caron et al. [45] reported data of the proteomic analysis of human cell line PRI, which is a model of in vitro EBV-transformed LCLs; their 2-DE protein map with annotations are accessible at URL: http://www-smbh.univ-paris13.fr/lbtp/index-.htm. However, their data are insufficient to study molecular mechanisms of immortalization, because they only analyzed the proteome of post-immortal LCLs. We have also constructed a proteome map accessible with annotations on our TMIG-2DPAGE server at URL: http://proteome.tmig.or.jp/2D/. In the database, we intend to show not only static information, such as protein identification and physico-chemical properties, but also the changes in protein expression during immortalization.

All data obtained in the research on immortalization of EBV-transformed LCLs, are also saved in our local server computer. Only some of the data from our research have been referred to in our previous paper [43]. Important information may be hidden in our data base, and should be obtained from the database in the future by linkage with other information. So we decided to make available our unpublished data in our web home page. The main frame of our TMIG-2DPAGE proteome database is a clickable 2-DE image map, which is linked to related data files and external web sites. We have already

established our original proteome database about age-related protein alterations in normal human diploid fibroblasts. The novel 2-DE protein map of EBV-transformed LCLs has been also linked to the proteome database. Many other databases constructed on local server computers by many other research groups independently, should be linked to each other to form a unit of global bioinformatics. The Human Proteome Organization (HUPO) is expected to have an early role in standardizing a consensus structure of a proteome database to avoid serious failure to include important data from the linkage in the global biomedical information network.

4. Conclusions

Differential proteome analysis of pre- and post-immortal LCL cells transformed by EBV provides a powerful tool to chase proteins participating in the process of immortalization. We actually found out that several non-viral proteins underwent down- and up-regulation according to the immortalization of LCL cells by the differential proteome analysis.

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