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Quantitative Proteomic Analysis of Human Breast Epithelial Cells with Differential Telomere Length

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Abstract

Telomeres play important functional roles in cell proliferation, cell cycle regulation, and genetic stability, in which telomere length is critical. In this study, quantitative proteome comparisons for the human breast epithelial cells with short and long telomeres (184-hTERT_L vs. 184-hTERT_S and 90P-hTERT_L vs. 90P-hTERT_S), resulted from transfection of the human telomerase reverse transcriptase (hTERT) gene, were performed using cleavable isotope-coded affinity tags. More than 2000 proteins were quantified in each comparative experiment, with approximately 77% of the proteins identified in both analyses. In the cells with long telomeres, significant and consistent alterations were observed in metabolism (amino acid, nucleotide, and lipid metabolism), genetic information transmission (transcription and translation regulation, spliceosome and ribosome complexes) and cell signaling. Interestingly, the DNA excision repair pathway is enhanced, while integrin and its ligands are downregulated in the cells with long telomeres. These results may provide valuable information related to telomere functions.

Keywords

Breast epithelial cells; ICAT; Mass spectrometry; Quantitative proteomics; Telomere length

Telomeres, the ends of eukaryotic chromosomes, play important functional roles in cell proliferation and cell cycle regulation [1]. Telomere dysfunction increases genomic instability, which leads to accelerated aging and initiation of cancer [2]. In most cancers, telomere length is maintained by telomerase, an enzyme that synthesizes telomere repeat sequences T₂AG₃ to replace those that are lost during DNA replication [3]. However, telomere length can be stabilized without detectable changes in telomerase activity [4]. Studies have shown that short telomeres, even in the presence of telomerase, limit tissue renewal capacity [5], and the shortest telomere is critical for cell viability and chromosome stability [6]. These results provide evidence that certain cellular mechanisms exist in response to telomere length. Therefore, it is quite intriguing to elucidate the cellular events and pathways that are associated with telomere length.

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Several efforts were made to ascertain global transcription profiles of human telomerase reverse transcriptase (hTERT)-immortalized cells [7,8]. Although a recent study was performed to screen genes that contribute to telomere length maintenance in yeast on a genome scale [9], little has been done to comprehensively quantify protein expression in the hTERT-immortalized cells. Measurement of the relative abundances of proteins in cells has been advanced by cleavable isotope-coded affinity tags (cICAT), multidimensional chromatography, and mass spectrometry (MS) [10–12]. In this study, human breast epithelial cells established from two distinct patients (184 and 90P) were transfected with the hTERT gene, and quantitative proteomic analyses were conducted to compare the cells bearing short and long telomeres, respectively (i.e., 184-hTERT_S vs. 184-hTERT_L and 90P-hTERT_S vs. 90P-hTERT_L). Significant and consistent changes were observed in DNA repair, integrin signaling and other protein pathways.

Materials and methods (see the supplementary data for the details)

The Cells

Parental human breast epithelial cell strains 184 and 90P [13] were transfected with the retrovirus vector pLXIN-hTERT to establish the 184-hTERT and 90P-hTERT cell lines. All the 184-hTERT and 90P-hTERT cells expressed high levels of hTERT mRNA, and strong and similar telomerase activity as measured by the methods described previously [14]. Telomere length was confirmed to be short (~3–5 kb mean telomeric restriction fragment, TRF) for the hTERT cells in early passages (~10 population doubling levels [PDLs], or less), and telomere elongation was observed mid to late passages of the cells, PDL dependently, with length more than 10 kb mean TRF [15]. Since the cyclin dependent kinase inhibitor p16^{INK4a} is associated with the senescent human breast epithelial cells [16] and expression of p16^{INK4a} coincides with the human senescent cell biomarker senescence-associated β -galactosidase [16], we examined the expression of p16^{INK4a} in the 184, 90P, 184-hTERT and 90P-hTERT cells. The expression of p16^{INK4a} was negative in all the cells examined, and promoter methylation was found for the gene *p16^{INK4a}*.

cICAT labeling of proteins

The 184-hTERT and 90P-hTERT cell lines were harvested with short telomeres (184-hTERT_S, 90P-hTERT_S) at early passages (~10 PDLs) and with long telomeres (184-hTERT_L, 90P-hTERT_L) at mid-late passages (~80 PDLs). Equal amounts of protein (800 μ g each) from the 184-hTERT_S and 184-hTERT_L samples were labeled with the light (cICAT-¹²C₉) and heavy (cICAT-¹³C₉) isotopic versions of cICAT reagents (ABI, Framingham, MA), respectively, using the method described previously [12]. The two samples were combined, buffer-exchanged into 50 mM NH₄HCO₃, pH 8.3, and digested with trypsin (Promega, Madison, WI). The proteome samples extracted from the 90P-hTERT_S and 90P-hTERT_L cells were cICAT-labeled and digested using the same procedure.

Affinity purification and fractionation of cICAT-labeled peptides

The cICAT-labeled peptides were isolated using avidin columns according to the method described previously [12], and biotin moieties were cleaved from the isolated peptides. The resulting cICAT-labeled peptides were fractionated using strong cation exchange liquid chromatography (SCXLC) into 96 fractions.

Microcapillary reversed-phase liquid chromatography-tandem mass spectrometry (μ RPLC-MS/MS)

A 10 cm $\times$ 75 μ m i.d. μ RPLC-electrospray ionization column, packed with 3 μ m, 300 Å pore size C18 RP particles (Vydac, Hercules, CA), was coupled online with an ion-trap (IT) MS

(LCQ Deca XP, Thermo Finnigan, San Jose, CA) to analyze each SCXLC fraction. A step gradient of solvent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) was used to elute the peptides: 2%–40% solvent B for 110 min and 40%–98% solvent B for 30 min at a flow rate of ~400 nL/min. The IT-MS was operated in a data-dependent MS/MS mode with dynamic exclusion to acquire MS and MS/MS spectra.

Peptide identification

The raw MS/MS data were searched using TurboSEQUEST (ThermoElectron, San Jose, CA) against the human protein database (35873 protein sequence entries) from the European Bioinformatics Institute (EBI) (www.ebi.ac.uk) to identify light and heavy cICAT-labeled peptides. SEQUEST criteria were $Xcorr \geq 2.1$ for $[M+H]^1+$ ions, ≥ 2.4 for $[M+2H]^2+$ ions and ≥ 2.9 for $[M+3H]^3+$ ions, and $\Delta Cn \geq 0.08$ for identification of fully tryptic peptides [17].

Quantitation and normalization

The identified peptides were quantified using XPRESS (ThermoElectron, San Jose, CA), which calculates the relative abundance ($^{13}C_9/^{12}C_9$, H/L, in this study) of the identified peptides. Normal distribution of each cICAT analysis was plotted in terms of the number of peptides as a function of binned $\log_2 ratio$. After nonlinear regression, the mean was used to normalize the cICAT dataset. To identify and quantify proteins, a peptide that is specific to a unique protein in the searched protein database was considered to be a valid peptide for protein identification [17], and average ratio was calculated for a protein if multiple peptides were identified.

Pathway analysis, gene ontology and Pearson correlation

Pathway analysis was performed using the Ingenuity Pathway Analysis (IPA, version 3.0, www.ingenuity.com) software. To perform quantitative data correlation between the two cell lines 184-hTERT and 90P-hTERT, Pearson correlation coefficient was calculated using JMP (version 5.0.1a, www.jmp.com) for the protein groups defined by Gene Ontology terms of molecular functions and protein complexes (GO, www.geneontology.org) and for the selected pathways revealed by IPA.

Results

Comparison of protein identification and quantitation from hTERT cells

In this study, cICAT-based quantitative proteomic analyses were performed for two pairs of protein samples from human breast epithelial cells with short and long telomeres (184-hTERT_S vs. 184-hTERT_L and 90P-hTERT_S vs. 90P-hTERT_L). Peptide identification was achieved at a confidence level of $\geq 95\%$, as revealed by searching a randomized sequence database as described previously [17]. A total of 2176 (from 4295 unique cICAT-labeled peptides) and 2195 (from 4367 unique cICAT-labeled peptides) proteins were identified and quantified from the 184-hTERT and 90P-hTERT cells, respectively. Among them, 1689 proteins were identified in both cell lines, representing an overlap of approximately 77% of the proteins identified in each comparative study. The quantitated proteins from the two cell lines exhibit normal distributions as a function of binned $\log_2 ratio$ (Fig. 1A) with similar coefficients of determination ($R^2 = 0.9864$ for 184-hTERT, and $R^2 = 0.9824$ for 90P-hTERT). The standard deviation (σ) of the two datasets is close; however, the 90P-hTERT cICAT dataset has a slightly higher σ (0.44) compared to the 184-hTERT dataset ($\sigma = 0.36$), suggesting a higher percentage of the proteins change in abundance. Since quantitation error and the real abundance change in protein expression are implied in the calculated abundance ratio (apparent ratio), and the quantitation error in the two cICAT analyses is the same (data shown later), more proteins in 90P-hTERT cells are impacted by the length of telomeres (details below).

To evaluate quantitation variability in these cICAT analyses, the coefficient of variation (CV, %) of the abundance ratio was calculated for each protein with multiple identifications from each cICAT analysis. The relative numbers of proteins (as a percentage of total proteins with multiple IDs, %) within each binned CV for each cell line are shown in Fig. 1B. This analysis indicates that the cICAT analyses of both cell lines are quite reproducible in terms of CV distribution with correlation coefficient of 0.9975 and P -value $<10^{-4}$. The highest percentage of proteins was observed with a CV of ~25% for both cICAT analyses. The average CV of all the proteins with multiple IDs is 26.58% and 26.55% for 184-hTERT and 90P-hTERT cICAT analyses, respectively.

Functional analysis of protein changes in the cells with long telomeres at a systematic level

For the cells used in this study, inactivation of *p16^{INK4a}* was observed for the hTERT cell lines as well as the parent 184 and 90P cells, which is in agreement with the previous observations that long lifespan and immortal growth of human breast epithelial cells accompany with loss of *p16^{INK4a}* expression [16]. Since this event and related genetic alterations had occurred prior to the hTERT transfection, the proteome changes observed in this study would mostly reflect the molecular events in the cells as a result of telomere lengthening after hTERT transfection.

We set $1.25 \leq \text{fold-change} < 1.5$ as slight abundance change, and ≥ 1.5 -fold as significant change, since most proteins were quantitated with a CV of ~25% or less. Since biologically effective abundance change of each protein varies individually, the setting of slight abundance change would facilitate the investigation of systematic effect of protein expression at the pathway level. Using such criteria, 540 (24.8% of the total) and 658 (30.0%) proteins were upregulated; and 430 (19.8%) and 530 (24.1%) proteins were downregulated in the 184-hTERT and 90P-hTERT cells with long telomeres, respectively (Supplementary Tables S1–S4). Commonly, 149 proteins were upregulated and 110 proteins were downregulated in both cell lines with long telomeres. Ingenuity pathway analysis revealed that a variety of molecular and cellular functions were impacted in the long telomere-bearing cells such as cell-to-cell signaling and interaction, small molecule biochemistry, protein synthesis, DNA replication, recombination, and repair, etc.

Pearson correlation measurements were performed to evaluate whether the protein pathways revealed by Ingenuity analysis and gene ontology-defined functional protein complexes were significantly perturbed in both cICAT comparisons. Significant correlations (i.e., P -values < 0.05) were found for proteins involved in metabolism, genetic information processing and cell signaling (Table 1, Supplementary Tables S5–S16). Correlated changes were observed in both cell lines in the protein pathways associated with amino acid metabolism (alanine and aspartate metabolism, glutamate metabolism, and glutathione metabolism), nucleotide metabolism (pyrimidine metabolism), and lipid metabolism (prostaglandin and leukotriene metabolism). In general, these metabolism pathways were enhanced in the cells with long telomeres. Changes in genetic information transmission, including transcription (transcription regulation), mRNA processing (spliceosome complex) and translation (translation regulation and ribosome proteins), were also observed in both cICAT studies. Spliceosome complex proteins, proteins involved in translation regulation, and both cytosolic and mitochondrial ribosome proteins were upregulated in the long telomere-bearing cells. In addition, a cell-signaling complex, the signalosome complex, was identified to be upregulated in the cells with long telomeres.

Upregulation of the DNA excision repair pathway

Since Pearson correlation is a kind of pair-wise linear correlation, it may not provide significant correlation when two datasets have coincident changes and the individual changes are nonlinear, especially in the complex biological systems. Ingenuity analysis indicated that the

DNA excision repair pathway was enhanced in both 184-hTERT and 90P-hTERT cells with long telomeres; however, Pearson correlation did not provide a significant correlation. As shown in Fig. 2, all the pathway nodes identified from the cICAT data analyses are enhanced with a minimum of one protein upregulated. Five and 3 proteins were significantly overexpressed with abundance ratios > 1.5 in the 184-hTERT_L and 90P-hTERT_L cells, respectively. DNA damage-binding protein 1 had an average abundance ratio of 1.38 and 1.39 with CV of 15.2% and 18.7% in the 184-hTERT and 90P-hTERT cells, respectively. The abundance change is considered to be slightly upregulated; however, the CV is relatively small.

Downregulation of integrin and its ligands

In contrast, integrin and several of its ligands were downregulated in one or both of the cell lines with long telomeres (Fig. 3). Collagen alpha 2(IV) was downregulated in the 184-hTERT_L cells; and fibronectin, laminin α -3 and β -3 were downregulated in the 90P-hTERT_L cells. Integrin beta-5 was underexpressed in the 184-hTERT_L cells, while integrin alpha-2, beta-6 and beta-8 were underexpressed in the 90P-hTERT_L cells. Integrin alpha-V was underexpressed (-1.8 -fold and -1.9 -fold, respectively) in both cell lines with long telomeres (the MS/MS spectra of representative peptides and the area of their extracted ion chromatograms of this protein from each cell line are shown in the supplementary Fig. S1 and supplementary Fig. S2). Interestingly, α -actinin 1 and 4, which interact with β -integrin, were downregulated in both cell lines. No proteins annotated as integrin or integrin ligands were upregulated in either of the cell lines bearing long telomeres.

Discussion

In this study, protein abundance alterations in functional protein pathways in human breast epithelial cells with differential telomere length have been analyzed. The high degree of overlap between two large datasets in protein identification provides the basis to discover common features that respond to telomere length. However, finding such features is complicated by the biological system itself since each protein interacts with others and any change in one protein or interaction node between different systems (e.g., cell lines) could result in a different response to the same stimulus. The complications in quantitative proteome analysis raise several issues and challenges. For instance, the same proteins in a pathway may change abundance in the same direction (all upregulated or all downregulated) or may change in different directions (combination of upregulation, downregulation or non-change) in response to a specific stimulus. Proteins in a pathway may show a linear or nonlinear change in relative abundance. As exemplified in this study, 5/7 of the common proteins in the DNA excision repair pathway were upregulated in both cell lines (in the same direction). Another situation is that the data shows concordance in activation or deactivation of a particular protein pathway; however, this conclusion may be obtained from different proteins. This case is illustrated in the telomere length-dependent integrin signaling pathway change observed in this study. While integrin and its ligands were downregulated in both 184-hTERT_L and 90P-hTERT_L cells, the conclusion is made mostly from dissimilar proteins found in the two cICAT experiments. However, the trend for integrin-related proteins (i.e., lower abundance in the cells with long telomeres) is consistent in both comparative analyses.

Previous findings have shown that interactions between hTERT and telomeres enhance the transcription of various genes that contribute to increased genomic stability and enhanced DNA repair [18,19]. Cells transfected with hTERT have been shown previously to counter DNA damaging agents better through the activation of DNA repair proteins [20]. The cICAT studies presented here show several proteins involved in DNA repair to be of higher abundance in cells with long telomeres although they were not reported previously to be related to telomere length. Because all the hTERT cells with either short or long telomeres have strong telomerase activity,

it is still not clear in the present study what role telomere length plays in the expression of these nucleotide repair proteins. Recent studies have shown that phosphorylated telomere repeat binding factor 2 (TRF2) translocates to DNA-damaged sites [21,22] and telomeres of the cells undergoing alternative lengthening of telomeres (ALT) [21], suggesting the certain relationship between telomere lengthening and DNA damage and repair.

The results also showed a consistent downregulation of integrin, its ligands (fibronectin, collagen, and laminin), and other proteins with which it interacts (vinculin, calpain, α -actinin, and zyxin) in cells with long telomeres. Zyxin is known to interact with α -actinin and helps to regulate actin filament assembly and functions in cell motility [23]. Calpain has also been shown to play a role in actin filament formation and cell migration [24]. A recent study indicated that cells with critically short telomeres expressed higher levels of transcripts for pro-collagens, integrin β 1, α -actinin, and a series of other proteins related to the extracellular cellular matrix, adhesion, and cytoskeletal dynamics [25]. Another study showed an increased abundance of fibronectin in fibroblasts obtained from centenarians, who had age-related decreases in their telomere length, compared to younger individuals [26]. These results agree with the general trend observed in this quantitative proteomic study in which a series of integrin-related proteins were downregulated in cells with long telomeres. The results obtained in this study may provide valuable information related to telomere functions in cell motility, cancer, and aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A) Normal distribution of the number of unique proteins quantified in the 184-hTERT and 90P-hTERT cells within binned logarithms of protein abundance ratios. B) Plot of relative number of quantified proteins (%) in binned coefficient of variation (CV) of abundance ratios of proteins with multiple peptides quantified from the 184-hTERT and 90P-hTERT cells, respectively.

Fig. 2.

A) Upregulation of the nucleotide excision repair pathway in both 184-hTERT (left half of each protein) and 90P-hTERT (right half of each protein) cell lines with long telomeres. Proteins (pathway nodes) with slight overexpression ($1.25 \leq \text{ratio} < 1.5$) are in light red, and those with significant overexpression ($\text{ratio} \geq 1.5$) are in deep red; blank means no proteins were identified. B) Actual changes in protein abundance observed in the two cICAT analyses comparing cells with long and short telomeres. Fold-change is calculated as long telomere/short telomere.

Fig. 3.

A) Downregulation of integrin and its ligands in the 184-hTERT and 90P-hTERT cell lines with long telomeres. B) Actual changes in protein abundance observed in the two cICAT analyses comparing cells with long and short telomeres. Proteins with slight underexpression ($0.67 < \text{ratio} \leq 0.8$) are in light blue, and those with significant underexpression ($\text{ratio} \leq 0.67$) are in deep blue. Gray means no changes in protein expression ($0.8 < \text{ratio} < 1.25$), and blank means no proteins were identified. Fold-change is calculated as long telomere/short telomere.

Table 1

Pathways, protein complexes and functions having significantly correlated expression alterations between the 184-hTERT and 90P-hTERT human breast epithelial cells with long telomeres.

Pathway and Protein Complex & Function	Mapping Method ^a	Correlation ^b	P-value	Count ^c
Alanine and aspartate metabolism	IPA	0.73	0.0270	9
Glutamate metabolism	IPA	0.97	<0.0001	11
Glutathione metabolism	IPA	0.89	0.0003	11
Pyrimidine metabolism	IPA	0.56	0.0018	28
Prostaglandin and leukotriene metabolism	IPA	0.90	0.0001	12
Ribosome, cytosolic	GO	0.70	<0.0001	29
Ribosome, mitochondrial	GO	0.65	0.0300	11
Signalosome complex	GO	0.97	0.0257	4
Spliceosome complex	GO	0.44	0.0232	26
Transcription regulation	GO	0.55	<0.0001	75
Translation regulation	GO	0.60	0.0002	33

^a IPA, Ingenuity Pathway Analysis; GO, Gene Ontology term-definitions for grouping of proteins based on pathway, complex, or functional relationship.

^b Pearson correlation coefficient.

^c The number of unique proteins observed for the calculation of Pearson correlation coefficient.